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IMBIBITIONAL INJURY OF PHASEOLUS VULGARIS, L. SEEDS

By

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ABSTRACT

IMBIBITIONAL INJURY OF PHASEOLUS VULGARIS, L. SEEDS

By

William David Wolk

Characteristics and bases of injury sustained by low moisture legume seeds during imbibition were determined using two cultivars of Phaseolus vulgaris, L. cvs. 'Tendercrop' (TC) and 'Kinghorn Wax' (KW).

Germination was inversely proportional to the rate of imbibition for low moisture seeds of both cultivars. TC, a soft seeded cultivar with a loosely adhering testa, took up water more rapidly than its embryo tissue could imbibe resulting in an accumulation of intercotyledonary water. At initial moisture levels <13% KW, a semihard seeded cultivar with a tightly adhering testa, imbibed slowly and did not accumulate excess water. The semihard seed characteristic of KW reduced water uptake and the tightly adhering testa retarded imbibition of the embryo tissue. Within whole seeds axes imbibed more rapidly than cotyledons but sustained less injury. It was concluded that the axis is more resistant to imbibitional injury than the cotyledon.

The seed moisture level marking the onset of imbibitional injury (breakpoint) was determined. At 20°C the breakpoints were 0.15 g H_2O/gdw (g/g) for TC and 0.11 g/g for KW. When seeds were imbibed at 5°C the breakpoints were 0.19 g/g (TC) and 0.16 g/g (KW). Below the breakpoint

germination changed 4.6%/0.01 g/g for all treatments. Imbibition rates were maximal at 0.07 g/g and 0.33 g/g after 20 min imbibition. Rates of electrolyte leakage were correlated with the imbibition rate maximum at 0.07 g/g but were unaffected by the maximum at 0.33 g/g. The transition from tightly bound to semi-bound water occurred at 0.09 g/g and 0.11 g/g for KW and TC, respectively. T1 values increased exponentially as seed moisture decreased from 0.47 g/g to 0.05 g/g. ^{13}C-NMR sugar signals increased at moisture levels above 0.14 g/g and plateaued at approximately 0.33 g/g seed moisture.

These results suggest that the breakpoint moisture level is a function of temperature while the injury process is similar at both 5° and 20°C. Imbibition and leakage rate maxima reflect transitions in the states of seed water. NMR data support the application of the Water Replacement Hypothesis to seeds.

To Roz, for her unwavering support throughout...

"These are the fascinations, these lurking forces of expansion, these necessities of upspringing in the seed, these beautiful determinations, to grow as tall as possible, to push into the light and the air and thickly flower there."

- Henry James

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Guidance Committee:

This dissertation was written in the journal format in accordance with departmental and university regulations. The thesis body is divided into five sections, Sections I, II, III and Appendices A and B. Sections I and II and the appendices are written in the style of Plant Physiology. Section two was prepared in the style of the Journal of the American Society for Horticultural Science.

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LITERATURE REVIEW

The deleterious effects by exposure to low, non-freezing temperatures during imbibition to the seeds of certain species is well documented. (Kidd and West, 1918, 1919; Kotowski, 1926; Harrington and Minges, 1954; Harrington and Kihara, 1960). The current line of reasoning being used to discover the mechanism of the injury started with series of papers published in the mid-to-late 1960's by Pollock and co-workers in which they determined that: 1) imbibitional chilling injury occurs in the earliest stages of water uptake, 2) the seed's chilling sensitivity is a function of its moisture content prior to the onset of imbibition and 3) the injury is characterized by and can be correlated with the leakage of large quantities of intracellular constituents (Pollock, 1969; Pollock and Toole, 1966; Pollock et al., 1969; Pollock and Manalo, 1970). These findings initiated a rapid and widespread interest into the subject of imbibitional chilling injury that continued into the early 1980's. During this period Pollock's original findings were confirmed for a large number of economically important crop species (Cal and Obendorf, 1972; Christiansen,1969; Obendorf and Hobbs, 1970, Simon and Wiebe, 1975, Bramalage et al., 1978; Hobbs and Obendorf, 1972.) During this time apparent differences in sensitivity were noted between different cultivars of the same species (Cal and Obendorf, 1972; Clay et al., 1976; Hobbs and Obendorf, 1972; Obendorf and Hobbs, 1970; Malf and

Tigchelaar, 1980; Pollock et al., 1969.) and even between different seed lots of a given cultivar (Cohn and Obendorf, 1978).

Investigations into the specific nature of imbibitional chilling injury were strongly influenced by hypotheses concerning the mechanism of chilling injury in hydrated plant tissues. These were based in large part on the so-called Lyons-Raison Hypothesis (Lyons and Raison, 1970a, 1970b) which held that chilling injury was a physiological dysfunction induced when the phospholipid fraction of cell membranes underwent a low temperature phase transition from the liquid crystalline to solid gel state. The transition temperature is, in part, a function of the degree to which the fatty acid moieties of membrane phospholipids are saturated (Lyons, 1973) and thus a great deal of chilling injury research was focused on qualitative and quantitative analyses of plant membrane lipids (Lyons, 1973.)

The subject of chilling injury in plants has been extensively reviewed by Lyons (1973). The rationale for accepting the membrane phase transition hypothesis as a starting point to explain the mechanism of imbibitional chilling injury was primarily twofold. First, chilling sensitive plants generally produce chilling sensitive seeds and resistant plants produce resistant seeds. Chilling sensitive plants are, by and large, of tropical and subtropical origin and, from an agricultural perspective,

warm season crops. Chilling resistant plants' centers of origin are the temperate to arctic regions and are cool season crops (Lyons, 1973). Exceptions to these general observations have been noted (Lyons, 1973). The second observation that encouraged researchers to associate the injury in seeds with the same causal mechanism as that hypothesized for chilling injury in growing plants was the solute leakage phenomenon. All seeds leak a variety of intracellular substances when first placed in water (Simon, 1974). If seeds are imbibed at non-chilling temperatures the leakage rate quickly declines over a period of 15 to 60 minutes (Larson, 1968; Simon and RajaHarum, 1972). If seeds are imbibed at chilling temperatures, however, leakage rates are greatly increased and the period of leakage is prolonged (Bramalage et al., 1978; Christiansen, 1967; Simon and Wiebe, 1975) and is negatively correlated with the seed vigor and germination (Bramalage et al., 1978; Yaklich et al., 1979). Increased leakage from chilling sensitive growing plant tissues had previously been noted and used as an indicator of injury (Rikin and Richmond, 1976, 1979; Wright and Simon, 1973).

The temperature dependence of a reaction rate constant, k, can be expressed

$$k = A \cdot e^{-Ea/RT}$$

where A is the frequency factor, R the ideal gas constant, T the absolute temperature and Ea the Arrhenius energy of

activation. Thus, when, in an Arrhenius plot, k is plotted as a function of 1/T, the slope equals -Ea/R from which Ea can be obtained. As the temperature decreases there is a sudden increase in activation energies for metabolic processes mediated by membrane bound enzyme systems such as those involved with mitochondrial respiration or the light reactions of photosynthesis in chilling sensitive tissues of growing plants. It occurs at the same temperature that membrane probes indicate a phase transition in membrane lipids (Lyons and Raison, 1970a). Chilling resistant plant tissues do not display the increase in E_a nor is there evidence that membranes in resistant tissues undergo a phase transition. Similar calculations for increases in E_a in chilling sensitive seeds have been reported for the non-physiological processes of imbibition and leakage (Leopold, 1980). Use of Arrhenius plots for diagnosis of membrane phase transitions has recently been questioned (Martin, 1986.)

In addition to the application of the membrane phase transition hypothesis to imbibitional chilling injury, an explanation was needed for the observation that low moisture seeds were more severely chill-injured than high moisture seeds. To this end Simon (1974) suggested that the membrane bilayer configuration in seeds becomes increasingly unstable as water is removed from the system until a point of desiccation is reached when an inverted hexagonal phase

becomes thermodynamically favored. In the hexagonal array phospholipids form the continuous medium arranged around groups of water molecules. Simon envisioned the water to exist in columns running through the phospholipids to form channels from the "inside" to the "outside" of the cytoplasm. This, he asserted, accounted for the large quantity of the leakage observed during the first minutes of imbibition. According to this scheme membranes had to reform into functional bilayers during the first stages of imbibition. Once the bilayers had been re-established leakage would decline which was in keeping with leakage data. This hypothesis, though not directly tested until approximately ten years after it was first published, was quickly accepted and widely quoted in literature dealing with imbibitional chilling injury.

Thus, the similarities of chilling injury between hydrated tissues and imbibing seeds were strong enough to influence the course of research taken by seed physiologists from the time of Pollock's work in the 1960's up and into the early 1980's. Included are two projects from this laboratory which investigated the relationship between the degree of membrane fatty acid saturation and imbibitional chilling resistance (Dogras et al., 1977; Wolk, 1980). Early studies on the correlation between chilling sensitivity and membrane fatty acid saturation for both hydrated plant tissues and imbibing seeds generally were

based on comparisons between chilling sensitive and chilling resistant material of different species. In order to eliminate species differences, the second study from our laboratory (Wolk, 1980) used two cultivars of a given species (Phaseolus vulgaris L.) 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 necessarily support the membrane phase transition hypothesis as the mechanism of low temperature injury in imbibing seeds. At approximately the same time a second research group (Priestley and Leopold, 1980) also began to question the importance of membrane lipids in imbibitional chilling based on similar findings in their laboratory. Concurrent with these reports came results of investigations of membrane structure in dry seeds which refuted the hexagonal array hypothesis. Simon had originally proposed his model based on studies of the behavior of bulk phospholipid/water systems. However, X-ray diffraction of both extracted (McKersie and Stinson, 1980) and in situ (Seewaldt et al., 1981) seed membrane lipids and ^{31}P-NMR (Priestley and Kruijff, 1982) studies of membrane structure in dehydrated pollen grains indicated that the bilayer configuration is maintained even under conditions of extreme desiccation.

These findings, taken together, the lack of correlation between membrane saturation and imbibitional chilling

sensitivity and the persistence of the bilayer configuration in dry seeds, marked the end of a fifteen year period of research in which these had been the two predominate hypotheses. More thorough reviews of low temperature imbibition and germination from this period of research have been published (Wolk and Herner, 1982; Herner, 1986).

With the rejection of its central hypotheses, both the number of laboratories focusing on the problem of low temperature imbibition and the number of publications per year devoted to it declined sharply at the outset of the 1980's. Those who remained in the field returned to some of the observations made during the earliest periods of the research: primarily those regarding solute leakage from seeds. Leakage was clearly a symptom of the injury and though it could not be explained by a phase transition or by hexagonal arrays of membrane lipids, the increase in membrane permeability induced by low temperature, especially in low moisture seeds, indicated some sort of membrane dysfunction. In a series of papers spanning a ten year period Powell and Matthews studied the leakage phenomenon as a way of understanding the injury process. In their first paper (Powell and Matthews, 1978) they broke with the membrane phase transition hypothesis a number of years before the X-ray diffraction and NMR data supporting their move became available and put forth the basic hypothesis they would further elucidate over the following years. They

suggested that increased leakage induced by imbibitional chilling was the result of membrane rupture. Imbibitional injury was sustained by the cells of naked embryonic cotyledons but not when the testa was left intact covering the cells. Embryos imbibed in a 40% solution of polyethylene glycol (PEG) were not injured. They concluded that the injury was caused by imbibition per se and not by low temperature. In subsequent studies they found a correlation between testa damage and imbibitional injury (Powell and Matthews, 1979) and suggested that imbibition rate was the prime factor causing the injury (Powell and Matthews, 1980). Noting that the pattern of leakage over time is the same for both living and dead seeds (Powell et al., 1986a) they further concluded that it was a purely physical phenomenon and reflected disruption of cell membranes caused by imbibition. More recently they have reported that white seeded cultivars of P. vulgaris tend to imbibe more rapidly and are more readily injured than those cultivars with pigmented testae (Powell et al., 1986a). The pigmented cultivars with which they work have the trait of having a testa that adheres very tightly to the cotyledon and they have correlated this characteristic with reduced imbibition rate, decreased leakage and increased emergence in the field (Powell et al., 1986b).

Taylor and Dickson (1987) determined that the semihard seed coat characteristic in snap beans, which delays the

onset of imbibition at low initial moisture levels, reduces imbibitional chilling injury. Water entry into semihard seeds takes place primarily through the chalaza and raphe rather than the hilum and micropyle (Holubowicz et al., 1988.)

Duke and co-workers centered their studies on the role of the testa in preventing cellular rupture during imbibition and tried to develop refined methods to both measure and interpret leakage data. In doing so they have confirmed several points first introduced by Powell and Matthews (1978). They reported that both seeds and embryos of bean, soybean, pea and peanut leak cytosol marker enzymes but only embryo tissues of these species leak mitochondrial marker enzymes (Duke and Kakefuda, 1981) supporting the hypothesis that the testa provides protection against membrane rupture. They suggested that leakage of electrolytes and UV absorbing compounds, the two most commonly used methods of measuring leakage, do not accurately reflect the state of membrane integrity because, they contend, 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 (Duke et al., 1983). Finally, they have determined that the testa epidermis is the layer essential in preventing the imbibitional injury observed in naked embryos or seeds with cracked seedcoats (Duke et al.,

1986).

In studies evaluating the effect of seed vigor on imbibitional injury, Woodstock and co-workers have determined (Woodstock and Tao, 1981) that low vigor soybean axes are susceptible to imbibitional injury while normal axes are not. The injury to the former is overcome if the axes are imbibed in a 30% PEG solution. They concluded that imbibitional injury is sustained only by low vigor seeds. In addition, they have measured toxic levels of ethanol and acetaldehyde in both high and low vigor soybean seeds that were imbibed by soaking under water (Woodstock and Taylorson, 1981). This observation holds strong implications for a great deal of research in the area of imbibitional injury. Though it had been clearly documented back at the turn of the century (Kidd and West, 1918, 1919) and then later explained (Orphanos and Heydecker, 1968) most researchers have tended to ignore the phenomenon of soaking injury. When seeds are imbibed under conditions favorable for germination, measurement of imbibition rates have inherently high levels of variation. This is, in part, due to the fact that imbibition rates are highly dependent upon the area of contact between the seed and the imbibition medium (Collins-George and Hector, 1966). Imbibing seeds by submersion eliminates a great deal of this variation but induces soaking injury whereby the seed is injured or killed by anoxia and/or the buildup of toxic metabolites as noted

by Woodstock and Tao (1981). Submersion can certainly be justified for certain experimental objectives dealing with the physical aspects of imbibition or the resistance of the testa to water flow. However, it seems quite reasonable to question the results of studies in which germination of soaked seeds is discussed as a function of factors other than soaking itself.

And so, by the early 1980's, a body of evidence strongly implicated rapid imbibition as a cause of imbibitional injury. A major flaw, however, in the studies supporting such a conclusion was that in every case rapid and slow imbibition rates had been imposed by soaking seeds in water or PEG solutions, respectively. Regardless of whether the imbibition rate affected the degree of injury, it was clear that the injury occurred early in tissue rehydration. A reasonable explanation for the mechanism of injury was lacking since the membrane phase transition/hexagonal array hypotheses had been abandoned. The first move toward developing a new hypothesis came in 1983 when Vertucci and Leopold 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, surprisingly, 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 key step in the injury process. Their research led them into the area of anhydrobiosis, a field that had been previously dominated by interest in animal and bacterial organisms which are capable of surviving extended periods of extreme desiccation. These studies are currently ongoing and are focused on the maintenance of cell structures during dehydration. The central hypothesis is the "Water Replacement Hypothesis" and has recently been reviewed by Clegg (1986), one of its principal proponents. Briefly, the water replacement hypothesis holds that certain polyhydroxyl compounds such as the sugar trehalose are structured in a way that allows their hydroxyl groups to hydrogen bond 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 et al., 1983). Animals such as nematodes (Madin and Crowe, 1975) and brine shrimp (Clegg, 1976) that regularly survive desiccation synthesize large quantities of trehalose in response to dehydration. While seeds are not known to contain trehalose (Kandler and Hope, 1966) Caffrey et al. (1988) have recently demonstrated <u>in vitro</u> the ability of raffinose/sucrose mixtures, sugars common in seeds, to preserve the bilayer configuration of phospholipid mixtures at low water

activities.

Like protein-water interactions (Rupley et al., 1983) the hydration water adsorbing to seed tissues appears to exist in three states: bound, semi-bound and loosely bound (Vertucci and Leopold, 1984) corresponding to adsorption at charged, polar and weakly interacting sites, respectively. The tissue moisture levels at which each of these types of sites become saturated can be determined from thermodynamic parameters derived from water sorption isotherm curves (Clegg, 1978). Vertucci and Leopold (1984) have hypothesized that imbibitional injury is dependent upon the types of sites being filled during water uptake. A test of this hypothesis serves as the basis for the third section of this dissertation.

Thus, the exact mechanism of imbibitional injury remains unknown. Though the membrane phase transition and hexagonal array hypotheses of ten years ago have been discarded, emphasis remains on the membranes of imbibing seeds because the dynamics of leakage indicate a membrane dysfunction. The focus has moved toward the physical binding of water to membrane components. There is now the additional consideration that during imbibition of low moisture seeds, water might possibly have to replace carbohydrates at membrane and other macromolecular binding sites.

Because soaking seeds in water or PEG solutions, which

have been used to induce rapid and slow imbibition, respectively, have effects on seeds quite beyond simply affecting imbibition rate, the question of the effect of imbibition rate on imbibitional injury remains to be properly tested. This is the objective of the first section of this dissertation.

The semihard seed characteristic reported by Taylor and Dickson (1987) and the tight adherence of the testa to the cotyledon discussed by Powell et al. (1986a, 1986b) appear to be distinct traits. One of the snap bean cultivars used in the research for this dissertation has the semihard characteristic and also has a tightly adhering testa. The second objective of this dissertation was to determine the individual roles of each of these traits in the avoidance of imbibitional injury.

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SECTION I

THE EFFECT OF THE RATE OF IMBIBITION ON GERMINATION OF LOW MOISTURE *PHASEOLUS VULGARIS*, L. SEEDS

ABSTRACT

Rates of imbibition rates and their effect on subsequent germination of low moisture (7.5% to 9%) Phaseolus vulgaris L. seeds (cvs. 'Tendercrop' and 'Kinghorn Wax') were determined when seeds were imbibed in four different ways to control water uptake rate. They were: 1) positioning the seed relative to the hilum, 2) incremental addition of water 3) varying the number of layers of filter paper on which the seeds were imbibed and 4) applying wax to the seed's hilum region. Each method of imbibition affected the rate of water entry in the seeds. Reduced rates of imbibition were significantly correlated with increased germination. The interaction of imbibition rate with initial seed moisture, imbibitional chilling and cultivar were studied. It is proposed that the development of a hydrophobic compound which, when applied to a seed to retard imbibition rate, would be beneficial to seed germination and might be important commercially.

INTRODUCTION

Orthodox seeds of many species are subject to injury during the early stages of water uptake (8) often resulting in seed death or a reduction of growth for surviving seeds (7). Low initial seed moisture and exposure to chilling temperatures are both causal factors in the process and can interact to increase the extent of the injury (8). Observations in a number of additional studies suggest that rapid rates of imbibition might also be a contributing factor to imbibitional injury.

Pea embryos imbibe more rapidly and lose more intracellular constituents during the process than intact pea seeds (5). Abaxial cells of pea cotyledons are killed when the seeds are imbibed in direct contact with water while adjacent cells with the testa left intact or adaxial cells on the same cotyledon not in direct contact with water during imbibition remain viable. When polyethylene glycol (PEG) is used as an osmoticum to retard the rate of imbibition of pea embryos, injury is decreased compared to embryos imbibed in water (9).

Soybean seeds imbibe more rapidly than pea seeds and imbibitional injury is proportionally greater in soybeans. If the testae of pea seeds are cut so their rates of imbibition approximate those of intact soybean seeds the amount of injury in pea seeds becomes equivalent to that of soybean seeds (12). Abaxial cells of soybean and bean seeds

with damaged or missing seed coats rupture during imbibition resulting in cell death (3). The testa epidermis serves as the primary barrier to water entry through the seed coat (4). When the epidermis is cracked, leakage of electrolytes during imbibition significantly increases and germination decreases.

