Suppressive Effects of Low Seed-Soaking Temperatures on Germination of Long-Term-Stored Rice Seeds

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Abstract: We investigated the effects of soaking temperature and duration on the germinability of seeds of rice (Oryza sativa L., cv. Koganemochi, Gohyakumangoku, and Koshihikari) that had been stored for a long period. The germinability of the seeds soaked at 5°C for 5 d was markedly lower than that of seeds soaked at 12°C for 5 d. The germinability of the seeds soaked at 5°C for 24 hr was not increased by subsequent soaking at 12°C for 4 d. On the other hand, the germinability of the seeds soaked either at 12°C for 24 hr or at 30°C for 80 min was similar to that of seeds soaked at 12°C for 5 d, even when followed by treatment at 5°C. Thus, the soaking temperature during the first 24 hr was most important for the germination of rice seeds that had been stored for a long period. Western blotting analysis revealed characteristic expression patterns of α-amylase isoforms in cultivars correlating with the germinability after soaking at a low-temperature.

Key words: α-Amylase, Germination, Long-term-stored seeds, Oryza sativa, Seed-soaking, Water temperature.

Rice seeds are soaked before germination to ensure uniformity of seedling growth by removing germination inhibitors (Mikkelsen and Sinah, 1961; Takahashi, 1967). In Japan, breeder seeds are stored for more than a year before planting to allow characteristics to be confirmed and to verify freedom from contamination. Seeds stored for a long period frequently produce stunted and abnormal seedlings depending on the soaking condition. This study was conducted to identify the optimum temperature and duration of soaking to ensure germination and seedling emergence after long-term storage of rice seeds.

In Niigata Prefecture, the seeds soaked in the open in late March to early April are often exposed to temperatures below 5°C. The three main cultivars grown in Niigata, Koshihikari (nonglutinous rice), Koganemochi (glutinous rice), and Gohyakumangoku (brewers’ rice) are affected differently by the low soaking temperature. Koganemochi and Gohyakumangoku seeds after long-term storage must be soaked at >10°C for high germinability (Sato et al., 2003). Dormancy is terminated after 100°C·day (Hirai et al., 2008). The initial soaking temperature has been reported to affect the germinability of the seeds after long-term storage: germination was improved by an initial temperature of 17.5°C (Kitano et al., 2010) or 20°C (Itayagoshi et al., 2011). Here, we investigated the effects of the soaking conditions in detail to determine the optimal conditions for germination of these cultivars.

α-Amylase plays a key role in degrading the starch reserves in germinating cereal seeds (Akazawa et al., 1988; Fincher, 1989; Jones and Jacobsen, 1991; Asatsuma et al., 2005). Germination and seedling growth were markedly delayed in α-amylase-suppressed rice seeds (Asatsuma et al., 2005). Therefore, we also examined the expression pattern of α-amylase isoforms in germinating and non-germinating seeds in rice cultivars affected differently by the low soaking temperature.

Materials and Methods

Cultivars tested were Gohyakumangoku and Koganemochi, which show low germinability in the nursery (seedling fields) in Niigata, and Koshihikari Niigata BL No. 2 (Ishizaki et al., 2005), which germinate well. All three cultivars were grown at the Niigata Agricultural Research Institute Crop Research Center, Nagaoka city, Niigata. Seeds harvested from 2009 to 2012 were air-dried and then stored for 1.5 or 2.5 years at 10 – 12°C. The investigation was carried out from January to March 2012 to 2014.

1. Influence of initial seed-soaking temperature on germination

Batches of 100 stored seeds were placed in bags of nonwoven fabric. Each bag was soaked in 8 L of tap water. Seeds were not sterilized. In the low-temperature treatment, the seeds were soaked at 5°C for 20, 40, 80, 120, or 360 min.
or 24 hr, and then at 12°C for another 5 d (Fig. 1A). In the 12°C treatment, the seeds were soaked at 12°C for 20, 40, 80, 120, or 360 min or 24 hr, and then at 5°C for another 5 d (Fig. 1B). In the high-temperature treatments, the seeds were soaked at 30 or 60°C for 5 to 120 min, and then at 5°C to 5 d from the start (Fig. 1C, D). Control seeds were soaked at 12 or 5°C for 5 d (Table 1). The water in all treatments was replaced on day 3.

