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## Freezing Avoidance in Rice and Wheat Seeds in Relation to Water Content<sup>1</sup>

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*Abstract* Mechanism of freezing resistance in rice and winter wheat seeds was investigated with differential thermal analysis (DTA). Rice seeds avoided freezing strain by preventing ice inoculation from outside and by deep supercooling. The results with wheat seeds were not clearcut enough to draw a conclusion. Both rice and wheat embryos yielded exptHERMS around the critical water content. Change in the state of water in seeds in relation to water content was discussed. Studies on seeds immersed in liquid nitrogen revealed that the critical water contents were 18 to 19% in rice, and 17 to 21% in wheat, respectively. The lower limit of freezable water is considered to be 19% or below in both seeds from the results of DTA.

### Introduction

To preserve the broad gene base needed in the future, a wide range of plant materials, that would otherwise be lost, must be preserved. Cryopreservation of plant materials in liquid nitrogen has been tried in seeds (7, 8, 9, 17), pollens (2, 4), hardy winter twigs (14, 15), etc. Based on the results of these experiments, it may be concluded that water content is the most important factor contributing to survival of materials cooled to the temperature of liquid nitrogen without cryoprotectants. Thus, the change in the state of water associated with water content appears to be an important problem.

Deep supercooling seems to be an important mechanism for the avoidance of freezing in xylem ray parenchyma of hardy deciduous trees (1, 12) and in florets of the genera *Rhododendron*, *Prunus*, etc (3, 13).

Recently it was also clarified that hydrated lettuce seeds avoided injury by supercooling, but not by tolerating freezing strain (5, 23). Little work has, however, been done so far from the viewpoint of the mechanism of freezing resistance in seeds. The present study was undertaken to elucidate the state of water and the mechanism of freezing resistance of rice and wheat seeds associated with water content, using differential thermal analysis (DTA), and based on the survival of seeds cooled to  $-196^{\circ}\text{C}$ .

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### Materials and Methods

Materials used in the experiments were seeds of winter wheat (*Triticum aestivum* L. cv. Mukakomugi) and rice (*Oryza sativa* L. cv. Horyu).

Water content of the seeds was adjusted to the desired level by leaving them in a water-saturated atmosphere at temperatures of 10, 15 and 26°C. Surface moisture on the seeds was rapidly wiped off by paper towel and the seeds were then used for various experiments. Dry weight was determined by drying the seeds at 105°C for 24 hrs. Although dry weight of some seeds did not reach a stationary state even in 24 hrs at 105°C, the 24 hr value was taken to represent the dry matter to compare with other work. The water content was all expressed as the percentage of fresh weight. 15 to 20 seeds enclosed with aluminum foil were cooled rapidly (cooling rate: 10<sup>3</sup> to 10<sup>4</sup>°C/min) by immersion in liquid nitrogen, and held there at least for 2 hrs. These seeds were then rewarmed either rapidly (rewarming rate: 10<sup>2</sup> to 10<sup>3</sup>°C/min) by immersion in water at 40°C, or slowly (10°C/min) by leaving the seeds in polyethylene bags at 0°C. Some seeds immersed in liquid nitrogen were held at -20°C for at least 16 hrs before being rewarmed in water at 40°C following removal from liquid nitrogen.

Viability was estimated by germinating at 26°C in dark for wheat, and at 30°C in light for rice alongside untreated control seeds, respectively. Normally germinated seeds on the seventh day were counted and germination rate was calculated as the percentage of germination of untreated control.

Rates of rapid cooling and rapid rewarming were detected with 0.1 mm copper-constantan thermocouples and recorded with oscilloscope. The rate was calculated from the time required for the temperature to fall or rise between -5 and -50°C.

Freezing events were observed by differential thermal analysis (DTA), either with an automatically controlled freezing system, DTA-1500L-S (Shinkuriko Co.), which regulated cooling rates between 0.6°C/min and 4.0°C/min, or with a simple system of placing a vacuum flask held at -3°C in a freezer at -40°C (cooling rate: 0.2°C to 0.3°C/min). Exotherm responses were amplified 40 times, and recorded on potentiometric recorders at 10 or 5 mV range, which were connected to 0.2 mm copper-constantan thermocouples in the samples. One, five or ten seeds were used for each DTA. In some tests seeds were ice inoculated by pouring water of about 5°C over the sample with a syringe just before the sample was cooled. In an attempt to determine the source of exotherms, an embryo was excised carefully from the seed and used in DTA, and the remainder of the seed was cooled and analysed independently. Samples for survival tests were removed from the freezer before or after the appearance of exothermic spikes on the DTA profile, then they were kept at room temperature until the viability tests.