From the studies cited above it can be hypothesized that the rate of imbibition affects seed germination. This hypothesis has not been directly tested. Furthermore, in each of these studies cited above information concerning effects of rates of have been confounded by the methods employed and, as a result, leave the question of the effect of rates of imbibition on germination unresolved. In each of the cases cited above, rapid imbibition was achieved by submerging the seed in water. This technique induces soaking injury in legume seeds, a separate and distinct phenomenon from imbibitional injury (6). Additionally, PEG has recently been shown to have the properties of a surfactant and at low concentrations actually increases the rate of imbibition of low moisture soybean cotyledons (13). Thus, each of these previous studies has one or more confounding factors and none have used the proper controls necessary to draw unequivocal conclusions regarding the effect of rapid imbibition on germination.

To test the hypothesis that rates of imbibition affect seed germination, experimental methods have been designed

that circumvent the inherent problems presented by the use of chemosmotic agents and submersion.

MATERIALS AND METHODS

Two snapbean (Phaseolus vulgaris, L.) cultivars, cvs. 'Tendercrop' and 'Kinghorn Wax' (Rogers Bros. Seeds, Twin Falls, ID), with standard germination in excess of 90% were used in all experiments. Seeds were stored at 5°C and 35% R.H. prior to use.

For all experiments seeds were brought to initial moisture levels of 7.5% to 9% over H_2SO_4 solutions in sealed desiccators. Seed moisture was determined by drying seeds 48 hr at 95°C in a forced draft drying oven. All moisture contents are expressed on a fresh weight basis.

Except where otherwise noted, seeds were germinated in the dark at 20°C in 15x100mm petri dishes, 10 seeds per dish, between two pieces of Whatman #1 filter paper wetted with 0.15% captan solution as a fungicide. In experiments using chilling stress, seeds were imbibed for 24 hr at 5°C as described above and then transferred to 20°C for germination.

Imbibition rates were controlled in four ways.

1) Incremental addition of solution: Seeds were imbibed with five ml captan solution added either in total at initiation or one ml every two hours until a total of five ml solution had been added.

2) Positioning of the hilum/micropyle/chalazal region: A six

cm circle of wire mesh was slightly bent into the shape of a flattened "w" and placed into a petri dish. A circle of seven cm Whatman #1 filter paper was folded similarly and placed over the wire mesh. Seeds were then placed, either hilum up or down, in the shallow grooves. A second piece of filter paper was used to cover the seeds. The imbibing solution wetted the filter paper by "wick" action. The seeds were then imbibed with 5 ml captan solution.

3) Varying the layers of filter paper: Seeds were imbibed on one, three or five 7.0 cm Whatman #1 filter paper circles with one additional filter paper used to cover the seeds. Seeds were imbibed with 5 ml captan solution for 24 hr at either 5°C or 20°C. After 24 hours imbibition seeds were transferred to new dishes at 20°C and germinated between two pieces of filter paper wetted with captan solution as needed.

4) Wax applied to the hilum/micropyle/chalazal region: A thin layer of a pliable wax was applied to the hilum region of each seed. Seeds were imbibed at 5° or 20° with 5 ml captan solution and germinated as described above.

Germination was considered as the protrusion and elongation of the radicle and was scored after five days at 20°C. Bartlett's tests of germination data were significant in all cases. Statistical analyses of germination data was performed on arc sin transformed data according to Steele and Torrie (10). Results from each

imbibition method were analyzed separately in completely randomized factorial designs. Duncan's multiple range test was used to separate means.

Rates of imbibition were determined by imbibing seeds under identical conditions as for germination experiments. Seeds were quickly blotted dry with tissue paper, weighed and replaced at designated sampling times during a 24 hr period except when wax was applied to the hilum region. For wax application experiments the level of imbibition was determined after 24 hr.

The rate of imbibition was determined using two replicates of ten seeds each for the incremental addition of solution experiment. All other rates of imbibition were determined with four replicates of individual seeds. Germination studies were replicated five times and data presented are the means of at least two experiments.

RESULTS

Studies on Imbibition: All methods used to control the rate of imbibition were successful to some degree. Positioning the hilum/micropyle/chalazal region down against the filter paper resulted in the most rapid uptake (Fig 1). Imbibition was essentially complete in eight hours for Tendercrop and in 12 hr for Kinghorn Wax. When the dorsal ridge, the region opposite the hilum, was placed against the filter paper ("up") forcing imbibition to proceed through

pores in the testa, imbibition was only 74% complete in Kinghorn Wax and 83% complete in Tendercrop after 24 hr. Complete imbibition is defined as 55% moisture.

Incremental addition of water retarded uptake compared to controls for at least the eight hour period while water was being added (Fig 2). After 24 hr at 20°C both controls and incremental additions for both cultivars had reached the same level of hydration. This would not be unexpected since both control and treated seeds were subject to equivalent imbibition conditions for the final 16 hr period prior to the 24 hr measurement. At 5°C, however, where low temperature itself retards imbibition (1) seeds receiving water in 1 ml increments were significantly less hydrated after 24 hr than those receiving the full 5 ml quantity at the start.

Differing the number of filter papers in the germination dishes afforded the greatest level of control over imbibition rate of all the methods tried and resulted in a wide spread of seed hydration levels after 24 hr for both cultivars at both 20° and 5°C (Figs. 3 and 4). At 20°C seeds imbibed on one filter paper were almost fully hydrated after 24 hr while those imbibed on five layers of filter paper were only approximately 50% hydrated (Fig. 3.) Low temperature reduced the rate of imbibition in Kinghorn Wax to a greater degree than it did in Tendercrop (Fig. 4.) At 5°C imbibition was reduced by approximately 27% compared to

Figure 1. The effect of the site of seed/water contact on rates of imbibition of two P. vulgaris cultivars. Seeds with hilum region touching wetted filter paper are designated "down." Seeds imbibed on the dorsal ridge with the hilum region up, away from the wetted filter paper are designated "up." Vertical bars indicate SE of the means.

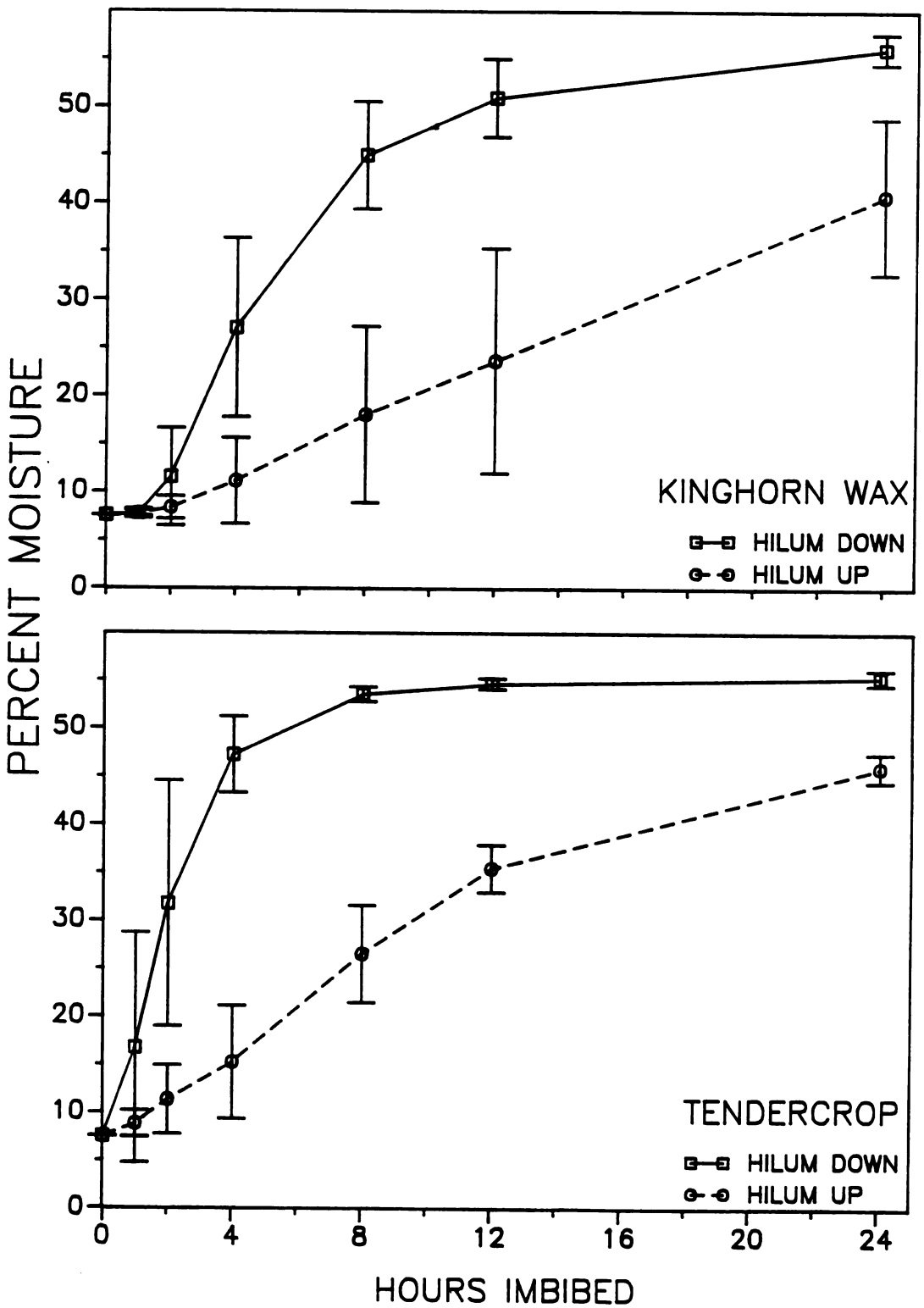
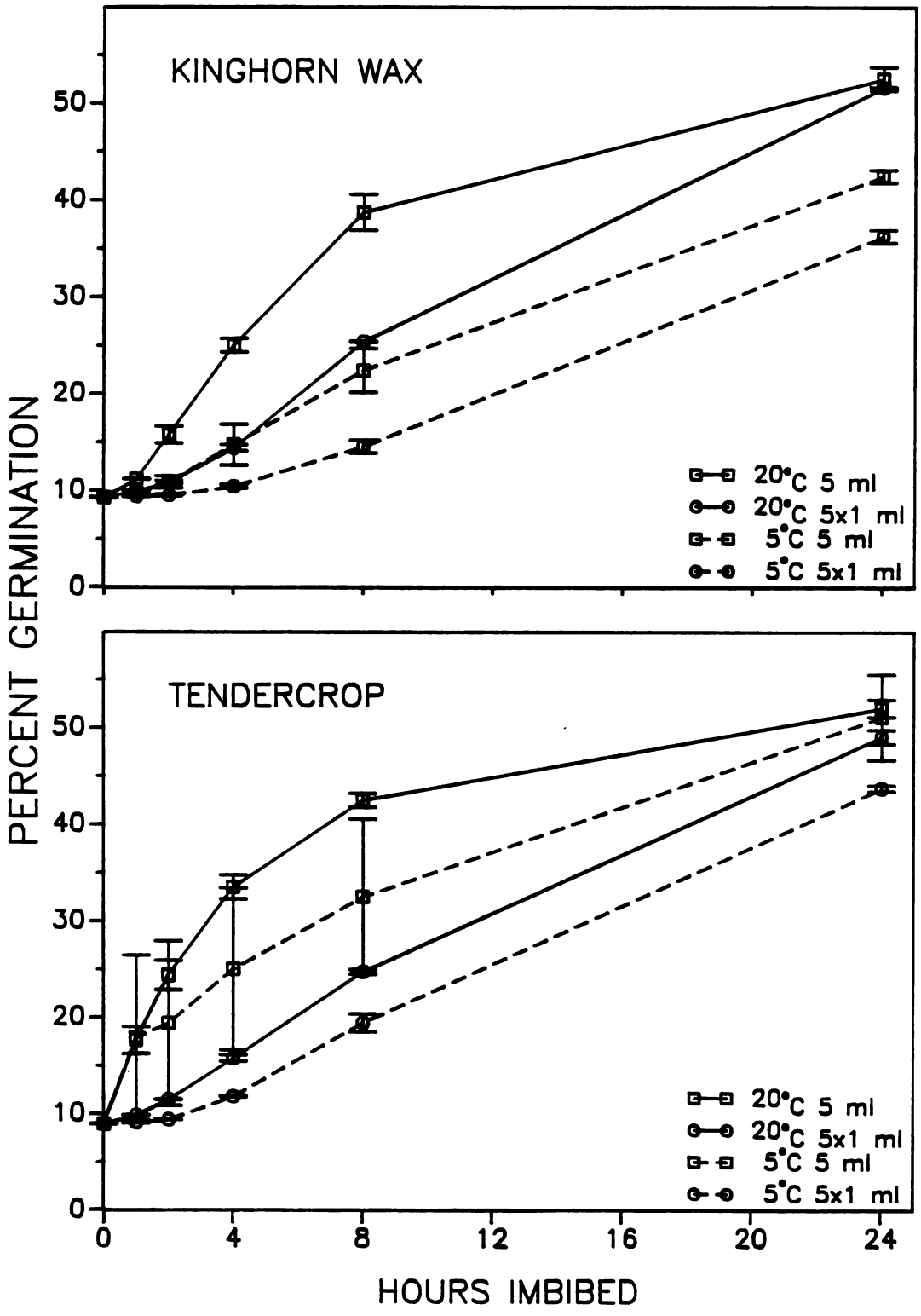

PERCENT MOISTURE
0
10
20
30
40
50
KINGHORN WAX
HILUM DOWN
HILUM UP
TENDERCROP
HILUM DOWN
HILUM UP
0
4
8
12
16
20
24
HOURS IMBIBED

Figure 2. The effect of incremental addition of water on rates of imbibition of two P. vulgaris cultivars at 5° and 20°C. Water was added to 10 seeds placed between 2 pieces of filter paper in a 15x100mm petri dish either as 1 ml every 2 hr until a total of 5 ml was reached (5 x 1 ml) or as 5 ml total at start (5 ml). Vertical bars indicate SE of the means.


KINGHORN WAX
TENDERCROP
PERCENT GERMINATION
HOURS IMBIBED
0
10
20
30
40
50
0
4
8
12
16
20
24
20°C 5 ml
20°C 5x1 ml
5°C 5 ml
5°C 5x1 ml

20°C for Kinghorn Wax and by 11% for Tendercrop.

Placing wax over the hilum region also caused a reduction in imbibition rate during the first 24 hr. The wax reduced imbibition levels in both cultivars equally. At20°C the wax reduced imbibition by 30% and by 20% at 5°C.

Germination: Altering the layers of filter paper to control imbibition rate had the additional main effect of temperature. In all cases F tests for main effects were very highly significant ($P<.001$). The interaction of cultivar and temperature was also significant in experiments testing for temperature effects. This significant interaction has been rationalized elsewhere (14, 15) and is related to a third factor, initial seed moisture content. Both cultivars exhibited a certain breakpoint initial moisture level below which germination declined linearly with continued decreasing moisture content. At 20° and 5°C Tendercrop's breakpoint was approximately 3% higher than that of Kinghorn Wax (15). Thus, when the moisture level chosen for germination experiments is below one or both cultivars' breakpoints the three way interaction term of cultivar x temperature x seed moisture will be significant. For all experiments in this study seeds of both cultivars were equilibrated to equal moisture levels prior to imbibition and germination. These ranged from 7.5% to 9% moisture over all experiments. Within a given experiment seeds of both cultivars were equilibrated to

Figure 3. The effect on rate of imbibition by seeds of two *P. vulgaris* cultivars on 1, 3 or 5 pieces of filter paper with a constant (5 ml) volume of water at 20°C.

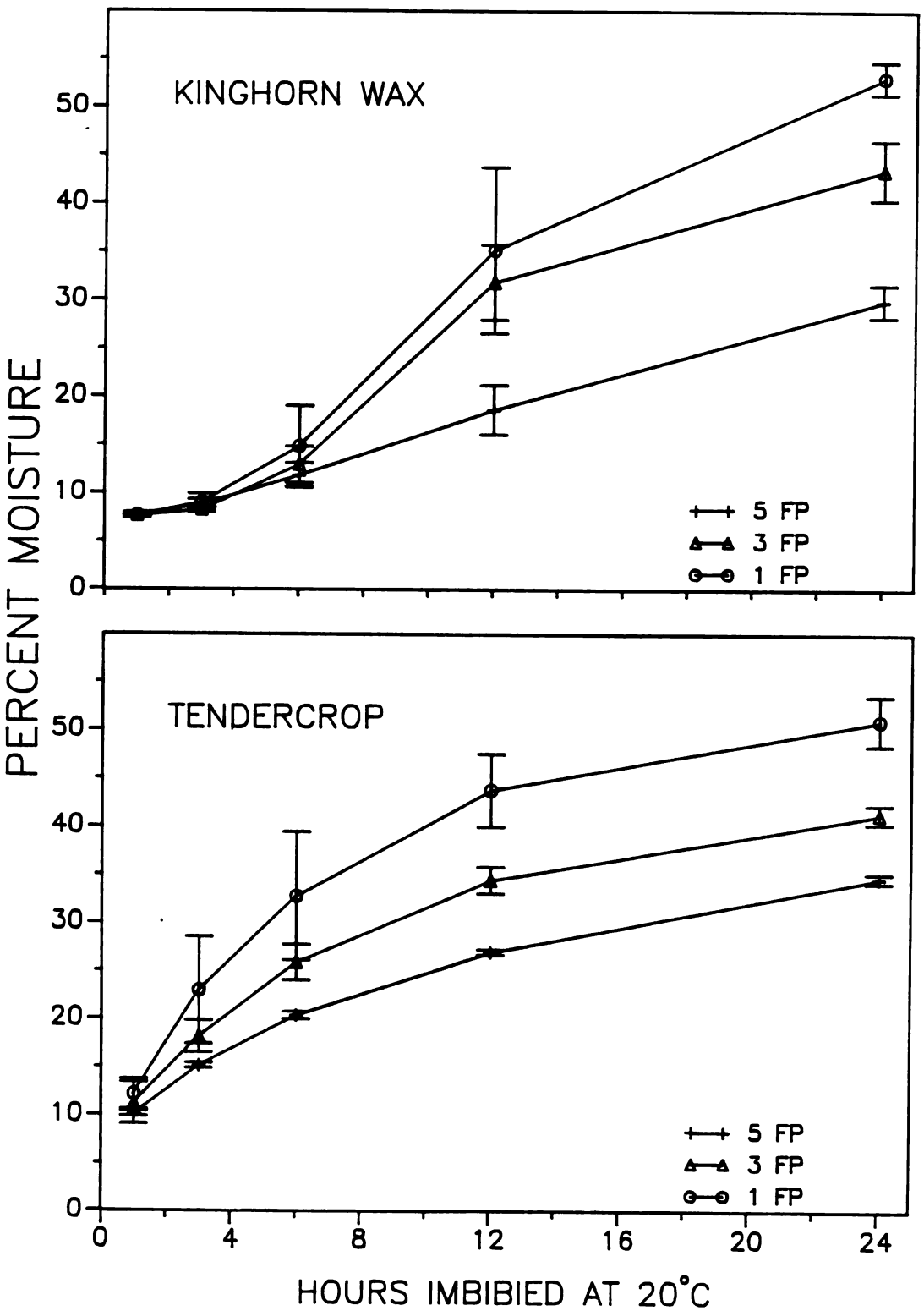
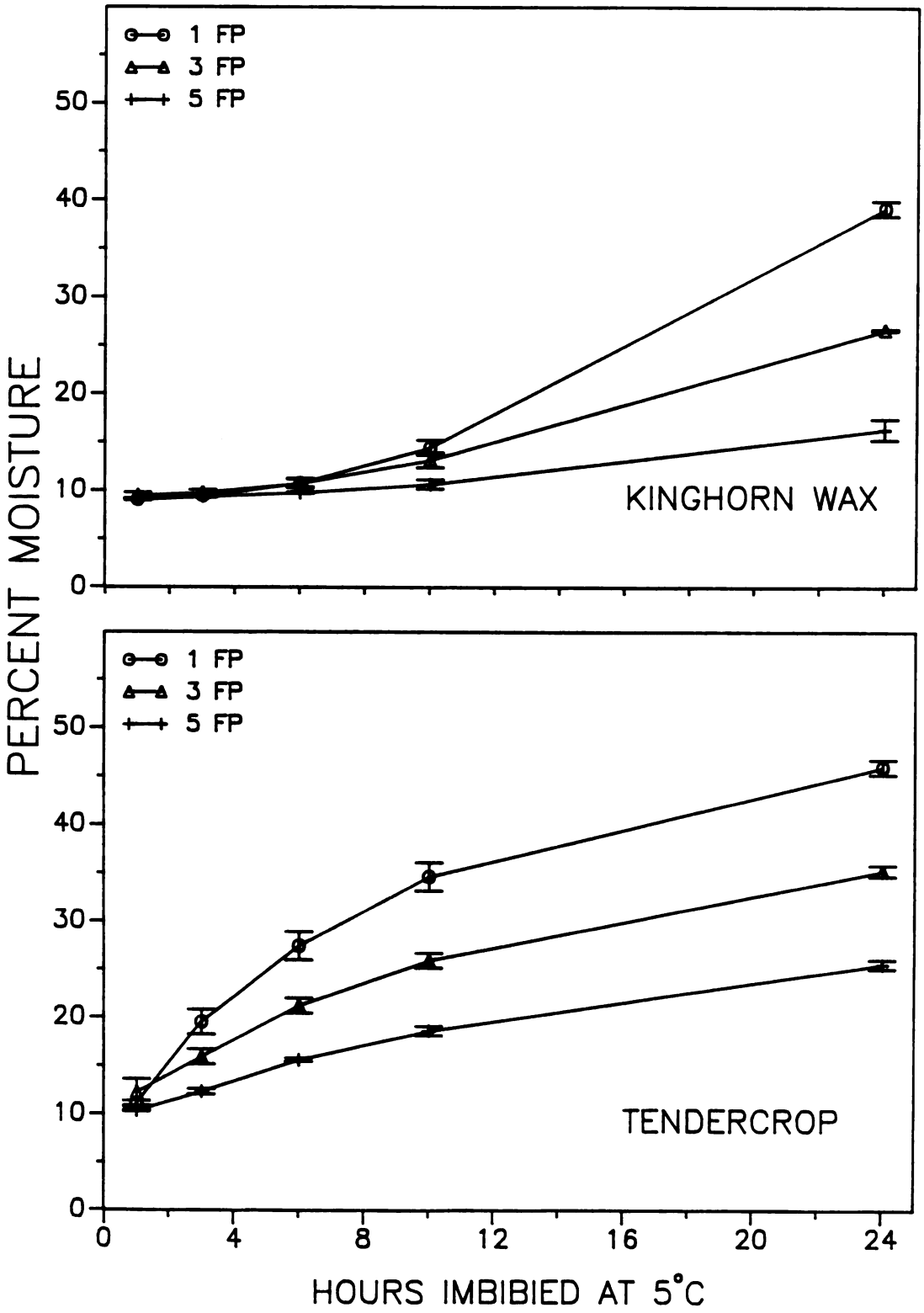

KINGHORN WAX
TENDERCROP
5 FP
3 FP
1 FP
PERCENT MOISTURE
HOURS IMBIBIED AT 20°C
0
10
20
30
40
50
0
4
8
12
16
20
24

Figure 4. Rates of imbibition by two P. vulgaris cultivars on 1, 3 or 5 pieces of filter paper with a constant (5 ml) volume of water at 5°C.


