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Chilling Stress to Soybeans during Imbibition[1]

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WILLIAM J. BRAMLAGE,[2] A. CARL LEOPOLD,[3] AND DAVID J. PARRISH[4]
Department of Agronomy, University of Nebraska, Lincoln, Nebraska 68583

ABSTRACT

Embryos, excised from seed coats of soybeans (*Glycine max* Merr. cv. 'Wayne'), leak profusely during the first minutes of imbibition. A discontinuity of temperature/leakage patterns occurs between 10 and 15 C; as embryos imbibe at 10 C or lower, disproportionately more solutes leak out per unit of water imbibed. Short periods of imbibition at or below 12 to 14 C reduce embryo germination and axis elongation; injury results from imbibition at 2 C for as little as 5 minutes. Humidifying embryos to 35 to 50% moisture before imbibition reduced leakage during imbibition and imparted some resistance to imbibitional chilling injury.

The period of profuse leakage is interpreted as a time of membrane reorganization. Imposing a low temperature during this period prolongs the rapid leakage, suggesting delayed or faulty membrane reorganization. Reduced cold sensitivity of embryos with an initial 35 to 50% moisture content is presumed to be due to at least partial membrane reorganization in the embryo before imbibition. These data collectively are taken to indicate that low temperature interferes with normal membrane reorganization during imbibition, probably by modifying the physical state of membrane phospholipids, and that the consequent abnormal organization of membranes is a basic cause of low temperature injury.

Soybean seeds are sensitive to low temperature during germination, injury occurring at about 10 C or lower. Hobbs and Obendorf (7) suggested that the greatest sensitivity occurs during the first hours of imbibition, and showed that injury was expressed both as reduced emergence of seedlings and as reduced vigor and yield of surviving plants. This problem is of major agricultural importance, since germination in a cold soil can markedly reduce productivity.

Based on theoretical and empirical considerations, Simon (19) proposed that membranes of dry seeds are dehydrated and leaky, allowing passive diffusion of cellular substances when the seeds are wetted. The leakage may be due to tubular channels in membrane phospholipids which open under conditions of low water content (1). Upon imbibition (rehydration), the normal lamellar phospholipid structure should reform, the membranes reorganize, and selective permeability be reestablished (19). Electron photomicrographs of soybean cotyledons during imbibition support this concept, and indicate that within 20 min of imbibition at 25 C, an extensive reorganization of membrane structures occurs in the outer layers of cells (24).

The sensitivity to chilling stress during imbibition may be due to low temperature alterations of the membrane reorganization processes. Chilling sensitivity may reside in the phospholipid composition of membranes, the chilling temperature being the point of lipid phase transition between a liquid-crystalline and crystalline-gel form (10). Simon (19) suggests that when dry seeds imbibe at chilling temperatures, the phospholipids are unable to change rapidly from the hexagonal (dehydrated) to the lamellar (hydrated) architecture because they are gelled in a rigid molecular shape; if this proposal is correct, membrane reorganization may be impeded and the imbibing embryo may suffer injury.

Parrish and Leopold (12) suggested that a period of declining rates of solute leakage from soybean cotyledons in the first few min of imbibition can be used to define the period of membrane reorganization. In the present study we have used this type of analysis to assess membrane reorganization at low temperature, and have found that membrane reorganization does indeed reflect the effects of chilling stress and that altered leakage patterns are correlated with chilling effects on germinability and growth of the embryo.

MATERIALS AND METHODS

Soybean (*Glycine max* Merr., cv. 'Wayne') seeds were obtained from the Nebraska Seed Foundation, and the same seed lot was used in all experiments. All tests reported here were conducted on cotyledons or embryos, not on intact seeds, since the soybean seed coat is a highly variable barrier to water uptake and solute leakage. Seed coats were removed carefully with a razor blade, with minimal injury to the embryos.

When cotyledons were used, the two cotyledons from each seed were used in paired treatments imbibed at 10 C increments, *e.g.* 15 and 25 C, within an experiment to reduce seed-to-seed variability in making a 10-degree temperature comparison. For each treatment, 10 cotyledons were imbibed in 25 ml of distilled H_2O. Treatments were replicated four times. "Dead" cotyledons were obtained by heating to 100 C for 24 hr.