### Results and Discussion

Most of rice seeds with water content below about 18% (fresh weight) germinated normally after immersion in liquid nitrogen regardless of rewarming conditions (Fig. 1), while as the water content was raised above 18%, the germination rate decreased according to thawing conditions. The seeds held at  $-20^{\circ}\text{C}$  overnight after removal from liquid nitrogen were killed even at 19% water content. On the other hand, 40% of rice seeds rewarmed rapidly remained alive at a water content of 19% and one sample survived even at 23%. Germination rate of rice seeds rewarmed slowly in air at  $0^{\circ}\text{C}$  was between the two cases described above. So in rice seeds with water contents in the range of 18 to 23%, germination rate of the seeds that were thawed rapidly was higher than that of thawed slowly.

One possible interpretation is that some freezable water remain in these seeds, but that ice crystals formed in cells during rapid cooling may have been small enough to be innocuous (10, 16, 18, 19, 20). When rewarming is carried out rapidly, the crystals may melt before they have time to grow to a damaging size, and the cells will then remain viable. Therefore, in the seeds which contain freezable water, a combination of rapid cooling and rapid rewarming makes it possible to maintain their viability after immersion in liquid nitrogen.

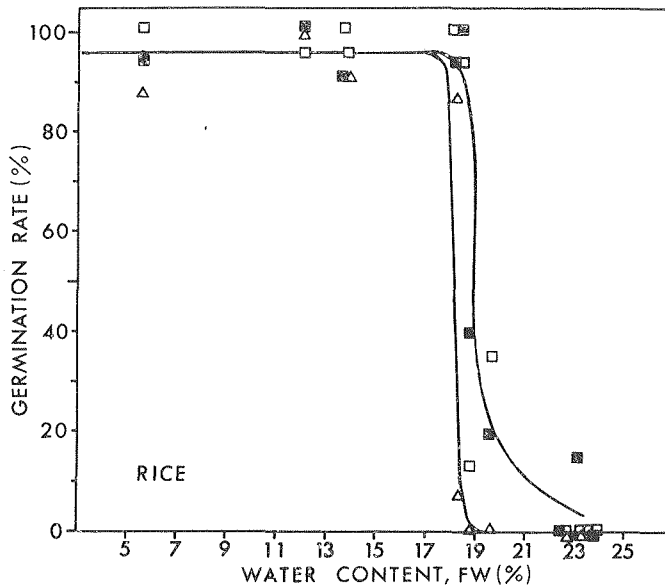


Fig. 1. Effect of water content on the survival of unhulled rice seeds rewarmed by different rewarming methods after immersion in liquid nitrogen.

■: Rapid thawing in water at  $40^{\circ}\text{C}$  ( $10^2$  to  $10^3^{\circ}\text{C}/\text{min}$ ); □: Slow thawing in water at  $0^{\circ}\text{C}$  ( $10^{\circ}\text{C}/\text{min}$ ); △: Held at  $-20^{\circ}\text{C}$  for 16 hrs before being rewarmed rapidly.

On the other hand, germination rates of seeds kept at  $-20^{\circ}\text{C}$  before being rewarmed could be a criterion in estimating the state of water in the seeds since  $-20^{\circ}\text{C}$  is considered as a favourable condition for growth of ice crystals (17, 19, 20). In the case of unhulled rice seeds, a boundary between free and bound water is considered to range between 18 and 19%.

In winter wheat seeds, nearly the same results were obtained after immersion in liquid nitrogen (Fig. 2). The boundary zone between free water and bound water seems to be between 17 and 21% water content, although the number of experiments was not sufficient to determine the boundary more precisely. However, a remaining question is why relative high viability was retained by wheat seeds with water content of 21 to 25% where little or no survival was obtained in rice seeds treated with the same method.