1 FP
3 FP
5 FP
KINGHORN WAX
TENDERCROP
PERCENT MOISTURE
HOURS IMBIBIED AT 5°C
0
10
20
30
40
50
0
4
8
12
16
20
24

equal (± 0.1%) moisture levels. In all cases the equilibrated moisture levels were below the breakpoint moisture levels for both cultivars and at both temperatures when chilling treatments were applied. However, because of the 3% difference in breakpoint levels between the two cultivars, Tendercrop's germination was always reduced compared to that of Kinghorn Wax. This difference will cause the cultivar x imbibition rate interaction term to be significant in all experiments. Even so, F values for main effects in all experiments were generally one or two orders of magnitude greater than those of the interaction terms. Thus, with the understanding of the interaction and with the large portion of treatment variance assignable to the main effects, it is reasonable to consider the main effect of imbibition rate on germination as well as its interaction with other factors. Table 1 summarizes these results. In all experiments, rapid imbibition decreased germination for each cultivar. The precipitous decline in germination observed when rapid imbibition was imposed by positioning the hilum region downward is most likely caused by soaking injury rather than imbibitional injury which is the assumed cause of reduced germination in the remaining rapid imbibition treatments. This is supported by the results of similar germination studies using higher moisture seeds. High moisture embryo tissue is more resistant to imbibitional injury than low moisture tissue (2). As legume

seeds become drier the testa increases its resistance to water flow (11). When high moisture seeds are imbibed with the hilum down they are more readily injured than low moisture seeds (data not shown). Because high moisture embryo tissue is more resistant to imbibitional injury the decreased germination can be attributed to soaking injury where the axis tissue is killed by anoxia when water trapped in the intercotyledonary cavity becomes depleted of oxygen.

Correlations of percent germination with average imbibition rate (delta %/hr) after 12 hr imbibition at both 20°and 5°C are highly significant when the results from all imbibition methods and both cultivars are combined (Fig. 5) Because germination has a maximum limit of 100% the three slowest imbibition means at 20°C, all with 100% germination, were excluded from the regression analysis.

Regression analysis for data presented in Figure 5 and for individual cultivars at different sampling times are presented in Table 2. In the individual analyses there is a sampling time with a maximal r^2 value for each cultivar x temperature treatment combination. At 20°C they are 8 hr and 12 hr for Tendercrop and Kinghorn Wax, respectively and 12 hr and 24 hr for the two respective cultivars at 5°C. These maxima occur at sampling times when the average moisture content for all imbibition method means for that cultivar x temperature combination is approximately 32%. This is close to the 36% moisture value that marks the

Table 1. Summary of four experiments determining the effect of imbibition rate controlled by various methods on the germination of two P. vulgaris cultivars, 'Tendercrop' and 'Kinghorn Wax' at 5° and 20°.

	TENDERCROP				KINGHORN WAX			
	PERCENT GERMINATION							
	20°		5°		20°		5°	
Imbibition Method	slow	fast	slow	fast	slow	fast	slow	fast
Waxed hilum	97a[z]	83b	56d	56d	99a	95a	86b	74c
Filter paper[a]	93a	82b	71b	22d	100a	99a	72b	64c
Incremental add	96a	63b	66b	36c	96a	83a	93a	69b
Hilum up/down	97a	22b	-	-	96a	10c	-	-

[z]Mean separation across rows. Duncan's Multiple Range P=0.05.

[a]Slow=5 filter papers, fast=1 filter paper.

MAIN EFFECTS[y]

	% GERMINATION	
Imbibition Method	slow	fast
Hilum up/down	96	16
Waxed hilum	94	68
Filter paper	94	57
Incremental add	85	66

[y]Main effects of imbibition method are significant at P<0.001

Figure 5. Correlation between imbibition rate and germination for two cultivars of P. vulgaris at 5° and 20°C. Combined data from different imbibition methods described in text.

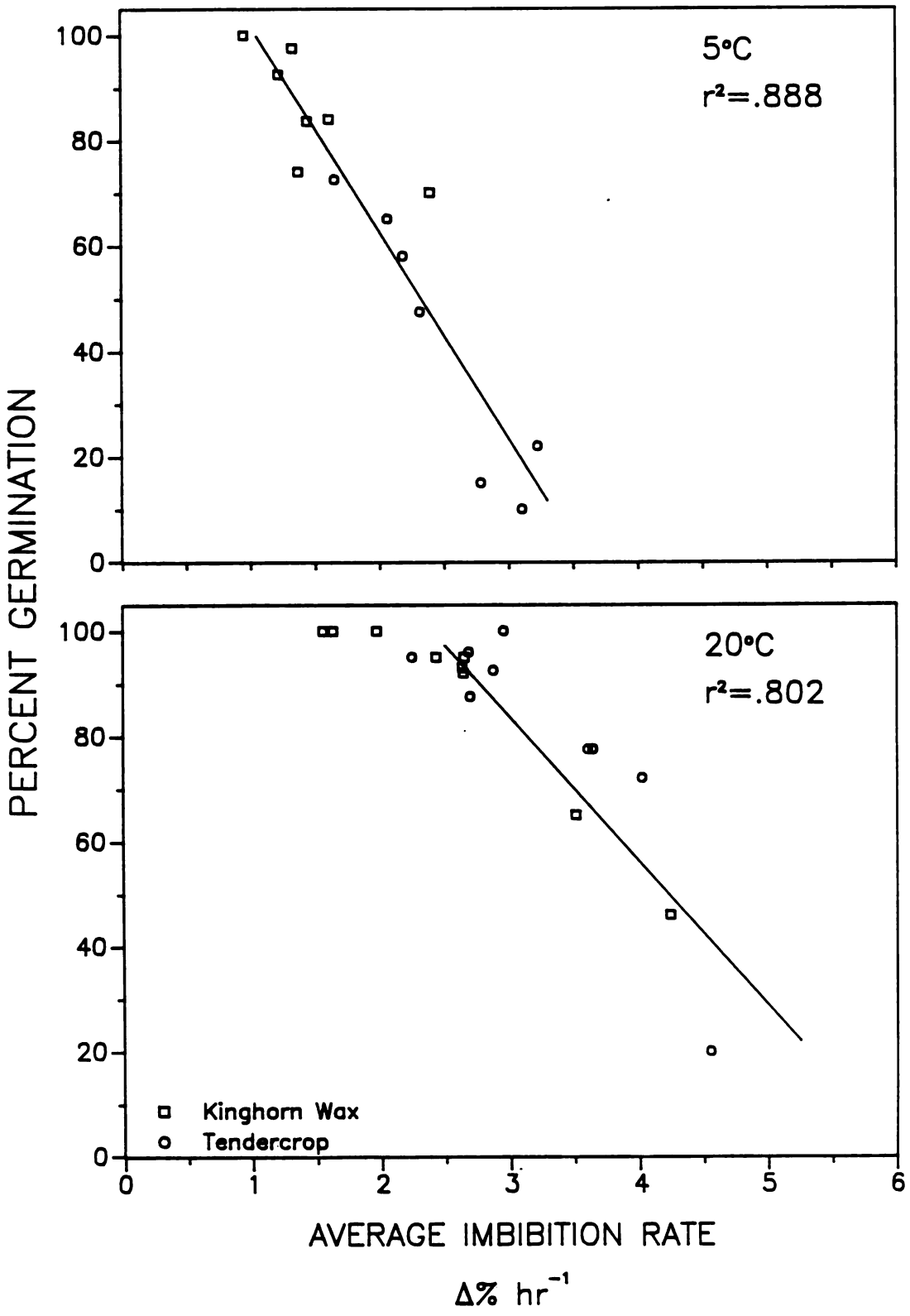

5°C
r²=.888
20°C
r²=.802
PERCENT GERMINATION
AVERAGE IMBIBITION RATE
Δ% hr⁻¹
Kinghorn Wax
Tendercrop
0
20
40
60
80
100
1
2
3
4
5
6

Table 2. Coefficients of determination and rate constants of correlations between germination and imbibition rates at 5° and 20° for two P. vulgaris cultivars, 'Tendercrop' and 'Kinghorn Wax', at 8, 12, and 24 hours of imbibition.

		TENDERCROP		KINGHORN WAX		TC+KW	
Hours	Temp	r^2	slope[a]	r^2	slope	r^2	slope[a]
8	20	.865**	-17.9	.359	-9.0	.628**	-11.8
12	20	.740	-28.2	.956**	-26.0	.755**	-23.2
24	20	.407	-53.4	.502	-32.3	.396	-42.7
8	5	.893**	-32.1	.581	-17.2	.911**	-30.5
12	5	.898**	-41.7	.608	-19.6	.888**	-39.2
24	5	.617	-54.2	.931**	-27.5	.599	-56.6

[a]Slope = % germination·(% moisture $hr)^{-1}$
** P≤0.01

midpoint of imbibition in terms of the total amount of water taken up during the process. The sampling time with the greatest r^2 also represents the time when there is a maximum range in moisture levels of seeds imbibing under the different experimental conditions.

DISCUSSION

The data clearly indicate an inverse relationship between imbibition rate and germination. For each cultivar at a given temperature and imbibition method, germination always decreased when imbibition rates increased above a particular minimum level.

Left unresolved by this study is the determination of a absolute effect of imbibition rate on germination. When the effects of imbibition rate on germination in two different species, peas and soybeans, were compared, Tully et al. found that similar imbibition rates produced similar germination results and implied that imbibition rate is the determining factor of germination at a given temperature. The situation is, however, significantly more complex.

Imbibitional injury increases as initial moisture level decreases. However, in P. vulgaris and most other legume species, decreased initial seed moisture also decreases imbibition rates (11) which serves as protection against low moisture induced imbibitional injury. At equal moisture levels, Kinghorn Wax embryo tissue imbibes more rapidly than

does Tendercrop embryo tissue (15) and yet, because of differences in their responses to low initial moisture, Kinghorn Wax has higher final germination percentages than Tendercrop. The sensitivity of embryo tissue to imbibitional injury as a function of initial moisture content must, therefore, be greater than its sensitivity to the same injury caused by rapid imbibition. Thus, the effects of imbibition rate are relative and may apply only when all other factors are equal.

Two factors limit the extent of interpretation that can be drawn from the present study. First, by using average imbibition rates as the independent variable in the regression analysis the slope of the germination response to imbibition rate will naturally increase (negatively) with time. Thus, it is only valid to compare slopes of the two cultivars at equal sampling times. However, because of the differences in uptake rates by the two cultivars the analyses are significant at different times.

The second confounding factor is the difference in sensitivity to initial moisture level between the two cultivars. As mentioned above, Tendercrop seeds at 10% moisture are approximately as sensitive to imbibitional injury as Kinghorn Wax seeds are at 7% moisture. The work presented here was completed prior to this determination and as a result these differences were not taken into account. An experimental design in which seed embryo tissue and whole

seeds are moisture equilibrated to levels of equal sensitivity and then germinated using the imbibition methods described in this report would not only eliminate this confounding problem but also reveal the specific extent to which the testa serves to protect the embryo as well as relative levels of resistance/sensitivity to the effects of imbibition rate in the embryo tissue itself.

Based on the general conclusion that germination can be increased by reducing the rate of imbibition and that this can be achieved by applying a physical barrier to water entry on the seed such as the wax applied in this study, an attempt was made to identify a compound that might be used for this purpose. In doing so, consideration was given to both ease of application and handling of treated seed so that this process might be applicable at a commercial level. To this end two commercial vegetable waxes with hydrophobic characteristics were obtained from the Decca Tiltbelt division of the Pennwalt Corporation, Monrovia, CA, and used as seed coatings. Neither compound successfully increased germination and further studies in this area were discontinued. However, the potential of increasing germination by the application of a compound to the seed that can retard water entry is apparent. The observation that the beneficial effects are greatest when chilling stress is applied would make the development of such a seed treatment particularly attractive to commercial growers.

Once a seed becomes sufficiently hydrated it gains a significant degree of chilling resistance (16) and such a treatment should help to improve stands of early spring plantings. Based on germination studies of artificially aged soybean axes (17) older seed with decreased germination potential might also benefit from reduced imbibition treatments.

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SECTION II

SEMIHARD SEED, ADHERENCE OF THE TESTA TO THE COTYLEDON AND DIFFERENTIAL SENSITIVITY OF THE AXIS AND COTYLEDON TO IMBIBITIONAL INJURY IN SNAP BEAN (*PHASEOLUS VULGARIS* L.)

ABSTRACT

Imbibitional injury of two snap bean (Phaseolus vulgaris, L.) cultivars, 'Tendercrop'(TC) and 'Kinghorn Wax' (KW), was investigated in terms of seed coat characteristics, initial moisture content and differential sensitivities of the axis and cotyledon. TC, a soft seeded cultivar with a loosely adhering testa, took up water more rapidly than seed tissues imbibed resulting in an accumulation of intercotyledonary water at all initial moisture levels. At initial moisture levels <13% KW, a semihard seeded cultivar with a tightly adhering testa, imbibed slowly with no accumulation of excess water. The tightly adhering testa of KW retarded imbibition of half-seeds imbibing directly through the adaxial cotyledon surface, possibly due to a physical restriction to swelling imposed by the testa. The loosely adhering testa of TC had no effect on imbibition of half-seeds. Axes within whole seeds of both cultivars imbibed more rapidly than cotyledons regardless of imbibition method. Excised axes were more resistant to imbibitional injury than either half-seeds or seeds. Axes excised from seeds after 24 hr imbibed at a chilling temperature of 5° had higher germination than axes left attached to seeds. It was concluded that the cotyledon

is more readily injured by rapid imbibition than the axis. Injury to the cotyledon is deleterious to axis germination An uninjured, fully imbibed axis might confer a degree of protection to the cotyledon. This suggests that the sequence in which embryo tissues imbibe might be a factor in the injury process.

INTRODUCTION

Imbibition of legume seeds has been determined to consist of two components: 1) the initial wetting reaction followed by 2) a flow of water through the wetted seed tissue (Vertucci and Leopold, 1983.) Imbibitional injury in legumes occurs immediately upon the onset of water uptake by the seed. Cells of pea embryo were killed in the first two minutes of imbibition (Powell and Matthews, 1978) and soybean embryos were injured when imbibed at low temperatures for five minutes (Bramalage et al., 1978.) Imbibitional injury in excised lima bean axes at chilling temperatures (Pollock and Toole, 1966) and low vigor soybean axes (Woodstock and Tao, 1981) is largely confined to the first five to ten minutes of imbibition. Within the potential range of resistance to imbibitional injury defined by its genome, a seed's immediate sensitivity is adversely affected by low imbibition temperature (Pollock and Toole, 1966), low initial moisture content (Pollock, 1969), rapid imbibition rate (Wolk, 1988) and low seed vigor (Woodstock and Tao, 1981.) These four factors are related in so far as optimizing any one factor serves to ameliorate, at least in part, the deleterious effects of the other three.

The role of the testa in limiting imbibitional injury is well documented (Duke and Kakefuda, 1981; Larson, 1968; Powell and Matthews, 1979; Tully, et al., 1981.) McCollum (1953) determined that the adherence of the testa to the

cotyledon influenced imbibition rate. Phaseolus vulgaris cultivars with tightly adhering testae imbibe more slowly (Atkin, 1959) and sustain less injury than do cultivars with loosely adhering testae (Powell et al. 1986.) The onset of imbibition is delayed in semihard snap bean seeds of low initial moisture content and these seeds are more resistant to imbibitional chilling injury than are soft seeded cultivars (Taylor and Dickson, 1987). The resistance to imbibitional chilling injury conferred by both tightly adhering testae and the semihard seed characteristic, which appear to be distinct traits, is attributed to the reduction of hydration rate effected by certain physical properties of the seed coat. Priestley and Leopold (1986) have achieved similar results by applying a thin coat of lanolin to reduce the imbibition rates of soybean and cotton seeds.

Whether the cotyledon and axis are equally or differentially sensitive to imbibitional injury is not clear. Cotyledon tissue is more readily injured that the axis when cacao seeds are imbibed at chilling temperatures (Ibanez and Casas, 1963; Ibanez, 1964.) Cacao is, however, a recalcitrant seed and therefore would not pass through an initial wetting phase as would the orthodox legume seeds of interest here. It may be that the injury cacao sustains is more similar to chilling injury in hydrated plant tissues than imbibitional injury. Several authors have utilized embryos, axes or cotyledons in imbibition studies and have

reported a range of initial moisture levels necessary to protect these seed parts from injury (e.g. Bramalage et al., 1978; Pollock, 1969; Simon and Wiebe, 1975; Vertucci and Leopold, 1984.) Differences in sensitivity based on the factors cited above as well as demonstrated species and cultivar differences preclude drawing conclusions as to the relative sensitivities of the cotyledon and axis from such reports.

The objectives of the present study were to determine the relative sensitivities to imbibitional injury of the axis and cotyledon in P. vulgaris seeds and the individual roles of the semihard seed characteristic and tightly adhering testa in the avoidance of such injury.

MATERIALS AND METHODS

Seed Material: Snap bean seeds, Phaseolus vulgaris L, cvs. 'Tendercrop' and 'Kinghorn Wax' (Rogers Brothers Seeds, Twin Falls, ID) with standard germination in excess of 95% were stored at 5°C and 35% R.H. until used. Embryos were prepared by carefully removing the testa with a sharpened spatula blade. A half-seed is designated as a cotyledon with intact testa and attached axis; a half-embryo is a half-seed with the testa removed. The moisture content of seeds used to obtain embryos, half-seeds, half-embryos and excised axes was increased in a humid, sealed chamber for 48 hr at 20°C to facilitate preparation. Moisture content of

seed parts was subsequently re-adjusted. For all experiments the moisture content of seeds and seed tissues was adjusted to desired levels in sealed desiccators over H_2SO_4 solutions at 20°C for 10 days. Moisture content was determined by drying seeds for 48 hr and all other tissues for 24 hr at 95°C in a forced draft drying oven. All moisture contents are expressed on a fresh weight basis.