Different moisture contents of cotyledons and embryos were achieved by placing them on weighing dishes in plastic boxes that were lined with wet paper towels and maintained at 25 to 28 C. An increase from 6 or 9% to 25 or 30% moisture required about 18 hr, and to 45 to 50% required about 42 hr. These humidification times were reasonably constant as long as temperature was constant, water was standing on the bottom of the boxes, and the embryos or cotyledons were evenly distributed in the dishes.

Leakage was measured as increase of $O.D._{280}$ of the imbibing solution (13, 14) measured in a Gilford spectrophotometer equipped with a sample return mechanism which allowed repeated measurement of the same solution at 30-sec intervals. Temperature of the imbibing solution was maintained within ±1 C during the imbibitional period, and the solutions were frequently agitated. Tests for seedling vigor gave no indication of O_2 stress in this system for imbibition times of at least 1 hr.

Water uptake was measured as fresh weight gain during the imbibitional period by placing the imbibed tissue onto filter paper and quickly blotting it free of surface water. Values are probably underestimates since measurable amounts of dry weight are

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[2] Permanent address: Department of Plant and Soil Sciences, University of Massachusetts, Amherst, Massachusetts 01003.

[3] Present address: Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, New York 14853.

[4] Present address: Agronomy Department, V.P.I. and S.U., Blacksburg, Virginia 24061.

leached out during imbibition. Dry weight was determined by drying 24 hr at 100 C, and moisture content, water uptake, and leakage were expressed on a dry weight basis.

Germinability of embryos was determined by placing 10 embryos between two pieces of filter paper wetted with about 7 ml of distilled H_2O and enclosed in a Petri dish. The dishes were maintained in the dark at 28 C for 3 days, with more water added daily. After 3 days, germination was determined as the per cent of the embryos whose axes had enlarged to at least 1 cm; heat-killed axes enlarged to no more than 0.5 cm. Lengths of the germinated axes were recorded.

RESULTS

When dry cotyledons are placed in water (Fig. 1) solutes leak profusely at first but leakage rates subside to a much lower, steady rate within 5 min. At 25 C, leakage rates from dead cotyledons decline more slowly than from live cotyledons, and at 5 C no decline occurs during the first 10 min of imbibition in dead material. The sustained rate of leakage from dead cotyledons is strongly influenced by temperature, while that of live cotyledons is only slightly affected. The lower leakage rates of dead than live cotyledons during the first min at 15 or 5 C correlated with slower wetting of the dead tissues, *i.e.* they gained weight more slowly than the live cotyledons, as reported earlier (23) for heat-killed pea cotyledons.

Leakage of solutes from initially dry cotyledons increases after 8 to 12 min at 25 C (12), and after longer times at lower temperatures. This upturn occurs as cracking and fragmentation of cotyledons become evident. Cracking can be avoided by humidifying soybean seeds to about 15% moisture before imbibition (11). However, with seed coats removed it was necessary to humidify to at least 25% moisture to avoid cotyledon cracking (data not shown). Humidification increased the imbibition rates (Fig. 2B) and reduced the initial leakage rates (Fig. 2A). Since cracking confounds other changes that may occur during imbibition of embryos, all subsequent studies employed tissue humidified to at least 25% moisture before imbibition.

When leakages at 25 and 5 C were compared (Fig. 3A), total leakage during the first 10 min from cotyledons at an initial 30% moisture content was greater at 5 C, even though leakage rate during the 1st min was much lower at 5 than at 25 C. The greater total leakage at the lower temperature was due to slower subsidence at 5 C, plus a higher rate of leakage once a steady rate was achieved. (The steady rate of leakage continued for at least 30 min.) For cotyledons at an initial 45% moisture content (Fig. 3B) there was, however, little difference in leakage during 25 and 5 C