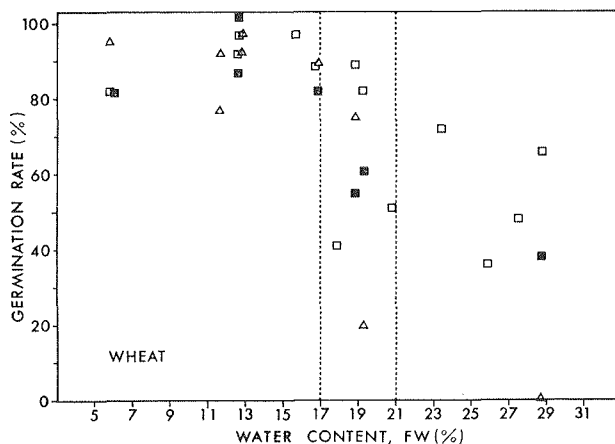


Fig. 2. Effect of water content on the survival of winter wheat seeds rewarmed rapidly ( $10^2$  to  $10^3^{\circ}\text{C}/\text{min}$ ) or slowly ( $10^{\circ}\text{C}/\text{min}$ ), or held at  $-20^{\circ}\text{C}$  for 16 hrs before being rewarmed rapidly, following immersion in liquid nitrogen (cooling rate,  $10^3$  to  $10^4^{\circ}\text{C}/\text{min}$ ).

■: Rapid rewarming; □: Slow rewarming; ▲: Held at  $-20^{\circ}\text{C}$  overnight.

Deviation in water content might be possible in wheat seeds. Thus, some seeds with high embryo water content might be damaged. Also, DTA experiments on different parts of wheat seed revealed that water was not equally distributed in wheat seeds (Table 1). In any case, more experiments are required to compare different periods held at  $-20^{\circ}\text{C}$  after immersion in liquid nitrogen, and to explain the differences in the response to rapid cooling to liquid nitrogen between rice and wheat seeds.

With rice seeds immersed in liquid nitrogen, some abnormal germinations, development only in the coleoptile and no growth in the radicle, were observed with water content over 19% (unpresented data). Most of them

**Table 1.** Exotherm temperature of excised embryos and the remainders of rice and wheat seeds hydrated to different degrees

Seed number	Embryo		The remainder of seed stripped off embryo*	
	Exotherm temperature	Water content (F. W. %)	Exotherm temperature	Water content (F. W. %)
Rice 1	-31	42.2**	None	17.0
2	-27	54.6**	-26	20.9
3	-27	30.0**	-26	21.3
Wheat 1	None	21.0	None	21.5
2	-33	23.5	None	22.0
3	-27, -47	41.0	-28	25.2

\* Rice seed stripped off embryo includes endosperm, testa and pericarp.

\*\* The embryo of a rice seed was too small (about 0.9 mg) to determine its water content accurately (error:  $\pm 20\%$ ).

were with seeds rewarmed rapidly following rapid cooling in liquid nitrogen, while there was no abnormal germination in 125 untreated control seeds. This indicates that there might be differences in sensitivity to rapid cooling in liquid nitrogen and rapid rewarming among different tissues, probably due to differences in their hydration (9).

Results of DTA with rice and wheat seeds are summarized in Figs. 3 and 4, respectively. The critical water contents at which the exotherms appeared on DTA profile were 19.2% in unhulled rice seeds, and 19.4% in intact wheat seeds. Exotherms at the critical water content were smaller than those of seeds with much higher water content. Thus, it is possible that the critical water content is slightly lower than this value. These figures, however, eventually coincide with the critical zone which divides the state of water, free or bound, estimated from the survival of seeds held at  $-20^{\circ}\text{C}$  before being rewarmed rapidly after removal from liquid nitrogen. The border line around 19% in the present data coincides with that of other materials. For instance, the upper limit of bound water content of soybeans was suggested to be 18.7% (F. W.) by the freezing curve method, and around 20% (F. W.) based on NMR spectra, electron microscopy, and scanning electron microscopy (11). A change in the state of water was also estimated during the drying of rice seeds to be 22% (F. W.) using dielectric constant test (25), and 22.2% (F. W.) in yeast using the calorimetric method (22). Therefore, it seems reasonable to consider that the state of water in rice and wheat seeds would shift from free to bound or vice versa at around 19%.