Imbibition Rates: Imbibition of whole seeds was determined by either submerging individual seeds in 8 ml deionized H_2O or imbibing 10 seeds between two 7 cm Whatman #1 filter paper discs wetted with 5 ml deionized H_2O in 100x15mm petri dishes. Seeds were blotted, weighed and quickly returned at the designated times.

To determine the rates of both water entry into the seed and seed tissue imbibition, pre-weighed seeds of known moisture content were submerged individually in 8 ml deionized water. At ten minute intervals, seeds were blotted, weighed, split open, excess intracotyledonary water blotted and the tissue was re-weighed. Imbibition rates from 10 to 60 min were calculated by linear regression.

To determine the moisture content of the axis relative to the cotyledon during imbibition, whole seeds were imbibed either by submersion or between moistened filter paper discs as described above. At 1, 2, 4, and 8 hr, seeds were removed, blotted dry and the axes excised. Axes and cotyledons were weighed individually, dried and re-weighed.

Imbibition rates of half-seeds and half-embryos were determined by placing 5 each, adaxial side down, on a single filter paper disc wetted with 2.5 ml deionized water in 100x15mm petri dishes. Care was taken to ensure that the testa did not touch the filter paper so that the sites of water uptake were restricted to the embryo tissues. The seed parts were blotted dry, weighed and returned at designated sampling times.

In all cases imbibition was determined with 5 individual seeds or seed parts and data presented are the means of two measurements.

Germination Experiments: Seeds and embryos were imbibed in the dark for 24 hr at either 5° or 20° in 100x15mm petri dishes, 10/dish, between two 7 cm Whatman #1 filter paper discs wetted with 5 ml of 0.15% captan solution as a fungicide. Half-seeds and half-embryos were imbibed on a single filter paper disc wetted with 2.5 ml 0.15% captan solution and otherwise like seeds and embryos. Axes were imbibed like half-seeds and half-embryos using 2.0 ml captan solution. Treatments imbibed at 5° were moved to 20° after 24 hr for germination. To determine the effect of the cotyledon on axis germination axes were excised from whole seeds after 24 hr imbibition at 5° and germinated in petri dishes as described above for axis imbibition. Germination or growth was determined after 5 days at 20°. All germination experiments were replicated 5 times and data

represent the means of two experiments. Bartlett's tests were significant for germination data. Statistical analysis of germination data was performed on arc sin transformed data. Data was analyzed in a completely randomized factorial design. The Student-Newman-Keul's Test was used to separate means.

RESULTS

Imbibition Studies: Illustrative examples of the dynamics of H_2O uptake and tissue imbibition by Tendercrop and Kinghorn Wax seeds at three initial moisture levels are presented in Figure 1. Water uptake and tissue imbibition rates determined by linear regression between 10 and 60 min for the entire range of initial seed moistures studied are plotted in Figure 2 as a function of moisture content. Water was taken up readily by Tendercrop seeds at all moisture levels while uptake by Kinghorn Wax seeds decreased in a linear fashion as initial moisture decreased from $<0.05\%\ min^{-1}$. At lower moisture levels uptake rates gradually diminished until a condition of impermeability was reached 15% to 12%. At 12% moisture water, uptake by Kinghorn Wax was
between 6% to 8% moisture. Both the pattern of imbibition (Holubowicz, 1988) and the reversibility of seed coat permeability (Taylor and Dickson, 1987) of Kinghorn Wax seeds are similar to those described for semihard snap bean

seeds (data not shown.) In addition, the Kinghorn Wax seed coat adheres tightly to the entire cotyledon except in the hilum/micropyle/chalazal region while only a very small portion, if any, of the Tendercrop seed coat adheres to its cotyledon. Water was imbibed into Tendercrop tissues more slowly than it entered the seed over all initial moisture levels tested thereby causing an accumulation of free water between the cotyledons. Water did not accumulate in Kinghorn Wax seeds until the initial moisture level was greater than 13% when the rate of water entry began to exceed tissue imbibition (Fig. 2.) Although a small quantity of free water was removed from lower moisture Kinghorn Wax seeds as indicated by the slight difference between water uptake and tissue imbibition curves in Figure 1, these rates did not actually begin to diverge significantly until moisture became >13% (Fig. 2.) The intercotyledonary water removed when lower moisture (<13%) Kinghorn Wax seeds were split and blotted did not accumulate but rather was water moving from the point of entry to the site where it would be imbibed into the seed tissue. Thus, in lower moisture Kinghorn Wax seeds where rapid imbibition would cause imbibitional injury (Wolk et al., 1988), Kinghorn Wax seeds took up water slowly and did not exceed the rate at which seed tissues imbibed. This was not the case with Tendercrop. Water entered Tendercrop seeds more rapidly than seed tissues could imbibe causing a

Figure 1. Dynamics of water uptake by whole seeds and imbibition by seed tissues of two P. vulgaris cultivars, 'Tendercrop' and 'Kinghorn Wax', at three initial moisture contents. Seeds were submerged and weighed at designated times for water entry. Tissue imbibition was determined by splitting the whole seed and removing excess water. Vertical bars indicate SE of the means.

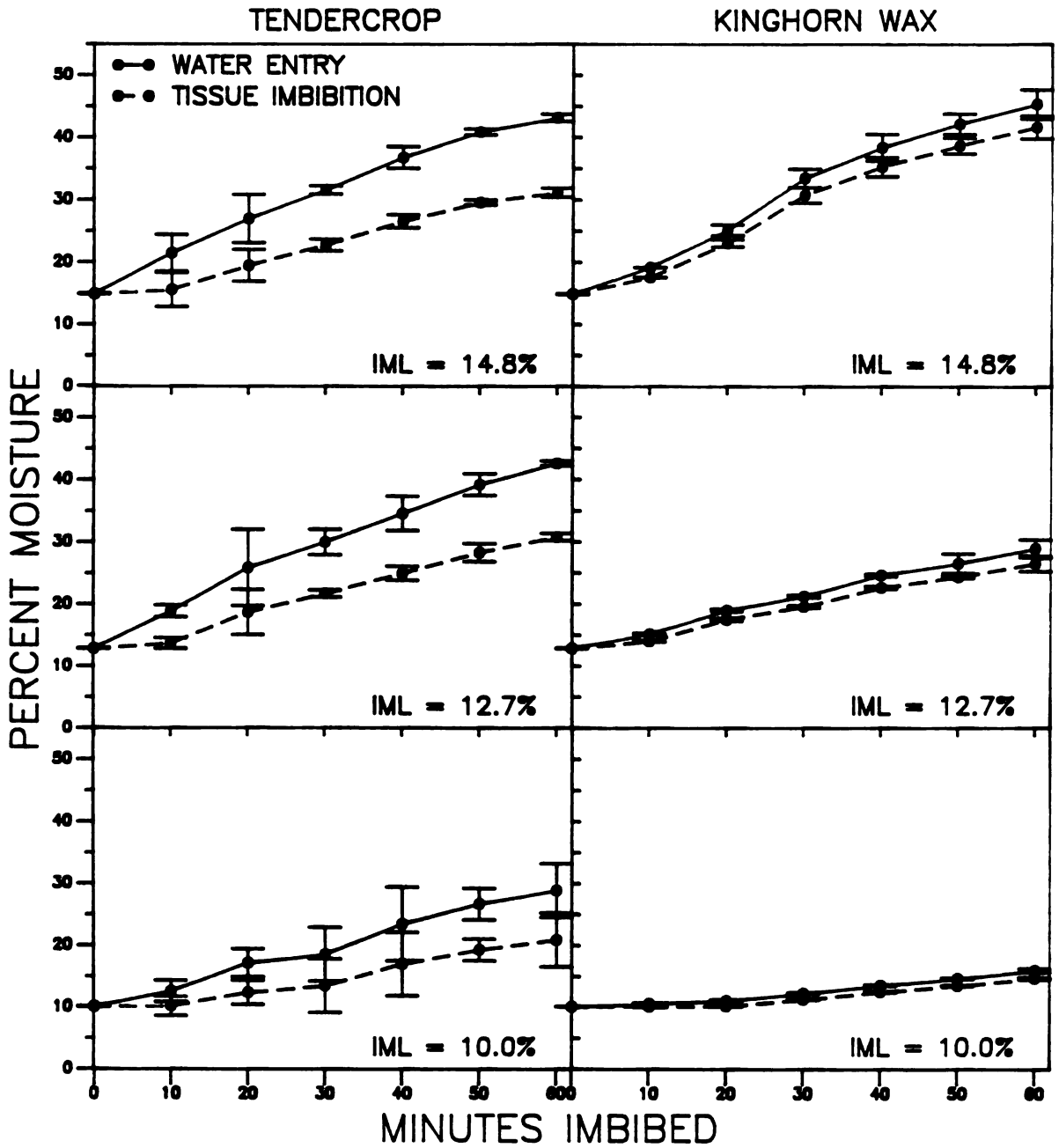
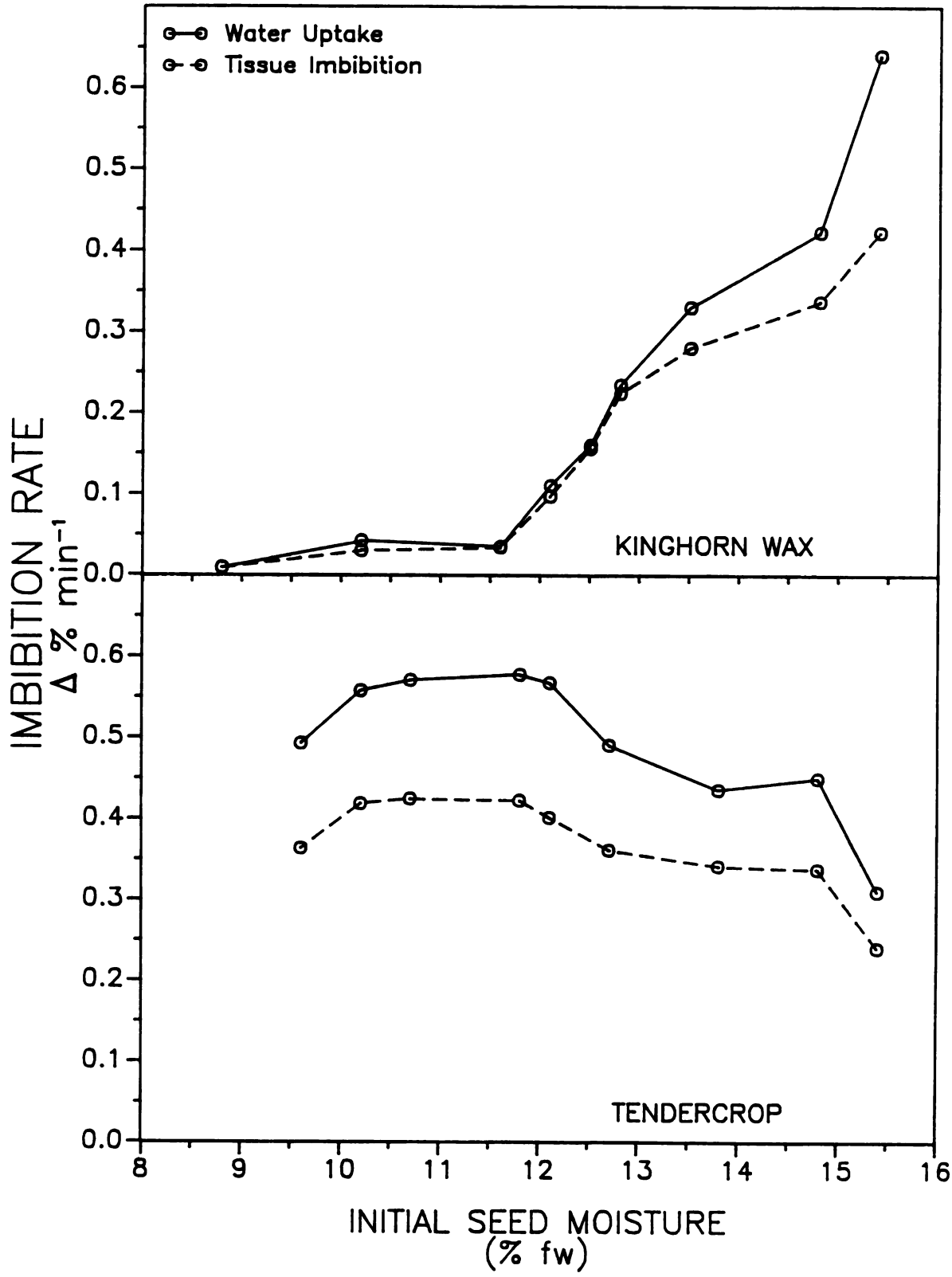

TENDERCROP
KINGHORN WAX
WATER ENTRY
TISSUE IMBIBITION
IML = 14.8%
IML = 14.8%
IML = 12.7%
IML = 12.7%
IML = 10.0%
IML = 10.0%
PERCENT MOISTURE
MINUTES IMBIBED
0
10
20
30
40
50
60

Figure 2. Rates of water uptake and tissue imbibition as a function of initial moisture content. Moisture contents during imbibition determined as described in Figure 1. Rates calculated by linear regression from 10 to 60 min imbibition.


Water Uptake
Tissue Imbibition
KINGHORN WAX
TENDERCROP
IMBIBITION RATE
Δ % min⁻¹
0.0
0.1
0.2
0.3
0.4
0.5
0.6
8
9
10
11
12
13
14
15
16
INITIAL SEED MOISTURE
(% fw)

significant accumulation of water between the cotyledons and allowing rapid tissue imbibition at all moisture levels.

Whole Tendercrop seeds imbibed equally whether they were submerged or between moistened filter papers (Fig. 3.) Kinghorn Wax seeds imbibed more slowly than Tendercrop seeds and imbibition of Kinghorn Wax seeds between moistened filter papers was retarded compared to submerged seeds. For seeds imbibed with either method, a linear relationship existed between axis and cotyledon moisture content during the course of water uptake (Fig. 4.) The slight delay in imbibition of Tendercrop axes relative to cotyledons might be due to the rapidity of water entry into Tendercrop seeds. In all cases when the moisture content of both the axis and cotyledon were increasing the axis imbibed more rapidly. Within the linear portion of the axis/cotyledon moisture relationship, axes imbibed 1.8 to 3.6 times more rapidly than cotyledons and approached full imbibition when the cotyledon was only 50% imbibed. Slopes are greater for the Tendercrop cultivar and for seeds that were submerged.

The testa had no effect on imbibition when Tendercrop half-seeds and half-embryos were imbibed through the adaxial surface on moistened filter paper at 5° and 20°C (Fig. 5.) The presence of the testa did, however, cause Kinghorn Wax half-seeds to imbibe more slowly than half-embryos even though water was taken up directly into the embryo tissue.

Germination: As with whole seeds (Wolk et al., 1988)

Figure 3. Whole seed imbibition by two P. vulgaris cultivars, 'Tendercrop' and 'Kinghorn Wax', at 20° between moistened filter papers or submerged. Seeds (10) were imbibed either between two 7 cm filter paper discs moistened with 5 ml H_2O in 100x15mm petri dishes or submerged individually in 8 ml H_2O. Vertical bars indicate SE of the means.

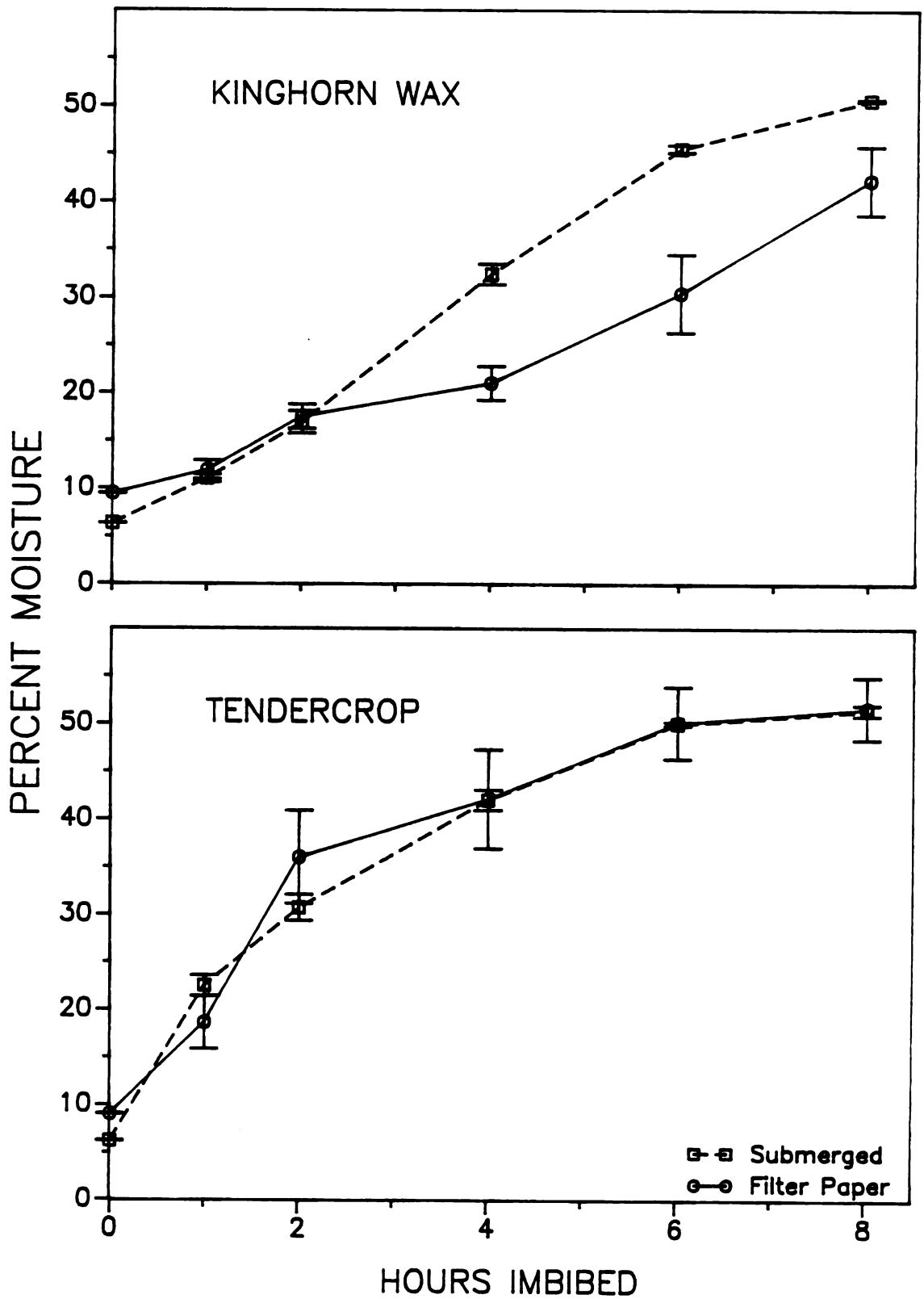

KINGHORN WAX
TENDERCROP
PERCENT MOISTURE
HOURS IMBIBED
0
10
20
30
40
50
0
2
4
6
8
Submerged
Filter Paper

Figure 4. Axis moisture as a function of cotyledon moisture during imbibition by two P. vulgaris cultivars, 'Tendercrop' and Kinghorn Wax'. Seeds were imbibed as with Figure 4. After 1, 2, 4, and 8 hr imbibition the moisture content of axes and cotyledons of individual seeds was determined. Linear regression is for range in which both axis and cotyledon moisture are increasing.