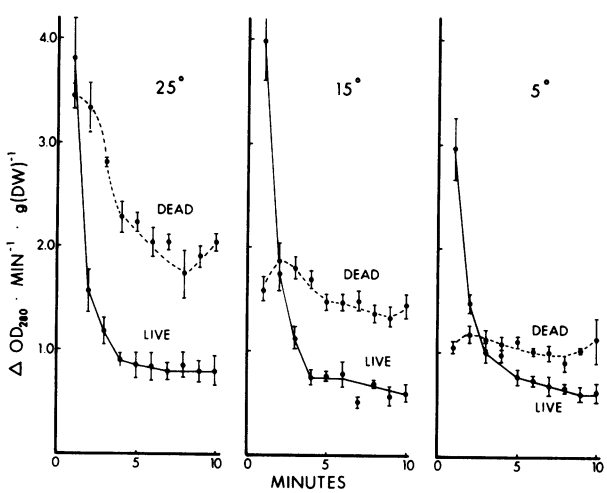

FIG. 1. Leakage of solutes from dry (6% moisture) soybean cotyledons at 25, 15, and 5 C. "Dead" cotyledons had been heated to 100 C for 24 hr. Leakage as rate of increase in O.D.$_{280}$. Mean ± SE of four replications.

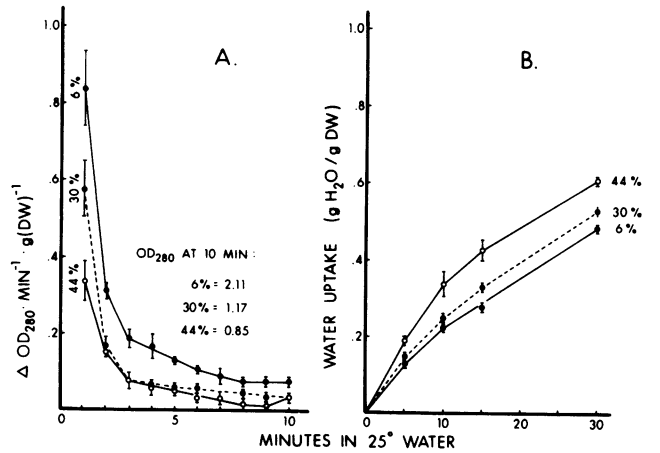

FIG. 2. A: Solute leakage rate and (B) water uptake (increase in fresh wt) during imbibition at 25 C of cotyledons at three different initial moisture contents. Mean ± SE of four replications.

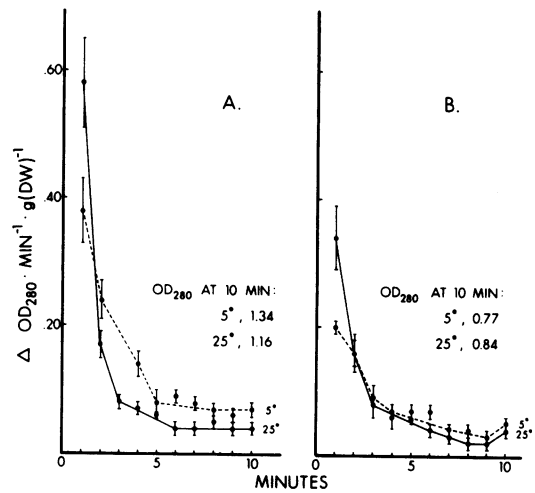

FIG. 3. Solute leakage rate during imbibition at 5 and 25 C, of cotyledons at initial moisture contents of (A) 30% and (B) 45%. Mean ± SE of four replications.

imbibition except during the 1st min. Thus, imbibition by drier cotyledons (30% moisture) at 5 C resulted in a disproportionately higher leakage rate than at 25 C, but this higher leakage rate did not occur if the initial moisture level was at 45%.

To examine further the effect of temperature on imbibitional leakage, cotyledons were imbibed at nine temperatures between 2 and 30 C for 20 min, and *A* was measured at 30-sec intervals. Figure 4 presents cumulative leakage (O.D. of imbibing solution) at four representative intervals during this time. When cotyledons initially at 30% moisture were tested (Fig. 4A), *A* increased almost linearly with temperature during the first 30 sec. The Q_{10} of leakage during this period was about 1.35, which was approximately the Q_{10} of water uptake during early imbibition (data not shown). Thus, solutes apparently diffused freely at all temperatures from the outer layers of cells as they first came in contact with water. However, this pattern did not persist; at 2 min, a discontinuity in the temperature/leakage pattern was evident between 10 and 15 C. By 10 and 20 min the discontinuity had become more pronounced; cotyledons at lower temperatures had all leaked more than those at 12 C. Similar data for cotyledons with an initial 50% moisture content (Fig. 4B) show evidence of a discontinuity between 10 and 15 C, but leakage was significantly increased only at the lowest temperature (2 C). Thus, the initial 50% moisture level before imbibition suppressed the low temperature effects on leakage during cotyledon imbibition.