Figs. 3 and 4 also show that the exotherm temperature tends to rise as the water content increases, both in rice and wheat seeds, and that effect of cooling rate on the exotherm temperature must be considered.

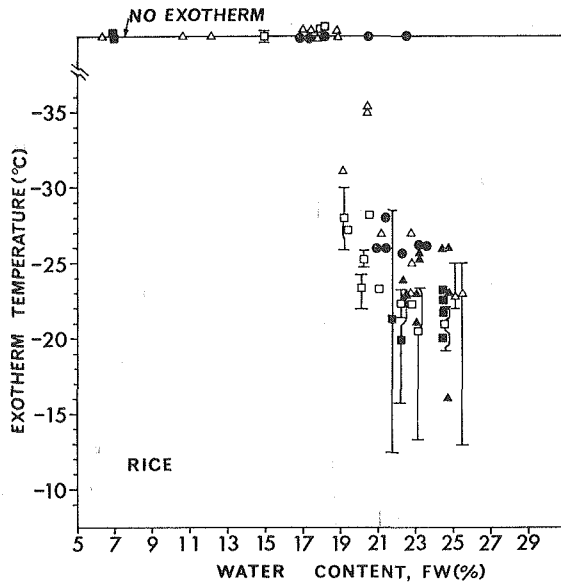


Fig. 3. The effect of water content on the exotherm temperature of rice seeds.

□: Slow cooling (0.2 to 0.3°C/min) without ice inoculation; ■: Slow cooling with ice inoculation; △: Rapid cooling (0.6 to 4°C/min) without ice inoculation; ▲: Rapid cooling with ice inoculation; ●: Seeds stripped off embryo and cooled rapidly; □̄: Average exotherm temperature of 5 or more seeds; □: Exotherm temperature of a single seed. The upper end of ordinate marked "No Exotherm" indicates no exotherm was detected during the cooling to -41°C (slow cooling), -50°C (rapid cooling).

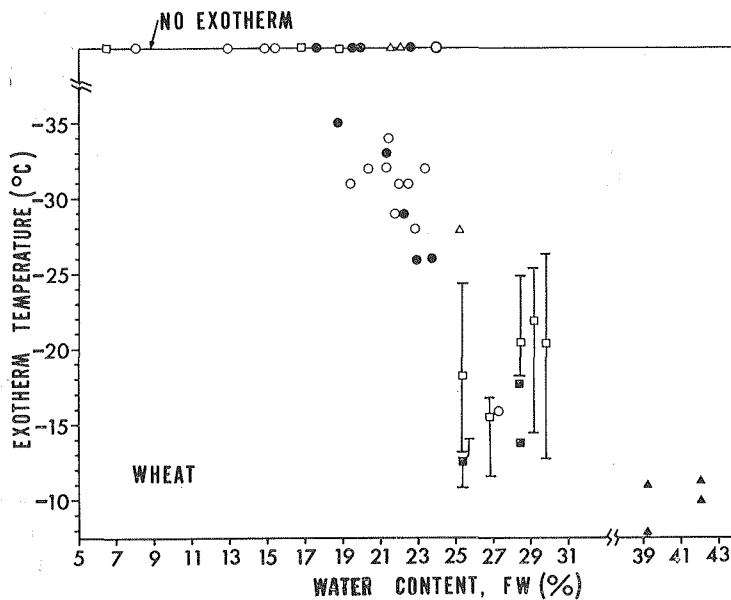


Fig. 4. Effect of water content on the exotherm temperature of winter wheat seeds.

○: Rapid cooling (0.6 to 4°C/min) without ice inoculation; □: Slow cooling (0.2 to 0.3°C/min) without ice inoculation; ■: Slow cooling with ice inoculation; ▲: Germinated seeds; △: Seeds with embryo excised, cooled rapidly; ●: Recooling of seeds killed by cooling to -50°C; □̄: Average exotherm temperature of 5 or more seeds; □: Exotherm temperature of a single seed. The upper end of ordinate marked "No Exotherm" indicates no exotherm was detected during the cooling to -41°C (slow cooling), -50°C (rapid cooling).