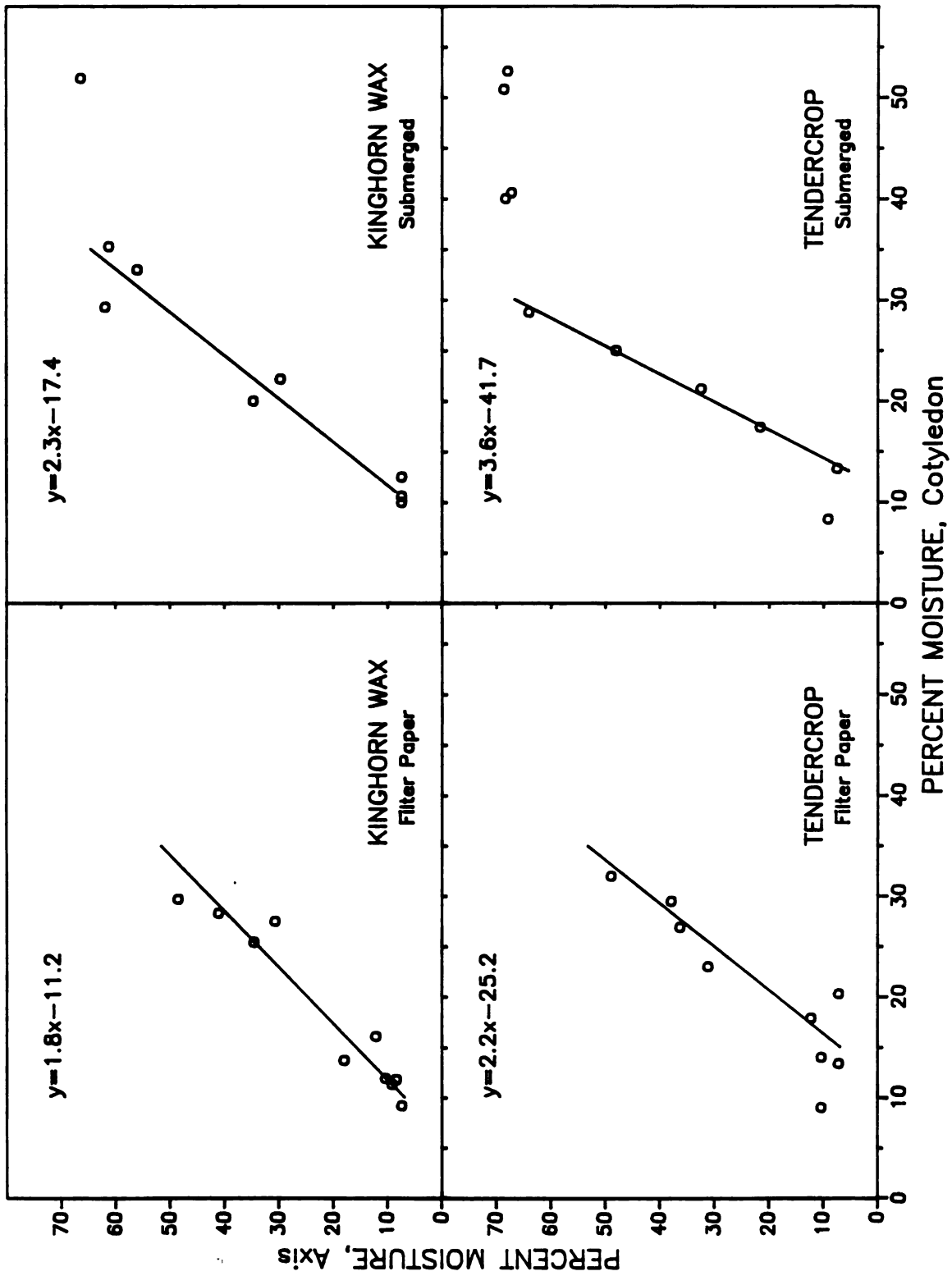
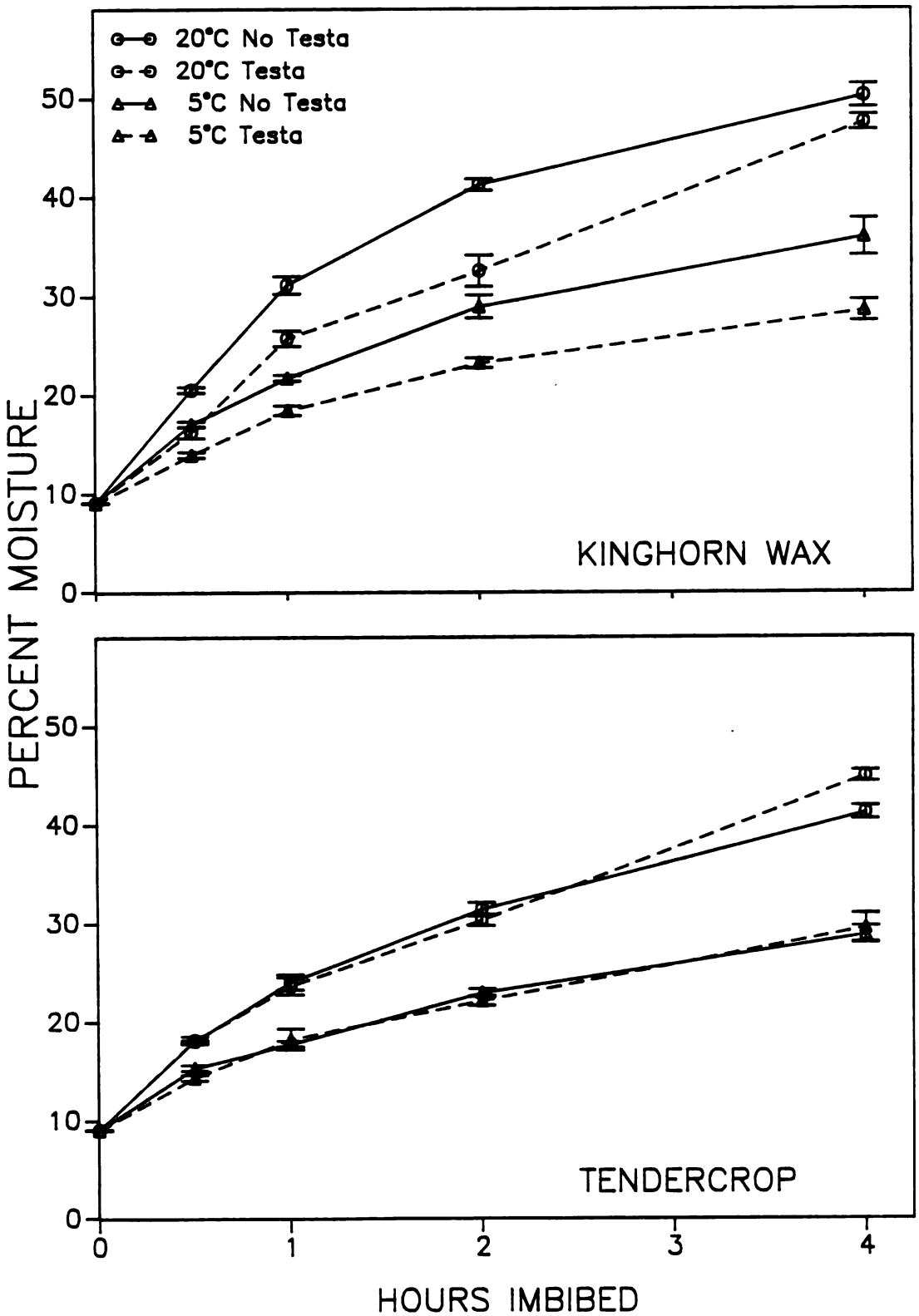

y=1.8x−11.2
KINGHORN WAX
Filter Paper
y=2.3x−17.4
KINGHORN WAX
Submerged
y=2.2x−25.2
TENDERCROP
Filter Paper
y=3.6x−41.7
TENDERCROP
Submerged
PERCENT MOISTURE, Axis
PERCENT MOISTURE, Cotyledon
0
10
20
30
40
50
60
70

Figure 5. Imbibition by cotyledons with attached axes by two P. vulgaris cultivars, 'Tendercrop' and Kinghorn Wax', at 5° and 20° with or without the testa. Cotyledons were imbibed through the adaxial cotyledon surface on 7 cm filter paper discs wetted with 2.5 ml H_2O in 100x15mm petri dishes. Vertical bars indicate SE of the means.


20°C No Testa
20°C Testa
5°C No Testa
5°C Testa
KINGHORN WAX
TENDERCROP
PERCENT MOISTURE
HOURS IMBIBED
0
10
20
30
40
50
0
1
2
3
4

germination of half-seeds decreased once initial moisture content dropped below a critical level (Fig. 6 a and b.) This breakpoint occurred at a higher moisture level for Tendercrop half-seeds than for Kinghorn Wax and at 5° compared to 20°C. Growth (fw/initial dw) of excised axes was relatively constant over all moisture levels at a given temperature at both 5° and 20°C for Kinghorn Wax and at 20° for Tendercrop (Fig. 6 c and d). Twenty-four hours imbibition at 5° decreased the growth of Kinghorn Wax axes equally at all moisture levels so that its 5° and 20° growth curves are approximately parallel. Tendercrop axis growth for 5° chilled axes parallels its 20° growth at higher moisture levels but declined as initial moisture dropped below 13%.

Axis germination was significantly higher than that of seeds of equal initial moisture content when imbibed for 24 hr at 5° (Table 1.) When axes were excised from seeds immediately after 24 hr imbibition at chilling temperatures germination was significantly higher than seed germination except for high moisture Tendercrop seeds where there was no difference. There was a trend for axes excised after chilling to germinate at intermediate levels between seeds and axes excised prior to the onset of imbibition.

The results of experiments in which high and low moisture seeds, embryos, half-seeds and half-embryos were imbibed at 5° or 20° for 24 hr and then germinated at 20°

Figure 6. Germination of half-seeds and growth of axes of two P. vulgaris cultivars, 'Tendercrop' and 'Kinghorn Wax', imbibed for 24 hr at 5° or 20° as a function or initial moisture content. Ten half-seeds (testa covered cotyledon with attached axis) were imbibed and germinated with the adaxial cotyledon surface against a 7 cm filter paper circle wetted with 2.5 ml of 0.15% captan solution in a 100x15mm petri dish. Axes were imbibed similarly using 2 ml 0.15% captan solution. Tissues imbibed at 5° were transferred to 20° after 24 hr for germination. Vertical bars indicate SE of the means.

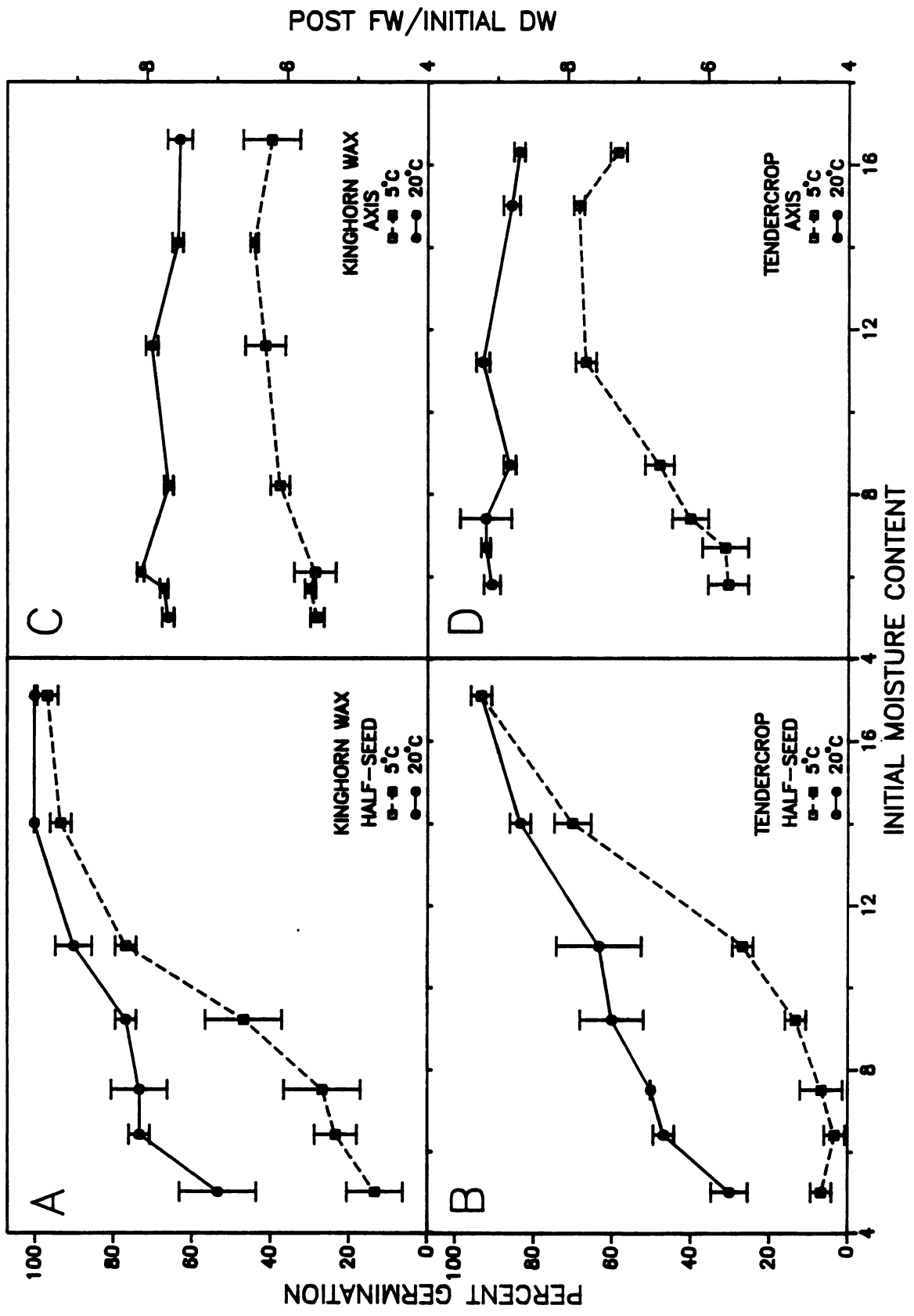

POST FW/INITIAL DW
PERCENT GERMINATION
INITIAL MOISTURE CONTENT
A
B
C
D
KINGHORN WAX
HALF-SEED
5°C
20°C
TENDERCROP
HALF-SEED
KINGHORN WAX
AXIS
TENDERCROP
AXIS

Table 1. The effect of the cotyledon on axis germination during chilling imbibition. Seeds and axes of *P. vulgaris* cultivars 'Tendercrop' (TC) and 'Kinghorn Wax' (KW) with high (13.5%) or low (6.5%) initial moisture content (IMC) were imbibed for 24 hr at 5°. After chilling, axes were excised from half the seeds (post-chill) and all material transferred to 20° for germination. Germination was scored after 5 days at 20°.

		PERCENT GERMINATION		
Cultivar	IMC	Seed	Post Chill	Axis
TC	13.5	84b[z]	82b	98a
KW	13.5	86b	100a	100a
TC	6.5	40e	50d	88b
KW	6.5	70c	96a	98a

[z]Means separated by Student-Newman-Keul, P=.01

Analysis of variance performed on arcsin transformed data.

Table 2. Germination of 'Tendercrop' and 'Kinghorn Wax' seeds (S), embryos (E), half-seeds (S/2) and half-embryos (E/2) at 20° with high (14%) or low (7%) initial moisture content imbibed for 24 hr at 5° or 20°. Seeds and seed parts were imbibed in 100x15mm petri dishes, 10 dish^{-1}. Seeds and embryos were placed between two 7 cm filter paper discs wetted with 5 ml of 0.15% captan solution. Half-seeds and half-embryos were placed adaxial side down on a single 7 cm filter paper circle wetted with 2.5 ml of captan solution. All 5° treatments were moved to 20° after 24 hr. Germination was scored after 5 days at 20°.

	TENDERCROP				KINGHORN WAX			
	PERCENT GERMINATION							
	HIGH		LOW		HIGH		LOW	
	20	5	20	5	20	5	20	5
S	100a[z]	85b	70c	35de	100a	99a	94a	58cd
E	85b	5f	10f	0f	100a	96a	46d	3f
S/2	100a	68c	54d	8f	100a	100a	93a	46d
E/2	98a	69c	41d	1f	100a	88b	55cd	8f

[z]Means separated by Student-Newman-Keul P=.01

Analysis of variance performed on arcsin transformed data.

are presented in Table 2. For these experiments the four way interaction of cultivar x initial moisture level x imbibition temperature x seed part germinated was significant. Within each treatment combination of cv x moisture x temperature whole seeds germinated as well as, or significantly higher than, any of the seed parts and, with the exception of chilled high moisture Kinghorn Wax, embryos had as low or lower germination than seeds or other seed parts. Tendercrop half-embryos germinated significantly higher than embryos except for the 5° low moisture treatment. There were no significant differences in germination between Tendercrop half-seeds and half-embryos. Kinghorn Wax half-seeds germinated equally as well as seeds and significantly higher than half-embryos except at the 20° high moisture treatment where both had 100% germination. Kinghorn Wax half-embryos germinated similarly to Kinghorn Wax embryos except for the 5° low moisture treatment.

DISCUSSION

Kinghorn Wax seeds and seed parts are more resistant to imbibitional injury than those of Tendercrop. Three factors appear to contribute to its higher level of resistance, two of which involve reduction of imbibition rate. First, a retarded rate of water entry into the seed prohibits an accumulation of intracotyledonary water (Figs. 1 and 2), a potentially lethal condition that can lead to anoxia of the

axis (Orphanos and Heydecker, 1968.) Tendercrop seeds imbibed equally either when submerged or between moistened filter papers (Fig. 3.) Thus a Tendercrop seed does not have to be submerged in order to accumulate excess water. This level of protection exhibited by Kinghorn Wax can be attributed to its semihard seed characteristic in which initial water entry occurs primarily through the raphe and chalazal end and migrates along the midline toward the seed's chalazal end (Holubowicz et al., 1988) away from immediate access to the intercotyledonary cavity. In addition, Kinghorn Wax embryo tissue imbibed more rapidly than Tendercrop's (Wolk et al., 1988) so that it accommodated a more rapid uptake without accumulating excess water. Second, the adherence of the Kinghorn Wax testa to the cotyledon reduced imbibition rate in a way that is distinct from the semihard seed mechanism as its presence reduced imbibition even when the embryo tissue and not the testa was in direct contact with water (Fig. 5.) The adherence of the testa to the cotyledon might provide a physical resistance to swelling which would reduce imbibition rate. At moisture levels where the semihard seed condition exists most of the testa is impermeable to water and can only become wetted and expand to accommodate seed swelling from its underside by water migrating along the surface of the cotyledon. Once hydrated, the Kinghorn Wax testa no longer adheres to the cotyledon. Third, Kinghorn

Wax's embryo tissue is inherently more resistant to imbibitional injury than Tendercrop's as evidenced by higher germination of its embryos and axes (Table 2.) The basis for this resistance is not known but may involve tissue water binding characteristics (Vertucci and Leopold, 1984; Wolk et al., in press.)

The more rapid imbibition of the axis tissue relative to that of the cotyledon (Fig. 4) may be due in part to their respective sizes. The axis and cotyledon constitute 2% and 98%, respectively, of the embryo's dry weight (Powrie et al., 1960.) The axis tissue may imbibe rapidly because of its position relative to the site of water entry as does that portion of the cotyledon in direct contact with incoming water. Powell and Matthews (1978) reported that the outer layers of cotyledon cells were killed by rapid imbibition at low initial moisture levels while inner tissues remained viable. Rapid imbibition at 20°, however, did not injure axes even at extremely low initial moisture levels (Fig. 6 c and d.) Over the same moisture range half-seeds showed significant injury. This difference can be ascribed to the presence of the cotyledon. This is further borne out when axes that were subjected to imbibitional chilling while attached to the cotyledon tended toward higher germination when they were excised after the chilling imbibition than those left attached to the cotyledon (Table 1.)

The data in Table 2 can be rationalized knowing that the tightly adhering testa of Kinghorn Wax retards imbibition rate in half-seeds and that the cotyledon is more easily injured than the axis. For embryos, half-seeds and half-embryos the embryo tissue imbibes water directly and thus rapidly without the direct effect of the testa. In the case of half-seeds and half-embryos, both the axis and adaxial cotyledon surface were in direct contact with water. In embryos the abaxial cotyledon surfaces imbibed water directly while the axis imbibed slowly as the wetting front moved from the cotyledon to the axis. There was a clear trend toward higher germination when imbibition took place through the axis/adaxial cotyledon surface of half-seeds and half-embryos, than through the abaxial cotyledon surfaces of whole embryos. This supports the conclusion drawn from Table 1 that injury sustained by the cotyledon had a deleterious effect on the axis. In germinated embryos, half-seeds and half-embryos a large distal portion of the cotyledon was frequently killed and only a small region in the immediate vicinity of the axis remained viable. It is possible that the presence of an uninjured axis might be beneficial to the cotyledon. If so, the sequence in which different seed parts imbibe could be a factor in the injury process.