To quantify the temperature/leakage discontinuity, Q_{10} values for leakage rates were calculated after representative intervals of imbibition (Table I). At 30 sec of imbibition by cotyledons with an initial 30% moisture content, a positive Q_{10} existed at all temperature intervals. By 5 min, however, clear differences existed among the Q_{10} values at different intervals. For live cotyledons, when both temperatures were above the discontinuity (approximately 12 C), the Q_{10} for leakage was at least 1; also, when both temperatures were below the discontinuity, the "Q_8" between 10 and 2 C (the best comparison possible among the data below the discontinuity) was above 1. However, each comparison that crossed the discontinuity (*e.g.* 15/5 C) produced a Q_{10} of less than 1, showing that leakage was greater at the lower than at the higher temperature. The low Q_{10} values persisted during the entire 20 min of observation, and were a function of living tissue, for the Q_{10} values of leakage from dead cotyledons were well above 1 throughout the 20-min period. When Q_{10} values were calculated for leakage from 50% moisture cotyledons, the only temperature comparison with a Q_{10} of less than 1 was 12/2 C.

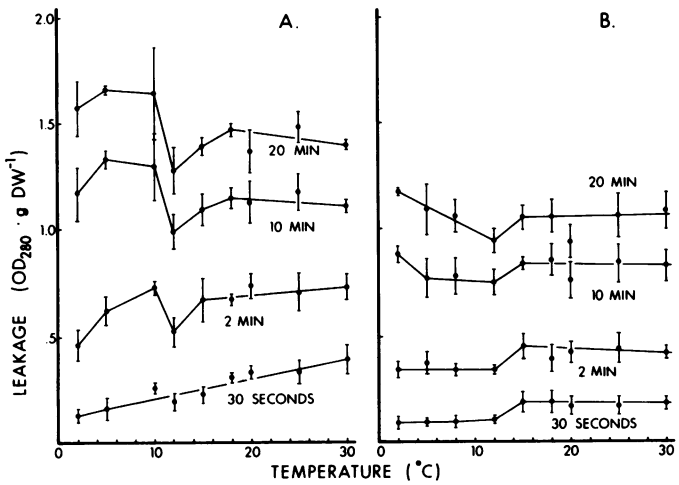

FIG. 4. Solute leakage (O.D.$_{280}$ of imbibing solution) from cotyledons at intervals during imbibition at various temperatures. Cotyledons initially at (A) 30% moisture or (B) 50% moisture. Mean ± SE of four replications.

Table I. Q_{10} values* of leakage rates ($\Delta OD_{280} \cdot min^{-1} \cdot g\ DW^{-1}$) of soybean cotyledons at 30% moisture before imbibition.

Ten cotyledons were imbibed in 25 ml water at from 2 to 30 C. OD_{280} was measured at 30 sec, 1 min, and each minute thereafter. Rates are calculated from increase in OD from the previous reading.

Imbibition time	Temperature comparisons (°C)							
	Live cotyledons						Dead cotyledons	
	10/2	12/2	15/5	20/10	25/15	30/20	15/5	25/15
30 sec	1.8	1.5	1.4	1.4	1.4	1.2	2.6	1.3
5 min	1.2	0.9	0.9	0.9	1.1	1.0	1.6	1.4
10 min	1.1	0.8	0.8	0.9	1.1	1.0	1.6	1.3
15 min	1.1	0.8	0.8	0.9	1.1	1.0	1.5	1.3
20 min	1.1	0.8	0.8	0.8	1.1	1.0	1.5	-

* The first column is the ratio of an 8 degree interval (i.e. between 10 C and 2 C), rather than the usual 10 degree interval.