As shown in Fig. 3, rice seeds cooled rapidly (0.6 to 4°C/min) supercooled to lower temperatures to some degree than those cooled slowly (0.2 to 0.3°C/min). But there was still a tendency for exotherms to shift to lower temperatures with decreasing water content. At the critical water content around 19%, exotherms appeared in the range of  $-26$  to  $-30^{\circ}\text{C}$  (Fig. 3).

Emphasis should be laid on elucidating the relation between the appearance of exotherms and the viability of seeds. Fig. 5 shows some examples of DTA profiles obtained with rice seeds. Seeds were all alive when they were taken out just before the occurrence of exotherms and three seeds were dead after the occurrence of three exothermic spikes (Fig. 5 a). The same result was obtained in the other thirty DTA experiments with rice seeds. Therefore, the exothermic spike is closely related to the death of rice seeds. Thus, cold resistance or killing temperature of hydrated rice seeds can be detected by differential thermal analysis.

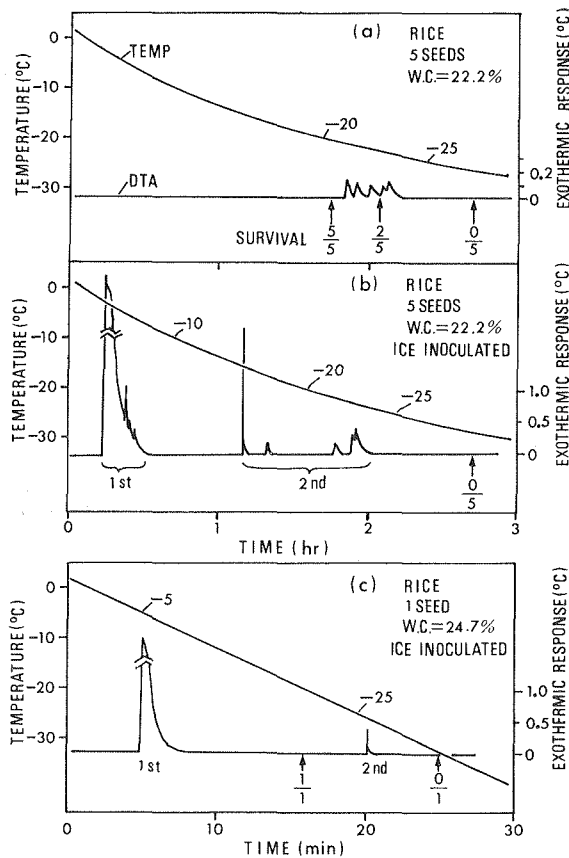


Fig. 5. Relationship between appearance of exotherm and survival of unhulled rice seeds.

Fraction under the DTA line indicates the survival rate of seeds taken out of the freezer at the temperature indicated by the arrow. Ice inoculation of seeds: Seeds were frozen in bulk water. 1st, first exotherm; 2nd, second exotherms.

One question arises as to whether the supercooling observed in rice seeds is affected by touching with ice. In order to check this some ice-inoculation experiments were conducted (Fig. 5b, c) by pouring water over seeds just before carrying out DTA. The large first exotherm (1st) and the small spikes on the shoulder around  $-5^{\circ}\text{C}$  resulted from freezing of bulk water on the seeds, because no decrease was observed in survival after the first exotherm. The second exotherms (2nd) in Fig. 5b, c, still appeared at nearly the same temperatures, ranging from  $-15$  to  $-25^{\circ}\text{C}$  (Fig. 3), regardless of the freezing of bulk water around the seeds. And seeds were all dead after the appearance of spikes. Thus, it may be considered that rice seeds have some mechanisms to prevent ice inoculation from outside and to deep supercool. It may be concluded that freezing resistance of hydrated rice seeds is attributed to freezing avoidance by deep supercooling.