Tendercrop half-seeds and half-embryos germinated equally while Kinghorn Wax half-seed germination was similar

to that of Kinghorn Wax whole seeds. This is consistent with the imbibition dynamics of each. There are no differences between imbibition of Tendercrop half-seeds and half-embryos (Fig.3.) The tightly adhering testa of Kinghorn Wax causes half-seeds to imbibe more slowly than half-embryos and therefore have greater resistance to imbibitional injury than half-embryos. The protection afforded by this characteristic appears to be quite substantial particularly in offsetting injury due to low initial moisture content.

The semihard seed characteristic delayed the onset of imbibition but did not appear to actually retard imbibition rate once water uptake was initiated (Taylor and Dickson, 1987.) Semihard seeds rehydrated as rapidly as soft seeded cultivars once they began to imbibe. Taylor and Dickson (1987) suggested that an increase in moisture content of embryo tissue by movement of water vapor from the environment might be partially responsible for the heightened resistance to imbibitional injury of semihard seeds. Our data do not indicate any significant increase in the moisture content of Kinghorn Wax seeds in which imbibition was delayed for 8 hr. Powell and Matthews (1978) suggested that the injury to imbibing seeds was caused by imbibition per se and not by low temperature. Their hypothesis was supported by the correlation of initial moisture content and germination of whole Tendercrop and

Kinghorn Wax seeds at 5° and 20° (Wolk et al, 1988). In experiments reported here, axes and half-seeds were clearly injured by low temperature (Fig. 6.) P. vulgaris is a chilling sensitive plant (Wilson, 1974.) Because the embryo tissues of axes and half-seeds imbibe much more rapidly than those of whole seeds they are fully hydrated for at least 12 hr of the 24 hr chilling treatment. Christiansen (1967) observed two periods of chilling sensitivity in cotton seeds. The first coincided with the onset of imbibition and the second after 18 hr of imbibition. Pollock (1969) reported that the resistance to imbibitional chilling conferred by high initial moisture content to Phaseolus lunatus axes dissipated after 24 hr at 5° and suggested that the imbibed axes were chill injured in the manner of fully hydrated tissues. We concur and offer a similar explanation for the decreased growth of high moisture axes imbibed at 5° for 24 hr and for the steeper germination slope of half-seeds imbibed at 5° compared to those imbibed at 20°.

We have shown that maximum rates of water entry into Tendercrop seeds, a soft seeded cultivar with a loosely adhering testa, can exceed the rate at which it is imbibed into the seed's tissues and lead to an accumulation of intercotyledonary water. Kinghorn Wax seeds have both the semihard seed characteristic and a tightly adhering testa. Water entry into Kinghorn Wax seeds with initial moisture contents <13% is slow and equal to the seed tissue

imbibition. As a result, throughout the range of initial moisture levels where rapid imbibition could be injurious, Kinghorn Wax seeds imbibe slowly and do not accumulate intercotyledonary water. We suggest Kinghorn Wax's tightly adhering testa might provide a physical resistance to swelling that causes a reduction in imbibition even when water is taken up directly into embryo tissue. Within whole seeds of both cultivars axes take up water more rapidly yet are more resistant to imbibitional injury than cotyledons. The injured cotyledon is deleterious to germination of the axis. The presence of an uninjured axis appears to protect the cotyledon tissue in its immediate vicinity from injury. The sequence in which seed parts rehydrate during imbibition may be a determinant in imbibitional injury. Fully hydrated half-seeds and axes are chill injured during a 24 hr exposure to 5°.

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SECTION III

DYNAMICS OF IMBIBITION IN PHASEOLUS VULGARIS L. IN RELATION TO INITIAL SEED MOISTURE CONTENT

ABSTRACT

The seed moisture level marking the onset of imbibitional injury (breakpoint) was determined for two cultivars of Phaseolus vulgaris L. cvs. 'Tendercrop'(TC) and 'Kinghorn Wax'(KW). At 20°C the breakpoints were 0.15 g H_2O/gdw (g/g) for TC and 0.11 g/g for KW. When seeds were imbibed at 5°C the breakpoints were 0.19 g/g (TC) and 0.16 g/g (KW). Below the breakpoint germination changed 4.6%/0.01 g/g for all treatments. Imbibition rates were maximal at 0.07 g/g and 0.33 g/g after 20 min imbibition. Rates of electrolyte leakage were correlated with the imbibition rate maximum at 0.07 g/g but were unaffected by the maximum at 0.33 g/g. The transition from tightly bound to semi-bound water occurred at 0.09 g/g and 0.11 g/g for KW and TC, respectively. T1 values increased exponentially as seed moisture decreased from 0.47 g/g to 0.05 g/g. ^{13}C-NMR sugar signals increased at moisture levels above 0.14 g/g and plateaued at approximately 0.33 g/g seed moisture.

These results suggest that the breakpoint moisture level is a function of temperature while the injury process is similar at both 5° and 20°C. Imbibition and leakage rate maxima reflect transitions in the states of seed water. NMR data support the application of the Water Replacement

Hypothesis to seeds.

INTRODUCTION

Orthodox seeds generally pass through a period of desiccation as the final stage of their development. Desiccation not only provides a mechanism for seeds to survive periods unfavorable for germination but is also a necessary step in redirecting metabolic pathways away from seed development and toward those for germination and growth [8]. Seeds of low moisture content, however, can be injured during the early stages of imbibition [17]. Imbibitional injury has been attributed to, at least in part, the disruption of cell membranes during the initial phase of rehydration and is characterized by a rapid loss of intracellular constituents [9,13,18].

Chilling temperatures [17] and rapid imbibition rates [25] can interact with low seed moisture and result in greater injury. Increasing seed moisture content by exposure to humidified air prior to imbibition of liquid water [17] or reducing imbibition rate [19] reduces or completely avoids imbibitional injury. The rapidity with which seeds are injured during imbibition suggests that the injury is sustained during the early wetting of seed tissue [17].

Imbibitional processes and injury in soybeans have been correlated with transitions in states of bound water in seed

tissue [24]. It has been hypothesized that sucrose, in the presence of raffinose, might serve as a substitute for water at low seed moistures [14] in the same way trehalose does in certain animal systems [5]. Recent evidence indicates that transition temperatures and lamellar repeat spacings of dry phospholipid/sucrose mixtures are similar to those of hydrated phospholipids and that sucrose plus raffinose protect membrane vesicles from disruption by drying [4].

In the present study we have measured germination and five parameters related to seed water as a function of initial moisture content in Phaseolus vulgaris, L. seeds and describe how each reflects hydration and injury processes.

MATERIALS AND METHODS

Seed Material. Phaseolus vulgaris L. seeds cvs. 'Tendercrop' and 'Kinghorn Wax' (Rogers Bros. Seeds, Twin Falls, ID) with standard germination in excess of 90% were stored at 35% RH and 5°C until used. Seeds in all experiments were pre-sorted by hand and excessively small, large, discolored and damaged seeds were discarded. Cotyledons were prepared by carefully peeling away the testa and removing the axis. All cotyledons used in imbibition and electrolyte leakage studies were ±25% of mean cotyledon weight within a given experiment.

Moisture Adjustment. Seed and cotyledon moisture contents were adjusted to desired levels in sealed, airtight

containers over various hygrostatic compounds, saturated salt solutions [26,27] or deionized water. For germination experiments seeds were held in desiccators over a series of H_2SO_4 solutions for 14 days or over H_2O for 8 days at 20°C. Cotyledons in spin-lattice relaxation (T1), leakage and imbibition studies and whole seeds used in NMR sugar analyses were suspended in nylon mesh bags from the lids of 500 ml jars over saturated salt solutions or concentrated acids for 14 days or over H_2O for 8 days at 20°C. Cotyledons used to determine the enthalpies of hydration were added singly into pre-weighed 1 ml polypropylene vials and placed on a plastic shelf in 500 ml jars over solutions as described above. Moisture contents of cotyledons used in enthalpy studies were first lowered to <0.04 g/g and then equilibrated over a period of 3 to 4 weeks at 20°±1° and 5°±1°C.

For all experiments, moisture content was determined by drying seeds for 48 hr or cotyledons for 24 hr at 95°C in a forced draft drying oven. All moisture contents are expressed on a g H_2O/gdw basis (g/g). Moisture contents cited from other sources have been converted to a g/g basis for comparative purposes.

Germination. Seeds were germinated in darkness in 15x100 mm petri dishes, 10 seeds per dish, between two 7.0 cm Whatman #1 filter paper discs wetted with 5 ml of 0.15% (w/v) captan solution. Dishes were held for 7 days at 20°C

or at 5°C for 24 hr followed by 7 days at 20°C. Additional captan solution was added as needed. Germination was scored as the protrusion and elongation of the radicle after 7 days at 20°C and expressed as percent germinated. Each point in Figure 1 is the mean germination from 5 dishes.

Imbibition. Weighed cotyledons of a known moisture content were submerged singly in 8 ml double deionized H_2O. Cotyledons were removed, blotted dry with tissue paper, weighed and resubmerged at 10 min intervals for 60 min. Imbibition rates are expressed as g/g min^{-1}. Five replicates were used in each experiment and data presented are the means of 3 experiments.

Electrolyte Leakage. Electrolyte leakage was measured by the increase in conductance of steep water with an ASAC-1000 Automated Seed Analyzer (Neogen Corporation, Lansing, MI.) Ten individually weighed cotyledons of known moisture content were submerged singly into cells of a seed analyzing tray containing 4 ml double deionized H_2O. Conductance was measured at 10 min intervals from 10 to 100 min. Leakage rates are expressed as μA gdw^{-1} min^{-1} from 20 to 70 min. Data presented are the means of 2 experiments.

Enthalpies of Hydration. The enthalpy of water binding was determined using a slight modification of the method described by Vertucci and Leopold [24]. Five cotyledons were brought to moisture equilibrium at 5°±1°C and 20°±1°C as described above. Water activities of equilibration

atmospheres were measured with a Model 911 Dew All Humidity Analyzer chilled mirror hygrometer (EG&G, Waltham, MS.) Sorption isotherms of equilibration data were used to calculate BET model parameters [3] and the differential enthalpies of hydration according to a form of the Clausis-Calpeyron equation

$$H=[RT_1T_2/(T_2-T_1)]\ \ln(aw_1/aw_2)$$

where aw_1 is the water activity at T_1 (°K) that renders the same cotyledon moisture content as does aw_2 at T_2 and R is the gas constant. Data presented are the means of 2 experiments.

NMR Experiments. For proton experiments seeds were sectioned and placed in a 5mm NMR tube. The tube was hydraulically inserted into a 20mm variable frequency probe in the center of a superconducting magnet. The magnet was interfaced with a Bruker AM400 spectrometer with a proton frequency of 400.13 MHz. Each collection used 2048 data points with a sweep width of 100,000 Hz. This minimized the acquisition time to 0.01 sec and minimized the delay during T1 measurements. The 90° pulse of 22 μsec was used. T1 was measured using progressive saturation. Five dummy scans preceded each collection pulse. The delay between pulses was progressively reduced: 15, 3, 1, 0.6, 0.3, 0.1, and 0.0001 seconds. Each free induction decay (FID) consisted of a single collection. These were Fourier transformed after a line broadening of 2 Hz. The peak heights were

fitted to the equation

$$M(t) = M_0 - M_0 \exp(-t/T1)$$

where M(t) is the peak intensity for the delay t, M_0 is the maximum intensity after full relaxation and T1 is the time constant of that recovery. T1 was calculated using a least squares fit of all the delays. No T1 exceeded 1 sec in these experiments. Thus, 15 sec far exceeds 5x T1, so these spectra are presumably fully relaxed. Addition of water confirmed that the major peak consists of the protons from H_2O.

For carbon experiments 15 to 20 seeds were placed in a 20mm NMR tube, entirely spanning the carbon receiver coil. The tube was placed in the magnet and the spectrometer tuned to 100.16 MHz. Two thousand data points were collected over a 50,000 Hz sweep width, using a pulse width of 35 μsec, an 85° pulse. The acquisition time was 0.02 sec with a recovery delay of 0.18 sec. Preliminary experiments found the T1 to be about 70 msec, for a total delay time of about 3x T1. The 85° pulse is the optimum signal-to-noise pulse under these conditions. Eight hundred FIDs were collected and a line broadening of 200 Hz applied before Fourier transformation. Peaks were identified by the addition of known compounds. Resolution can only identify peaks by their major subgroup, $-CH_2-$ for oils and -CH(OH)- for sugars. Maximum peak heights in the areas of interest were used to estimate relative amounts of oils and sugars.

RESULTS

Germination. Both 'Tendercrop' and 'Kinghorn Wax' have a critical initial moisture content below which germination declines linearly as the moisture content decreases. (Fig.1) For seeds germinated at 20°C these points were 0.11 g/g and 0.15 g/g

Kinghorn Wax and Tendercrop, respectively. Twenty-four hr imbibition at 5°C shifted the breakpoint of Kinghorn Wax to 0.16 g/g and Tendercrop to 0.19 g/g. At moisture levels higher than the breakpoint, Kinghorn Wax had a germination rate of 100% at both 20°C and 5°C while Tendercrop germinated at 97% and 93% at the two temperatures. The slope of the decline in germination as initial seed moisture decreased was approximately 4.6% per 0.01 g/g for both cultivars at both temperature treatments.

Imbibition Rates. Imbibition data is presented in Figure 2. From 0 to 10 min imbibition rates were inversely proportional to initial moisture content (Fig. 2A). After a short period imbibition rates declined in such a way that a maximum appeared for each cultivar at approximately 0.33 g/g (Fig. 2B). Later a second maximum appeared at 0.06 g/g to 0.07 g/g (Fig. 2C). During the course of imbibition the maximum first appearing at 0.33 g/g gradually shifted to lower moisture levels as imbibition rates on the high moisture side of the peak slowly declined (compare Figs. 2B and 2C). The lower moisture maximum and the minimum between

the two peaks, at 0.1 g/g for Kinghorn Wax and 0.17 g/g for Tendercrop, remained fixed throughout the 50 min period.

Electrolyte Leakage. Electrolyte leakage for individual cotyledons followed a pattern similar to imbibition. After an initial 20 min period of rapid leakage electrolyte loss declined and became constant for more than 90 min. When plotted as a function of initial moisture content rates of electrolyte loss from 20 to 70 min were maximal for Kinghorn Wax at 0.07 g/g and 0.09 g/g for Tendercrop (Fig. 3).

Enthalpy. Differential enthalpies of water binding are plotted in Figure 4. Enthalpy values determined for P. vulgaris cotyledons corresponded closely to those for soybean seed tissue [24] and to hydration of globular proteins [20]. The large negative enthalpy peak at 0.07 g/g (Fig. 4) occurs as the final charged sites having the highest affinity for water are filled during hydration [1]. Moisture contents at which primary binding sites for water are completely filled were approximately 0.9 g/g and 0.11 g/g for Kinghorn Wax and Tendercrop, respectively. BET Vm values [3], determined by Pauling [16] as the point at which primary binding sites become saturated, were 0.07 g/g for both Tendercrop and Kinghorn Wax. Between 0.11 /g/ and 0.21 g/g enthalpy values were generally <-1 kcal/mol and are indicative of semi-bound water attracted to polar sites on macromolecular surfaces [20]. Above 0.21 g/g enthalpy

Figure 1. The effect of initial seed moisture on the germination of two cultivars of P. vulgaris. Seeds were germinated at 20°C continually (20°) or at 5°C for 24 hr and then moved to 20°C (5°). Moisture contents at intercepts, determined by least squares regression, for 20°C and 5°C, respectively are: Tendercrop 0.15 and 0.19 g/g, Kinghorn Wax 0.11 and 0.16 g/g.

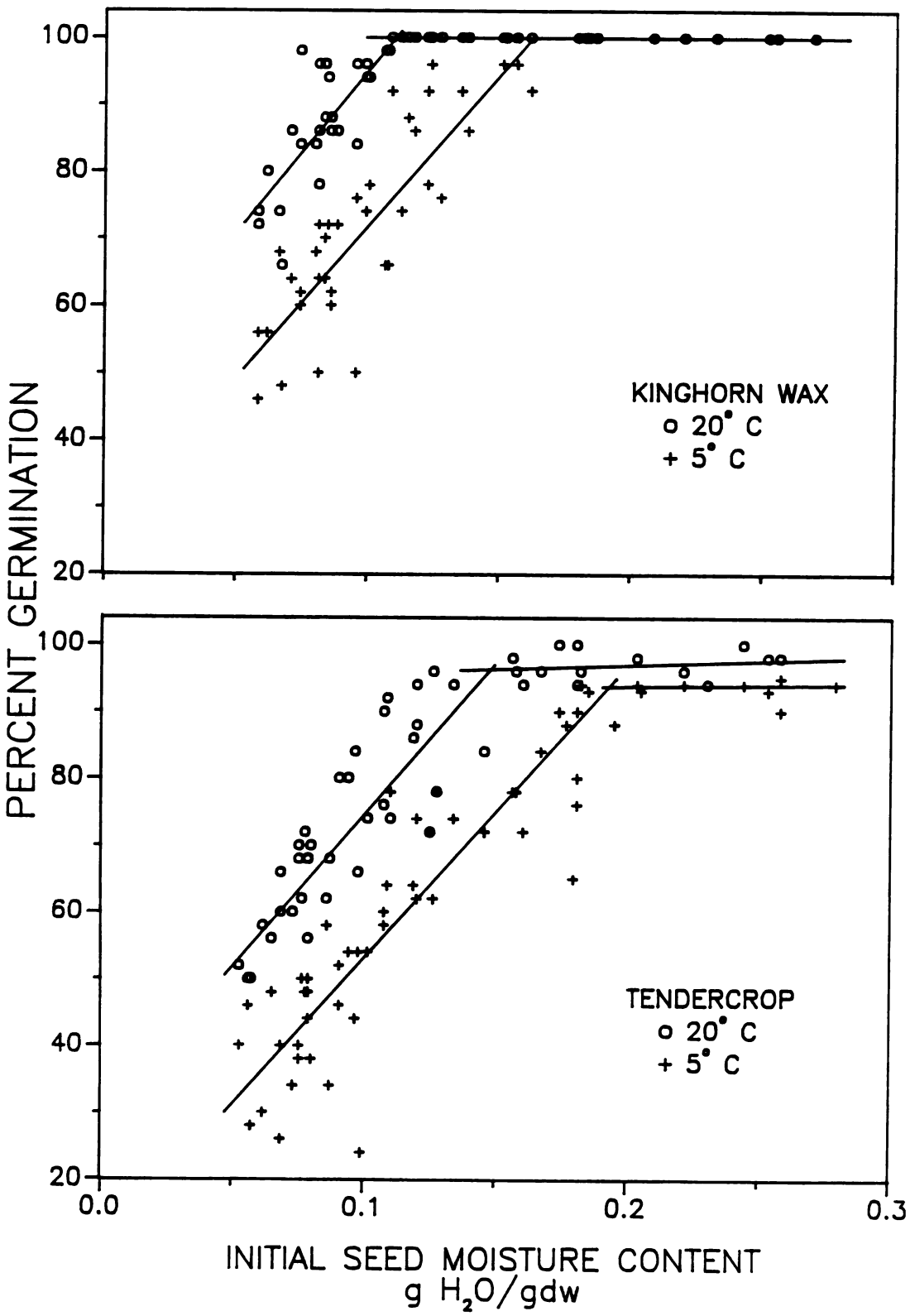

PERCENT GERMINATION
100
80
60
40
20
KINGHORN WAX
o 20° C
+ 5° C
TENDERCROP
o 20° C
+ 5° C
0.0
0.1
0.2
0.3
INITIAL SEED MOISTURE CONTENT
g H2O/gdw

Figure 2. Effect of initial moisture on imbibition rates of Tendercrop (TC) and Kinghorn Wax (KW) (P. vulgaris) cotyledons at 20°C. Average imbibition rates from: A. 0-10 min B. 10-20 min and C. 40-60 min.