To determine whether these leakage phenomena correlate with imbibitional chilling injury to 'Wayne' soybeans, embryos at 30% moisture were imbibed for 30 min in water at from 6 to 25 C and then were germinated on moist filter paper at 28 C for 3 days (Table II). Germination was reduced by imbibition at 12 C or less, with the greatest reduction at the lowest temperatures tested. Elongation of axes of embryos that did germinate was sharply reduced, with some injury resulting from 30-min imbibition even at 14 C. Thus, the inception of injury was approximately in the temperature range where the discontinuity in leakage occurred (Fig. 4). Growth of the germinated embryos was reduced more by imbibitional chilling than was germination itself. To ascertain that leakage was correlated with this damage, both water uptake by the embryos (increase in fresh weight) and leakage of solutes into the imbibing solution (ΔO.D.$_{280}$) were determined at the end of the 30-min imbibition period (Table II). The ratios of leakage to water uptake show that at lower temperatures, disproportionately greater amounts of solutes were lost per unit of water uptake.

To learn how quickly imbibitional chilling damage could occur,

Table III. Effects of duration of chilling at 2 C during initial imbibition of embryos.

Ten embryos were imbibed in 25 ml water at 2 C for designated time, then transferred to moist filter paper enclosed in a petri dish and maintained for 3 days at 28 C. Control (0 time in cold) placed on moist filter paper without being imbibed in water; controls elongated to 3.2 ± 0.3 cm, and 2.9 ± 0.5 cm, respectively, for 23 to 30% moisture and 35 to 50% moisture embryos. Mean ± S.E. of 6 replications.

Imbibition time at 2 C	Germination	Elongation of axes of germinated embryos
(min)	(%)	(% of growth of unchilled control)
Embryos initially at 23 to 30% moisture		
0	100	100
5	89 ± 5	76 ± 10
10	75 ± 10	79 ± 8
20	74 ± 10	63 ± 5
30	61 ± 2	66 ± 6
40	50 ± 5	51 ± 5
60	30 ± 6	47 ± 5
Embryos initially at 35 to 50% moisture		
0	92 ± 3	100
20	83 ± 3	104 ± 15
40	71 ± 6	120 ± 42
60	75 ± 7	78 ± 10

Table II. Effects of temperature applied during the first 30 min of imbibition by soybean embryos.

Ten embryos, initially at 30% moisture, were imbibed 30 min in 25 ml water at the temperature indicated, then transferred to moist filter paper enclosed in a petri dish at 25 C. Leakage was measured as OD_{280} of imbibing solution after 30 min; increase in fr wt was determined at end of the 30 min treatment. Embryos imbibed in 25 C water elongated to 3.3 ± 0.2 cm after 3 days at 28 C.

Imbibition temperature	Germination	Elongation of axes of germinated embryos	Leakage/water uptake ratio
(°C)	(%)	(% of growth of 25 C control)	($\Delta OD_{280} \cdot min^{-1} \cdot g\ DW^{-1}$) (g increase in fr. wt. · $g\ DW^{-1}$)
6°	77 ± 6	66 ± 7	1.67 ± 0.10
8°	91 ± 3	69 ± 5	1.64 ± 0.15
10°	88 ± 7	64 ± 6	1.35 ± 0.16
12°	88 ± 7	77 ± 12	1.29 ± 0.09
14°	98 ± 2	87 ± 4	1.10 ± 0.07
16°	98 ± 2	99 ± 10	0.89 ± 0.09
25°	98 ± 2	100	0.69 ± 0.03

embryos at 30% moisture were imbibed in 2 C water for 5 to 60 min before germination on wet filter paper at 28 C (Table III). Even 5 min of imbibition at 2 C reduced both germination and subsequent elongation of axes. Since increased humidification of embryos before imbibition suppressed the low temperature effect on solute leakage (Figs. 3 and 4), embryos initially at 35 to 50% moisture were also tested for germinability after imbibitional chilling (Table III). These embryos were subject to imbibitional chilling injury at 2 C, but germination and embryo growth showed less damage than when embryos were initially at only 23 to 30% moisture.

DISCUSSION

Solutes apparently diffuse freely from dry cells upon first contact with water, and this is evidently not diffusion from injured cells (20). Since leakage subsides more rapidly from living than from dead cotyledons (Fig. 1), we take the leakage pattern to be a manifestation of membrane reorganization in the outer layers of cells. As such, our data support Simon's (19) concept of imbibitional chilling injury, since an abrupt increase in leakage was correlated with the temperature at which chilling injury was initiated, and chilling injury resulted from even a brief exposure to low temperature at this stage of development.