The results with winter wheat seemed more complicated (Fig. 6). In some cases, occurrence of exotherms was closely related to the death of wheat seeds (Fig. 6a), while in other cases the exotherm temperature was not necessarily the killing temperature (Fig. 6b). One possible explanation is that distribution or localization of water within a seed might vary from one seed to another, and that some of the exotherms might have been derived from freezing of water which had nothing to do with the viability of the seed. Another possibility is that one seed might have released more than one exotherm. The third possibility is that wheat seeds could tolerate

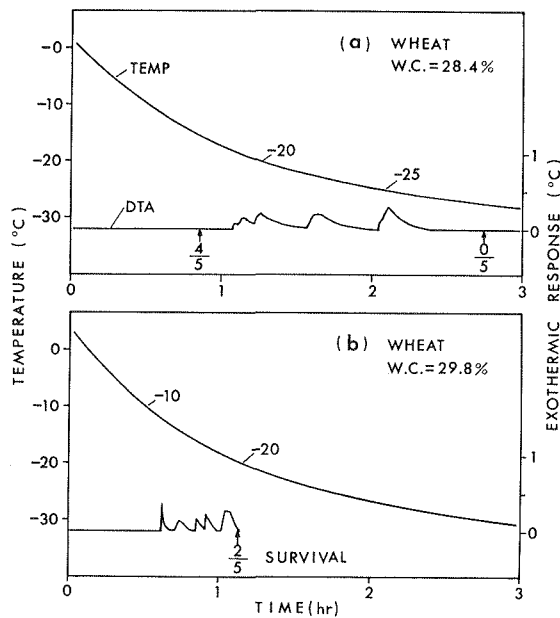


Fig. 6. Relationship between appearance of exotherm and survival of hydrated wheat seeds cooled at  $0.2$  to  $0.3^{\circ}\text{C}/\text{min}$ .

Fraction under the DTA line indicates the survival rate of seeds taken out of the freezer at the temperature pointed by the arrow.

freezing, thus they were alive even after the occurrence of the exotherm, which would be interesting, but it is unlikely, because sudden freezing at  $-20^{\circ}\text{C}$  easily causes intracellular freezing which is lethal. Combination of the first and the second possibilities seems to be reasonable because in some fully hydrated seeds, both rice and wheat, two exotherms were exhibited by one seed (one example is shown in Table 1). Some hardy winter wheat seedlings tolerate freezing from  $-10$  to  $-22$  when hardened well (6, 21, 24). Thus, it would be interesting if the type of freezing resistance changes with the development of wheat seeds.

Some experiments were conducted to elucidate the source of the exotherm. Embryos were carefully excised from rice and wheat seeds and their exotherms were detected together with the remaining parts of the seeds from which embryos were excised. Examples are shown in Table 1. At relatively low water content, the embryo was the source of the exotherm both in rice and wheat seeds. As water content increased, however, exotherms were also detected from embryo-excised seeds at nearly the same temperature as embryo-derived exotherms. In excised embryos of high water content, two exotherms, small and large, were observed (Table 1, wheat 3). This is supported by the fact that intact rice or wheat seeds yielded two exotherms when they were fully hydrated (unpresented data). These changes in exotherm source are expected to represent qualitative and quantitative changes in water distribution. Around the critical water content, water seemed to be more localized in the embryo. This supports the finding that water content of the embryo is considerably higher than that of the entire seed when a wheat seed is soaked in water (9).

Water imbibed lettuce seeds with damaged endosperm did not deep supercool (23). In the present experiments, however, embryos and endosperms were supercooled to  $-15$  to  $-25^{\circ}\text{C}$ , though they were slightly damaged during excision. This would indicate that integrity of the embryo or endosperm is not indispensable to supercooling ability at least when seeds are not inoculated with ice.

One interesting observation is that hydrated wheat seeds which had been killed by cooling below  $-50^{\circ}\text{C}$  exhibited exotherms in the same temperature range as the living seeds (see closed circles in Fig. 4). Thus, deep supercooling of wheat seeds seems not to be an exclusive property of living seeds. The same characteristics were reported with hydrated lettuce seeds (23) and xylem ray parenchyma cells in apple twigs (12).

The results with winter wheat seeds were rather complicated and more work is required. It is also necessary to try hydrating seeds in different ways, for example, imbibition in water.

Many seeds of deciduous trees in temperate and boreal zones fall in autumn and overwinter in various forms. Supercooling as a freezing resistance mechanism can be expected in those seeds which overwinter, under decayed leaves or snow cover, or in seeds remaining in the corns or fruit

coats on the trees during the winter. Further research in this direction is also required.

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