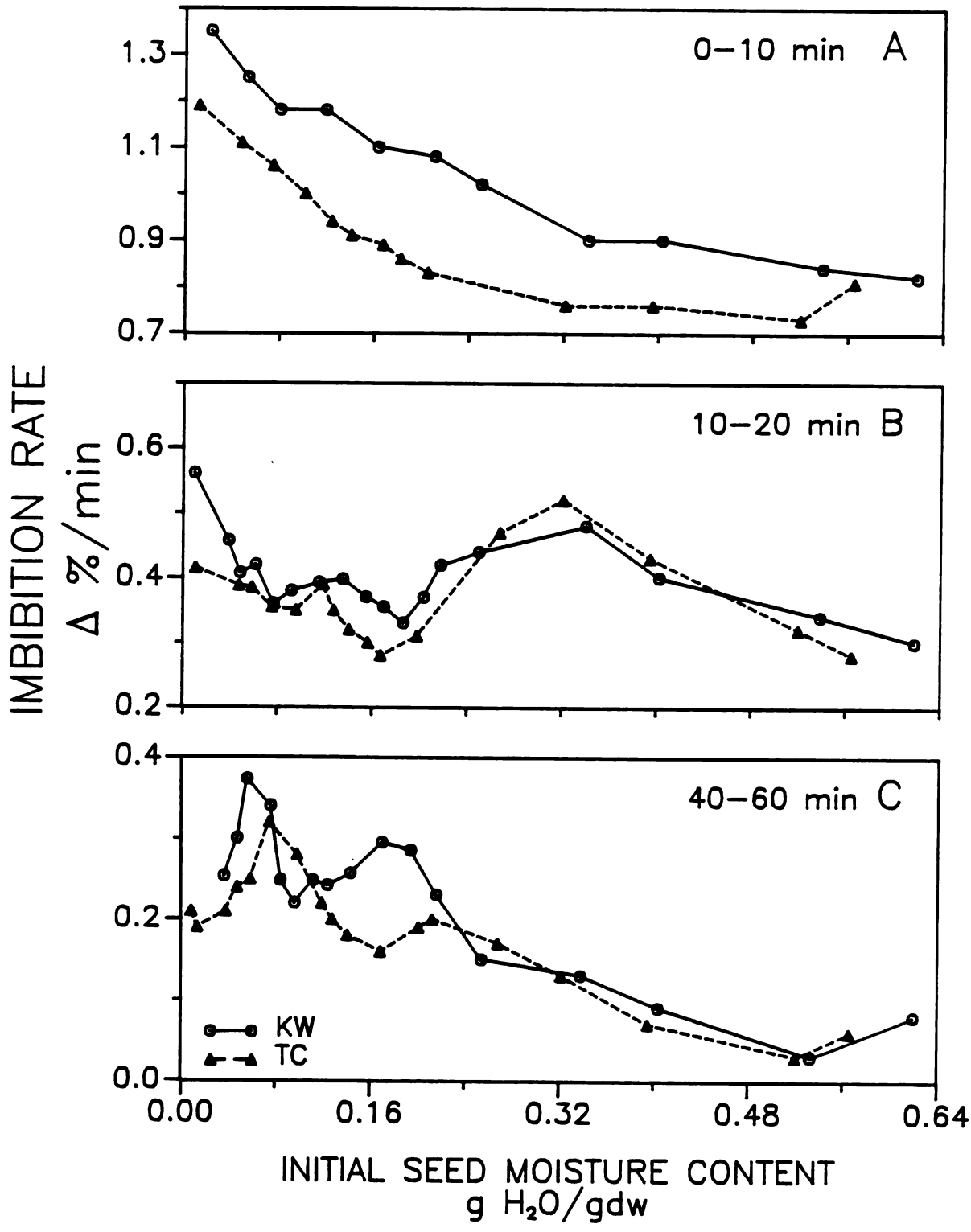

0–10 min A
10–20 min B
40–60 min C
KW
TC
IMBIBITION RATE
Δ %/min
1.3
1.1
0.9
0.7
0.6
0.4
0.2
0.4
0.2
0.0
0.00
0.16
0.32
0.48
0.64
INITIAL SEED MOISTURE CONTENT
g H₂O/gdw

Figure 3. Effect of initial moisture content on rates of electrolyte leakage between 20 and 70 min imbibition from cotyledons of two P. vulgaris cultivars at 20°C. TC=Tendercrop and KW=Kinghorn Wax.

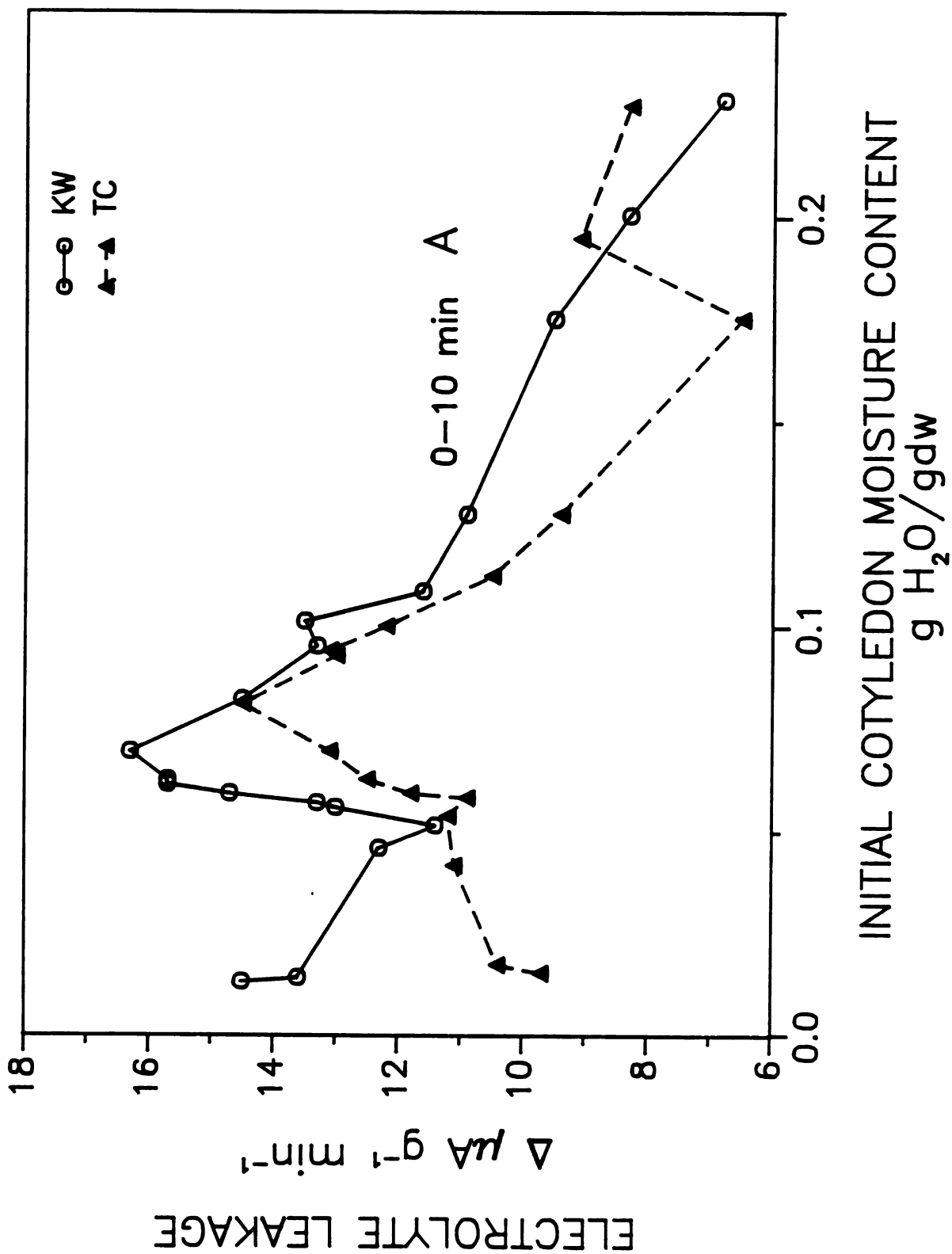
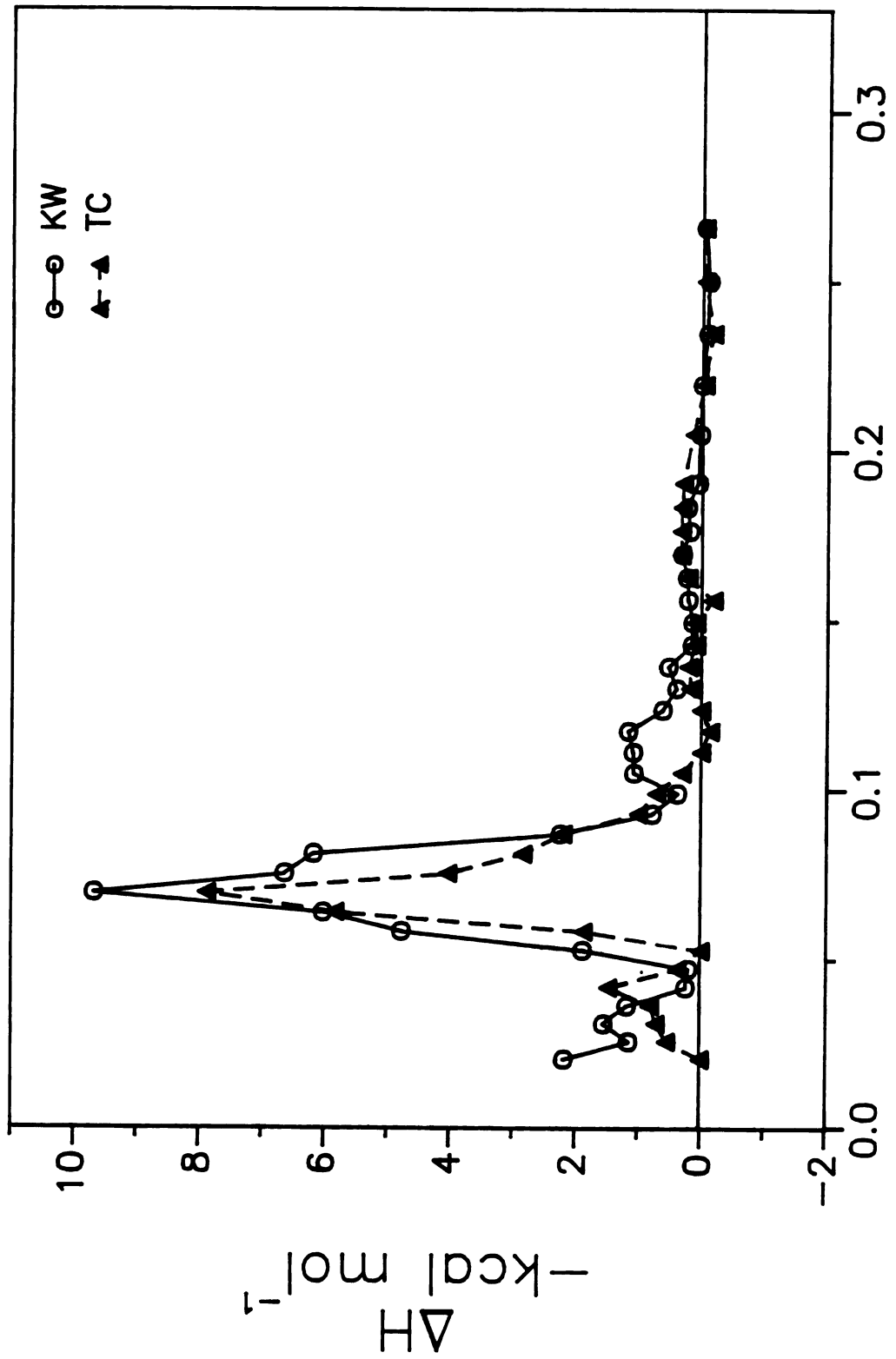

ELECTROLYTE LEAKAGE
Δ μA g⁻¹ min⁻¹
18
16
14
12
10
8
6
0.0
0.1
0.2
INITIAL COTYLEDON MOISTURE CONTENT
g H2O/gdw
0–10 min A
KW
TC

Figure 4. Differential enthalpies of water binding of *P. vulgaris* cotyledons at different moisture contents. TC=Tendercrop and KW=Kinghorn Wax.


KW
TC
10
8
6
4
2
0
−2
0.0
0.1
0.2
0.3
ΔH
−kcal mol⁻¹
COTYLEDON MOISTURE CONTENT
g H₂O/gdw

values were close to zero. At these moisture levels water is loosely bound, covering nonpolar surface areas [20]. NMR Experiments. The relaxation times of water protons increased sharply when seeds were hydrated from 0.03 to 0.05 g/g (Fig. 5). As moisture content continued to increase above 0.05 g/g T1 values decreased exponentially through the highest moisture levels attainable with humidified air (ca. 0.52 g/g). Representative ^{13}C-NMR spectra at two seed moisture levels, 0.18 g/g and 0.05 g/g, are presented in Figure 6. The sugar signals in ^{13}C-NMR spectra were more intense for the 0.18 g/g sample than they were for the 0.05 g/g sample while oil signals remained approximately constant at both moisture levels. Sugar-to-oil signal ratios are plotted as a function of initial moisture content in Figure 5. Below 0.14 g/g ratio values were <0.5. As initial moisture content rose above 0.14 g/g ratio values increased rapidly until they plateaued at approximately 0.33 g/g. The sugar signals correspond to chemical shifts for aqueous solutions of sucrose, raffinose and stachyose [6]. There were no differences in either ^{1}H or ^{13}C-NMR data between the cultivars. Traces in Figure 5 were obtained using combined cultivar data. T1 values for moisture contents ≥ 0.05 g/g were fit to the equation $y=1048 \exp(-13.1x +221.3)$ with $r^2 = 0.99$. Sugar/oil data were fit to $y=2.2/(1+\exp(-19.2+0.3x-48.3x^2-280x^3))$ with $r^2 = 0.98$.

Figure 5. Relaxation times (T1) of water protons (circles and solid line) and sugar-to-oil signal ratios (triangles and broken line) for seeds of two P. vulgaris cultivars as a function of equilibrated seed moisture content. Tendercrop= filled symbols, Kinghorn Wax=open symbols. Trace for T1 values >0.05 g/g is $y=1048 \exp(-13.1x+221.3)$, $r^2=0.99$. Trace for sugar/oil values is $y=2.2/(1+\exp(-19.2+0.3x-48.3x^2-280x^3))$, $r^2=0.98$.

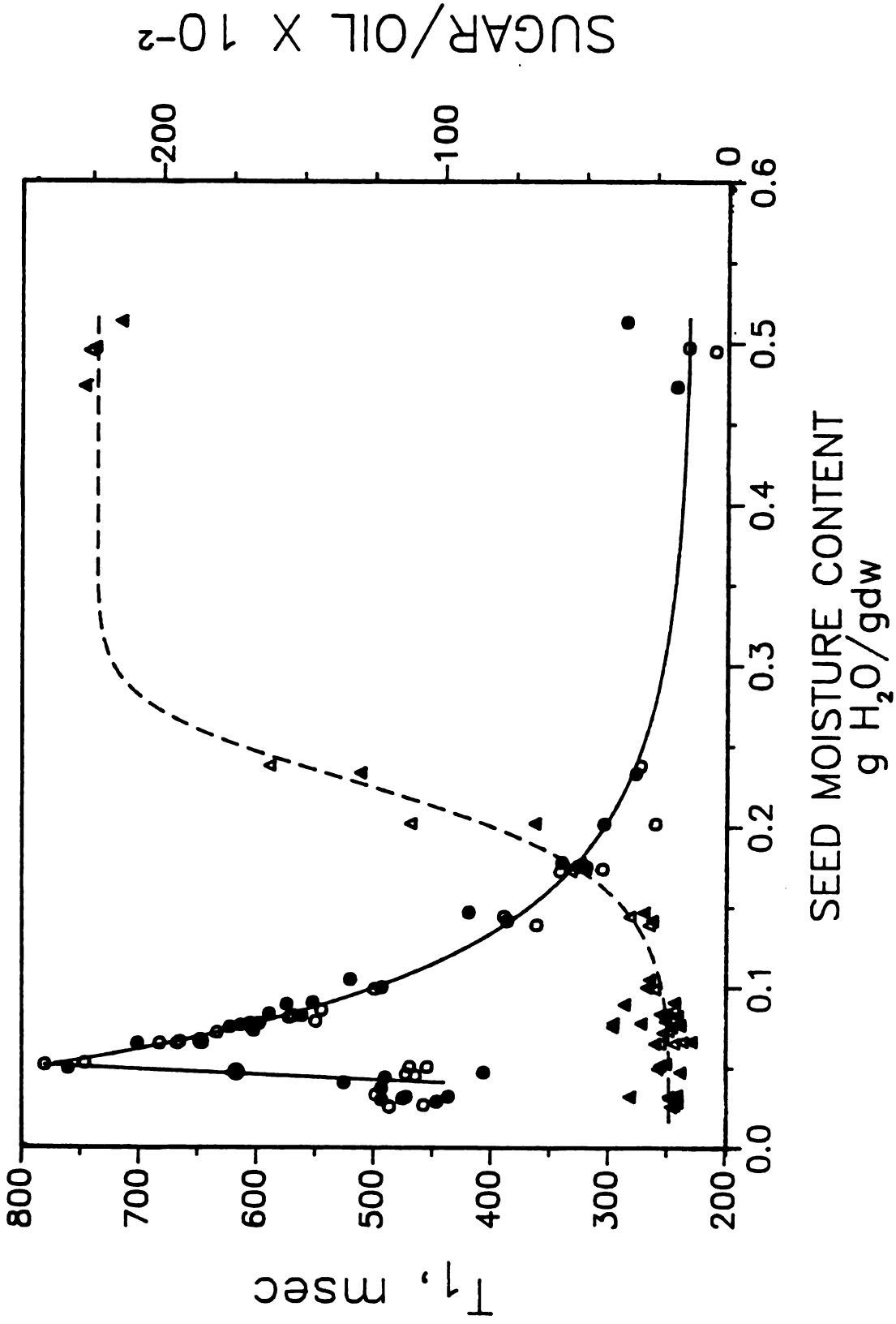
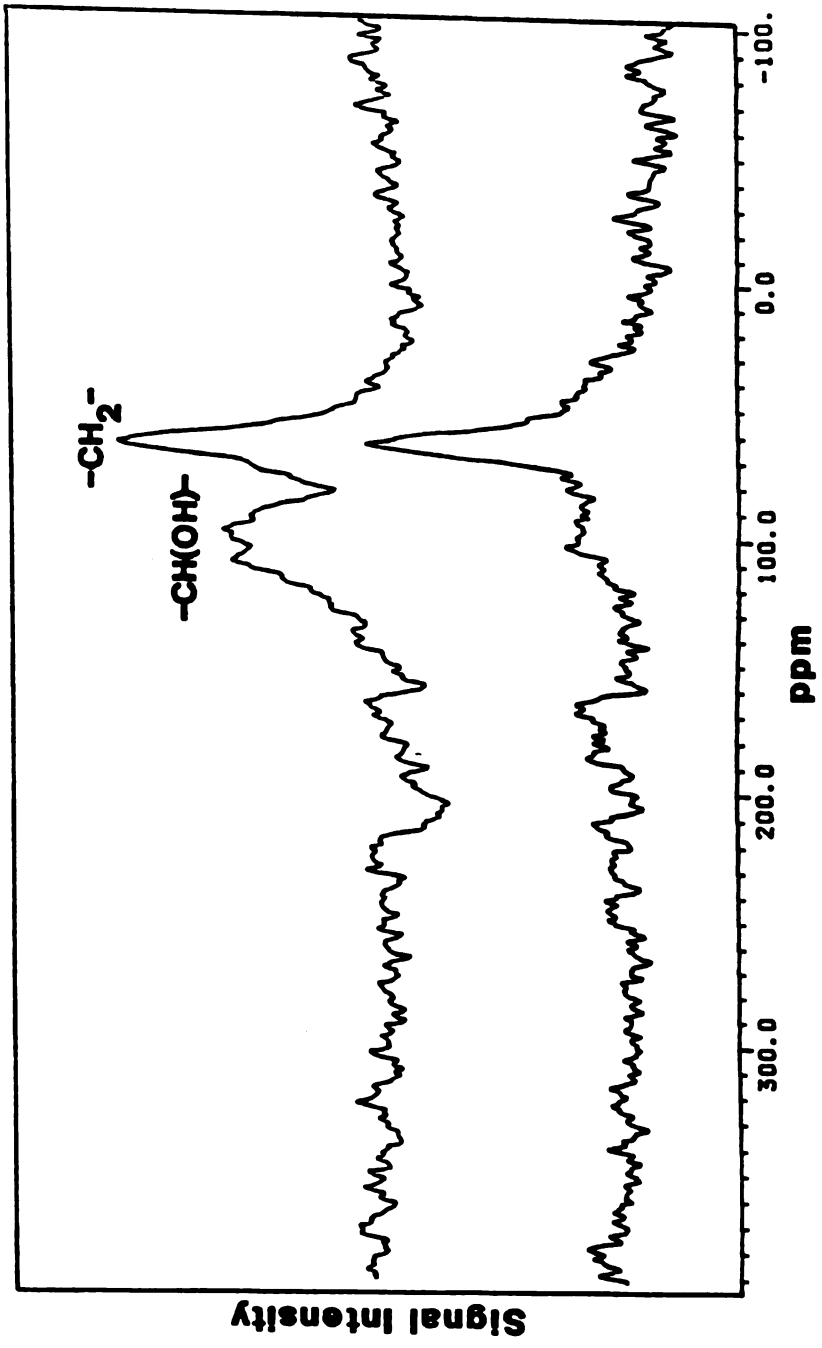

SUGAR/OIL X 10-2
200
100
0
T_1, msec
800
700
600
500
400
300
200
0.0
0.1
0.2
0.3
0.4
0.5
0.6
SEED MOISTURE CONTENT
g H_2O/gdw

Figure 6. ^{13}C NMR spectra of P. vulgaris cotyledons at two moisture levels. Upper curve = 0.18 g/g, lower curve = 0.05 g/g.