There is strong evidence that the locus of chilling injury to plants is in the phospholipid composition of cellular membranes, and that a phase change occurs at the chilling point, altering the functions of both the membranes and their associated enzyme systems (10, 15). In recent studies with cotton seeds, emergence from a cold soil was positively correlated with the unsaturated/saturated fatty acid ratio of total (2) and membrane (5) lipids. Furthermore, Raison and Chapman (16) demonstrated that in mung bean hypocotyls, breaks in the Arrhenius plots of membrane electron spin motion, of activation energy of mitochondrial succinate oxidase, and of hypocotyl growth rates all occur at the same temperature (15 C). We believe that the correlation between the inception of chilling injury and the alteration of imbibitional leakage patterns in our results is due to alteration of the molecular ordering of membrane lipids at low temperature, which impedes membrane reorganization and produces injury to the imbibing embryo.

A greater leakage of solutes during imbibition may both deplete the tissues of soluble food reserves, and stimulate growth of pathogenic microorganisms (18). Most of the solutes leaked are sugars and amino acids (17) which can probably be replenished during later hydrolysis. Excluding pathological considerations, a greater solute leakage may delay germination or retard growth, but it should not cause embryo failure; real damage from chilling during imbibition is more likely a result of internal disorganization. Imbibition is a period of great transformation of membranes in soybean embryos: plasma membrane integrity is being restored, the ER is reforming, and mitochondria are returning to a hydrated configuration (6, 21, 22, 24). If membrane restoration is impaired by a chilling temperature, as our data indicate, extensive internal disruption may result. In addition, delayed sequestering of cellular materials may produce destructive autolysis from hydrolytic enzymes (proteases, phosphatases, lipoxygenases, etc.), particularly if membrane components are exposed to prolonged attack before they achieve their normal configuration. The longer it takes to achieve cellular reorganization, the more lysis and loss of functionality may be expected. Let it be noted that although subsidence of leakage (Fig. 1) and occurrence of the temperature/leakage discontinuity (Table I) were functions of living tissue, at 5 C living cotyledons leaked at rates more nearly like those of dead cotyledons after the first 2 min of imbibition (Fig. 1), which suggests severe stress in the living tissue at this damaging temperature.

Most reports of chilling injury during seed imbibition have employed 6 to 12 hr of chilling to demonstrate damage, but our results demonstrate that severe chilling injury can occur during the first few min of imbibition. Soybean embryos thus respond to imbibitional chilling very much as do excised lima bean axes (13). Intact seeds have a longer response time due to the presence of the seed coat; we found that soybean seeds with intact seed coats required at least 90 min at 2 C before injury was sustained (data not shown). The seed coat delays water penetration, reduces leakage of solutes (9), and perhaps creates an osmotic solution within the seed of sufficient concentration to show imbibition even more (13, 20).

Chilling injury can be greatly reduced if seeds have a high moisture content before imbibition at low temperature. Chilling resistance is imparted at 13 to 16% for corn seeds (3), and at 13% for cotton (4) and soybean (7) seeds; chilling sensitivity increases linearly as soybean seed moisture declines from 13% (7). However, lima bean axes require 20% moisture for chilling resistance (13), and soybean embryos appeared to acquire chilling resistance abruptly at about 35% moisture (data not shown). Since membrane phospholipids adopt the normal lamellar configuration rather than the hexagonal (leaky) configuration (19) at about 20% moisture, humidification to about 20% moisture probably allows at least partial reorganization of membranes before imbibition, thereby lessening the destructive effects of membrane phase changes during imbibition at a chilling temperature. The lower moisture requirement for chilling resistance of seeds (13–16%) than of embryos (20–35%) may be attributable to the modulating effect of the seed coat. The extraordinarily high moisture requirement (35%) of soybean embryos is more difficult to resolve, but may correspond to the extraordinarily high moisture content required for germination of soybean seeds; soybeans require 50% moisture whereas corn and sugarbeet seeds require 26 to 31% (8).

In these tests we have demonstrated that imbibitional chilling injury to soybean embryos can occur during the first minutes of hydration. Chilling stress evidently interferes with the ability of the embryo to be transformed from a quiescent body into a metabolically active organ. Our data are consistent with the view that if the membrane lipids are in a crystalline gel state at the time of hydration, there results a faulty arrangement of cellular membranes, which may be expressed as damage to the seed in terms of subsequent viability and vigor.

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