Signal intensity
300.0
200.0
100.0
0.0
-100.
ppm
-CH2-
-CH(OH)-

DISCUSSION

Both cultivars exhibit a well defined breakpoint moisture level below which germinating seeds are injured during the course of imbibition (Fig. 1). Above the breakpoint germination is constant as moisture content changes. Below the breakpoint germination drops linearly as a function of decreasing moisture. Kinghorn Wax's resistance relative to Tendercrop's is exhibited by its having a lower critical moisture point. Temperature appears to determine the initial moisture level at which the injury is initiated. Identical values for the slopes of germination below the breakpoint suggest that the injury process, once initiated, is similar at both 5° and 20°C.

Water is imbibed into a seed due to the difference in water potential between the seed (ψ_s) and the water potential of its environment (ψ_e) [15]. The rate of imbibition is influenced by additional characteristics of the seed and environment and has been equated with an analogy of Ohm's Law [2]

$$F=(\psi_e-\psi_s)/(I+i_1+i_2)$$

where F=imbibition rate, i_1=internal impedance of the seed tissues, i_2=impedance of the imbibition matrix and I=external impedance. When cotyledons without testa are submerged in deionized water the equation simplifies to

$$F=-\psi_s/i_1.$$

Thus, when i_1 is constant F will vary inversely with ψ_s.

However, because F does not vary as a linear function of ψ_s in imbibing P. vulgaris cotyledons, i_1 must not be constant. The increase in T1 values as tissue moisture declines is similar to that found in dehydrating cysts of the brine shrimp Artemia salina where T1 values also reached a low moisture maximum [5]. Clegg et al.[5] found that the low moisture T1 maximum in Artemia cysts was accompanied by a two-fold increase in the diffusion coefficient of water. If the diffusion coefficient increases similarly in seed tissue at the low moisture T1 maximum it could account for the increase in imbibition rates seen at 0.7 g/g. Vertucci and Leopold [23] concluded that early imbibition consists of two components; a wetting reaction when initial seed moisture content is <0.32 g/g followed by a flow of water through the wetted tissue. We suggest that the peak imbibition rate first appearing at 0.33 g/g is indicative of the endpoint of the wetting reaction. At moisture levels slightly below this point imbibition rates are reduced due to the impedance of the wetting reaction. Above 0.33 g/g initial moisture imbibition rates are a function of $\Delta\psi$, the driving force for imbibition. The gradual shift of the 0.33 g/g peak to lower moisture levels during the course of imbibition occurs as higher moisture cotyledons approach saturation and their imbibition rates decrease. Kinghorn Wax imbibes more quickly than Tendercrop which may account for its greater apparent movement.

A point of interest is the minimum between the two imbibition peaks. These fall at 0.1 g/g and 0.17 g/g for Kinghorn Wax and Tendercrop, respectively. The minimum for each cultivar occurs at a moisture level that closely corresponds to the moisture level of its 20°C germination breakpoint. It marks a critical change in rehydration processes which appears to be closely associated with imbibitional injury. The nature of the change is not clear and is not indicated by any of the parameters we measured other than imbibition rate itself. It occurs at moisture levels where enthalpy values for Kinghorn Wax may indicate a slight increase in water binding not present in Tendercrop but there is not a corresponding enthalpic increase for Tendercrop at 0.17 g/g, its imbibition minimum.

High leakage rates under imbibitional stresses of low initial seed moisture have been interpreted to result from low moisture induced liquid-crystalline-to-gel phase transitions of seed membranes [7]. Others [9] have suggested that leakage of electrolytes is a process of passive diffusion through cell membranes that closely follows imbibition kinetics and, therefore, is not necessarily a reflection of membrane condition. If the latter was the case we would predict a bimodal distribution of leakage rates similar to that seen with imbibition. However, while leakage rates follow imbibition rates quite closely at low moisture levels, they are clearly unaffected

by the second imbibition maximum at higher moisture levels. At low moisture levels the membrane is not an effective barrier against electrolyte loss, possibly due to a hydration dependent phase transition, and ions leak from cells as rapidly as they are hydrated. At some moisture level between 0.07 g/g, the maximum leakage moisture level, and that at which imbibition rates begin to increase, the membrane begins to become an effective barrier against electrolyte loss and leakage rates decline accordingly.

T1 data from Artemia cysts differ in two ways from the values obtained in this study. First, T1 values from Artemia cysts continually decreased as moisture content declined from 0.6 g/g to 0.2 g/g and second, after reaching a low moisture maximum they plateaued at approximately 0.05 g/g and remained elevated as moisture content approached zero. The low moisture T1 maximum in Artemia has been used as evidence to support the Water Replacement Hypothesis (WRH) [5]. The WRH holds that sugars substitute for water in the maintenance of membrane structure at low moisture content. The increase in T1 has been interpreted to result from greater rotational and translational freedom of water molecules once they have been displaced [5]. A similar mechanism of desiccation tolerance has been suggested for seeds in which sucrose, in the presence of stachyose and/or raffinose, would act to replace water [14] and has recently been shown to be feasible [4]. Ishida et al.[11] have

reported an increase in T1 values at low moisture levels as soybean seeds dehydrate during maturation at moisture levels comparable to those we have detected in P. vulgaris cotyledons.

The free sugars in dry P. vulgaris seeds are sucrose, raffinose and stachyose [12]. The sugar signals in the ^{13}C NMR spectra correspond to the chemical shifts of these three carbohydrates in aqueous solution [6]. Free water is first detected by NMR in soybean tissue at 0.14 g/g [21], the same moisture content at which the intensity of the sugar peaks begins to increase (Fig. 5). Thus, the increase in sugar signal intensity probably results from pre-existing sugars entering into solution as free water first becomes available. Proton relaxation times and levels of free sugars measured as a function of initial moisture level are virtually identical for both cultivars and do not indicate differences in sensitivity to imbibitional injury.

We might speculate on further significance of the 0.33 g/g level. If we view the T1 maximum at 0.05 g/g in terms of the WRH the process of water replacement would begin at about 0.33 g/g and proceed first replacing loosely bound and then semi-bound water. The data leave open to question whether the bound water fraction below 0.05 g/g would be replaced. Work with phospholipid bilayers indicates that even under conditions of severe dehydration some water remains bound in the bilayer [10]. Seewaldt et al. [21]

found an abrupt increase in the bilayer spacing of membranes in soybeans when seed moisture was increased from 0.21 to 0.3 g/g. This is the range of moisture levels at which free sugars are rapidly entering into solution. The shift in spacing may be a response to the replacement of water from sites on the membrane surface. This would suggest the interesting possibility that the wetting reaction may involve the rebinding of water molecules onto sites occupied by sugars in the dry tissue. Additionally, the high moisture freezing limit for P. vulgaris seeds, i.e. the moisture level above which seeds are killed by exposure to liquid N_2, is 0.37 g/g [22]. Thus, the peak in imbibition rate at about 0.33 g/g occurs at a moisture level corresponding to a number of other phenomena involving hydration water that could be interrelated.

We have identified specific moisture levels for the onset of imbibitional injury in two cultivars of P.vulgaris. These breakpoint moisture levels are a function of imbibition temperature. The injury process appears to be similar at both 5° and 20°C. There are two initial moisture levels at which cotyledon imbibition rates are maximal. The first occurs at the same approximate moisture level of the enthalpic peak of water binding and close to that of the T1 maximum and might be explained by an increase in the diffusion coefficient of water associated with the increase in T1. The second occurs at 0.33 g/g and may indicate the

endpoint of the wetting reaction. The moisture level of the minimum falling between the two imbibition maxima is different for each cultivar and corresponds to its 20° germination breakpoint moisture level. Our data support the hypothesis that imbibitional injury is related to transitions in bound water in seed tissues [24]. Imbibitional injury may involve the rehydration of cell structures from which water has been displaced by sugars during desiccation.

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APPENDICES

APPENDIX A

MEASUREMENT OF CONTROLLED RELATIVE HUMIDITIES USED FOR SEED MOISTURE EQUILIBRATION

INTRODUCTION

Moisture contents of seed tissues were equilibrated in atmospheres of known relative humidity in determining the enthalpies of water binding reported in Section 3. As detailed in the Materials and Methods of Section 3, this was accomplished by holding seed tissue in sealed 500 ml glass jars over saturated salt, base and concentrated acid solutions at 5° and 20° until moisture equilibrium was reached. Published values for relative humidities created by many of the compounds used were often lacking at one of the temperatures, most often 5°, and in several instances different sources reported conflicting values. In order to obtain the missing values and use humidity values determined from a consistent source, the relative humidities of all solutions used were measured at 5° and 20°.

MATERIALS AND METHODS

Saturated salt and base solutions were prepared according to Winston and Bates (1960.) Briefly, enough solid was dissolved into boiling water so that the solution was approximately 95% saturated when cooled. Care was taken to avoid precipitation of salt crystals during cooling. After cooling, excess solid was added to achieve a saturated solution. Salts and bases used to achieve humidities below 25% were prepared as a thick slurry in order to avoid the formation of a unsaturated layer on the surface. Jars were

sealed and allowed to equilibrate at 5° and 20° for 30 days prior to use. Saturated salt and base solutions were used repeatedly over a two year period. On occasion it was necessary to add chemical to low humidity jars or water to high humidity jars. All water used was 2x deionized. Concentrated acid solutions were replaced after each use. Temperature was maintained (±1°) in constant temperature storage rooms.

Dew or frost points of equilibrated atmospheres were measured with a Model 911 Dew-All digital chilled mirror humidity analyzer (EG&G Environmental Equipment, Waltham, MS.) Frost points were converted to dew points and dew point to vapor pressures. Relative humidity was computed according to the relationship

$$RH = (\text{dew point v.p.}/\text{v.p. of ambient temp}) \times 100$$

A peristaltic pump was used to circulate an airflow of 10 to 15 ml min^{-1} through the jar and analyzer in a continuous 1 m circuit of 1/4" i.d. polyvinyl chloride tubing. The time required for the mirror temperature to stabilize was dependent on the humidity and temperature of the sample. Mid-range humidities (30% to 70%) and samples at 20° stabilized more quickly (5 to 20 min) than extreme humidities and samples at 5° (30 min to 4 hr.) Mirror temperature was monitored with a strip chart recorder to more accurately determine when the temperature had

stabilized.

RESULTS

Relative humidities of atmospheres measured over saturated salt, base and concentrated acid solutions are presented in Table 1.

Table 1. Relative humidities over saturated salt, base and concentrated acid solutions at 5° and 20°.

	RELATIVE HUMIDITY			RELATIVE HUMIDITY	
COMPOUND	20°	5°	COMPOUND	20°	5°
H_2SO_4	0.75	1.0	NaI	37.6	41.2
NaOH	6.0	10.4	$CaCl_2$	31.3	36.9
LiBr	6.5	9.3	K_2CO_3	42.9	40.2
KOH	9.2	13.9	$Mg(NO_3)_2$	52.9	53.7
$ZnBr_2$	7.8	10.7	$CuCl_2$	64.7	63.7
H_3PO_4	8.6	12.5	KI	67.3	69.0
$ZnCl_2$	5.4	10.8	NaCl	72.7	71.6
LiCl	11.3	13.8	$(NH_4)_2SO_2$	77.3	77.0
$CaBr_2$	17.4	23.0	KCl	80.0	82.7
KAc*	22.2	26.2	KNO_3	90.2	90.6
$MgCl_2$	33.3	32.3			

*KAc = Potassium Acetate

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APPENDIX B

GROWTH OF AXES IMBIBED AT DIFFERENT TEMPERATURES

INTRODUCTION

Axes excised from legume seeds have been used in several studies concerning imbibitional chilling in order to determine various responses of embryo tissue to low temperature (Pollock and Toole, 1966; Pollock, 1969; Leopold and Musgrave, 1979; Ashworth and Obendorf, 1980; Woodstock et al., 1984.) In each of these investigations a single low temperature, 5°, 10° or 15°, has been used as the chilling treatment. Wolk et al. (1988) determined specific breakpoint initial moisture contents for imbibitional injury in snap Phaseolus vulgaris seeds at 5° and 20°. The objective of this study was to determine if P. vulgaris axes of a given initial moisture content exhibited a similar breakpoint in response to temperature.

MATERIALS AND METHODS

Axes were excised from seeds of two P. vulgaris cultivars, 'Tendercrop' and 'Kinghorn Wax' (Rogers Brothers Seeds, Twin Falls, ID.) Seed moisture content was increased at 5° over water for 40 hr to facilitate excision. Excised axes were held a t 5°, 50% R.H. for 48 hr to adjust moisture content to ca. 8%.

Axes were imbibed on a temperature gradient bar modeled after Fox and Thompson (1971.) A temperature gradient ranging from 0° to 24° was maintained by circulating -2° and 25° polyethylene glycol solutions through chambers at each

end of the bar. Groups of 5 axes of each cultivar were placed into 1/2" x 1/2" x 3" plexiglas cells lined with moistened filter paper at locations along the bar at designated temperatures. The bar was encased in expanded polystyrene for the duration of the temperature treatment. Temperatures were monitored every 8 to 12 hr. After the requisite time on the bar, axes were transferred to 7 cm Whatman #1 filter paper discs wetted with 1.5 ml of 0.15% captan solution in 100x15 mm petri dishes and placed in darkness at 20°. After 4 days axes were removed and weighed. Growth is expressed as fresh wt/ initial dry wt. Each experiment was replicated twice on separate temperature gradient bars and data presented represent the means from two experiments.

RESULTS AND DISCUSSION

Growth of axes imbibed for 24 or 48 hr at different temperatures is presented in Figure 1. Growth of Tendercrop and Kinghorn Wax axes declined when they were imbibed below 12.5° and 14.5°, respectively, for 24 hr as determined by least squares analysis. After 48 hr Tendercrop's growth declined at temperatures below 16.0° and Kinghorn Wax's below 17.5°.

Although Kinghorn Wax axes did not grow as rapidly as Tendercrop's at warmer temperatures, they were less affected by lower temperature as indicated by the lower slope of

their decline (Table 1.) This is consistent with data presented in other sections of this dissertation which indicate that Kinghorn Wax is more resistant to imbibitional injury than Tendercrop. It is interesting that growth of Kinghorn Wax axes appear to begin to decline at higher temperatures than did that of Tendercrop. These differences were not statistically significant, possibly due to minimal replication. The same problem that was discussed in Section 2 of this dissertation arises in this study. That is one concerning the difference between imbibitional injury and chilling injury. Axes were held on the bar long enough to sustain both types of injury. To resolve this problem axes should be imbibed in petri dishes at non-chilling temperatures and then transferred to the temperature gradient bar.

Figure 1. Growth (fresh wt/dry wt) of axes of two cultivars of P. vulgaris, as a function of imbibition temperature. Axes were imbibed for either 48 hr (A) or 24 hr (B) at designated temperature and then transferred to 20°C for germination and growth. Weights were taken after 4 days at 20°. Vertical bars indicate SE of the means.

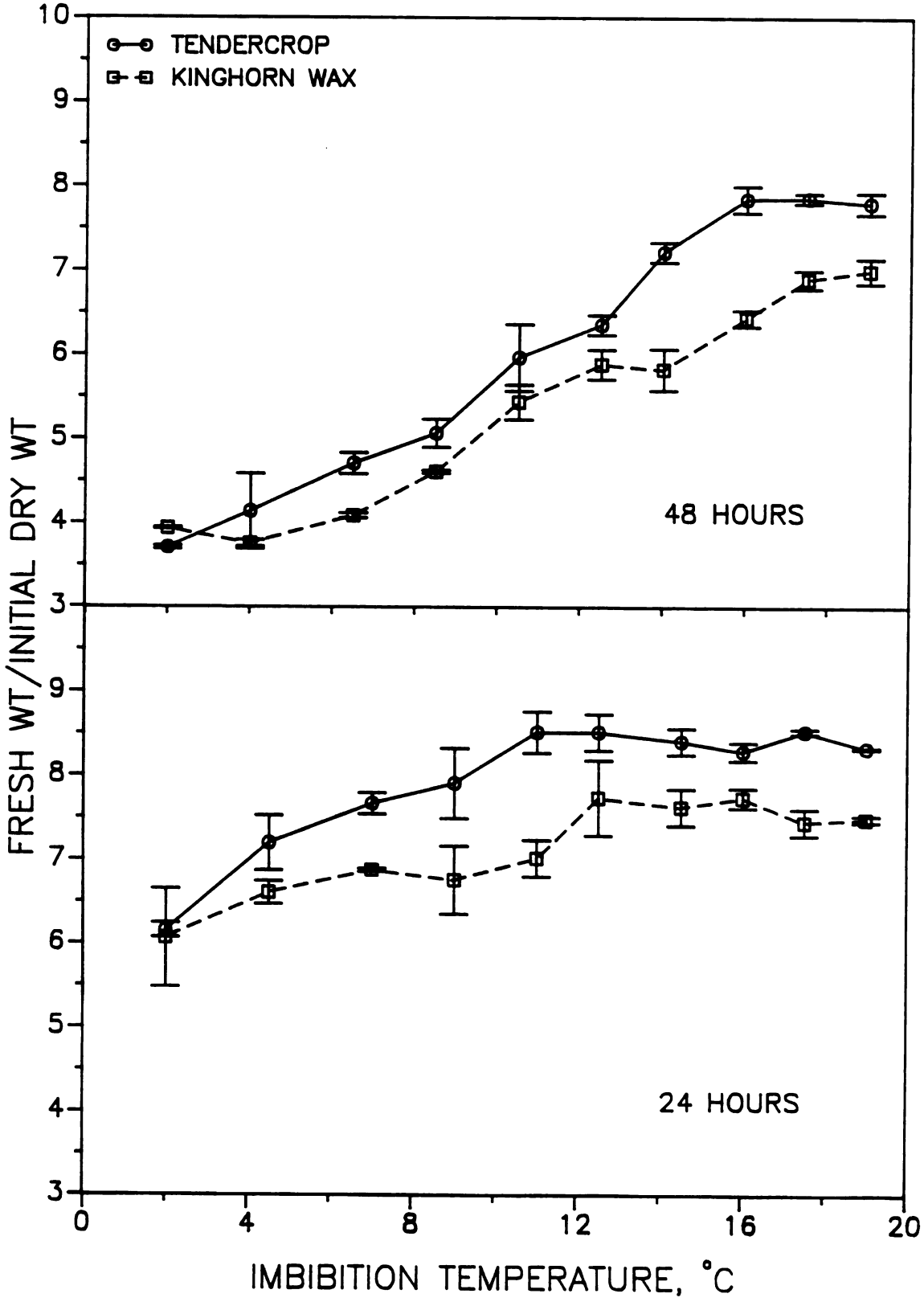
TENDERCROP
KINGHORN WAX
48 HOURS
24 HOURS
FRESH WT/INITIAL DRY WT
IMBIBITION TEMPERATURE, °C
10
9
8
7
6
5
4
3
0
4
8
12
16
20

Table 1. Regression data for temperatures of decline in axis growth of two *P. vulgaris* cultivars, 'Tendercrop' (TC) and 'Kinghorn Wax' (KW.)

Cultivar	Hours Imbibed	r	slope
TC	24	.98**	0.24
KW	24	.91*	0.12
TC	48	.99**	0.30
KW	48	.98**	0.21

**P=0.01
*P=0.05

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