After soaking, seeds were spin-dried in a dehydrator for 1 min to remove surface water, and then used in a germination test. Each batch of 100 seeds was placed on filter paper in a plastic dish (85 mm × 160 mm × 33 mm inside) and incubated with 9 mL of tap water at 25°C under light (17.5 μmol m⁻² s⁻¹). Three replicates were used for each treatment. On day 7, normal seedlings with the first leaf and a root (> 5 mm length) were counted to estimate the germination rate.

### 2. Influence of initial seed-soaking temperatures on emergence from soil

Emergence tests were performed between January and March in 2012 to 2013. Seeds stored for 1.5 and 2.5 years were treated with a hydration agent (1:7.5 dilution of pefurazoate, fludioxonil, and copper oxychloride; Hokko Chemical Industry Co. Ltd., Japan), and then 11.3 g of seed was packed in a nonwoven fabric bag. The seeds were immediately soaked at 5, 12, or 30°C in 40 L of tap water in an incubator set at 4°C (Sanyo Electric Co. Ltd., Japan). The air temperature was alternated between 4°C (night) and 14°C (day) every 12 hr to simulate the temperature in Nagaoka city. The seeds were incubated for 5 d at designated temperatures, and the water temperature was monitored by a data logger (TR-51; T&D Co., Japan). The water was replaced on day 3. Control seeds were soaked at 12°C for 5 d. The seeds were kept in a water tank at 30°C for 18 hr to sprout. The sprouted seeds were spin-dried in a dehydrator for 1 min and spread on fine soil (Chibyonawashiro; Honen Agri Co. Ltd., Japan) 15 mm deep, including fertilizer (N, P, K, Mg: 0.44, 0.66, 0.55, 0.11 g kg⁻¹), in a plastic dish (85 mm × 175 mm × 40 mm) with six 2-mm holes in the bottom. These seeds were covered with another 5 mm of soil. After moistening with tap water, the seeds were incubated first at 30°C for 36 hr in darkness and then at 20°C for 72 hr under light (17.5 μmol m⁻² s⁻¹). All treatments had two replicates. The seedlings in the central 8.5 mm × 8.5 mm of the dish were collected and

| Cultivar          | Strage period | Germination (%) |
|-------------------|---------------|-----------------|
|                   | 12°C/5 d | 5°C/5 d |
| Koshihikari       | 1.5 yr | 95.7 ± 1.5 | 96.6 ± 0.2 |
|                   | 2.5 yr | 94.6 ± 2.2 | 88.7 ± 5.1 |
| Koganemochi       | 1.5 yr | 87.0 ± 7.8 | 55.9 ± 21.6 |
|                   | 2.5 yr | 58.3 ± 15.1 | 12.4 ± 8.9 |
| Gohyakumangoku    | 1.5 yr | 95.4 ± 0.6 | 62.0 ± 6.2 |
|                   | 2.5 yr¹ | 76.7 ± 5.4 | 37.0 ± 23.8 |

Means and standard errors were obtained from experiments in 2012 to 2014 (*n* = 3; †2013 and 2014 only).
classified into normal, delayed (< 6 mm shoot length), or non-germinated. The emergence rate was defined as the ratio of normal seedlings to total seeds.

3. Western blotting analysis

After storage for 2.8 years, 100 g of unsterilized dry seeds of each cultivar were packed in a woven polypropylene bag and soaked in 8 L of tap water at 5ºC for 5 d. The water was replaced on day 3. The soaked seeds were then incubated at 30ºC for 36 hr in a water tank, and the germinated and non-germinated seeds were separated, husked, frozen immediately in liquid nitrogen, and stored at –80ºC. Just before analysis, the germinated, non-germinated and dry seeds were powdered in liquid nitrogen with a mortar and pestle, and the powder was suspended in 400 mL of 50 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid buffer (pH 7.5). The extraction of each sample (the germinated, non-germinated or dry seed) was separately carried out three times. The suspension was centrifuged at 15,000 × g for 10 min at 4ºC. An aliquot of the supernatant (10 μg protein) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% separation gels according to Laemmli (1970). After electrophoresis, the separated proteins were transferred to polyvinylidene fluoride membranes (Hybond-P; GE Healthcare, USA) (Towbin et al., 1979). The membranes were incubated in 15 mM phosphate-buffered saline (pH 6.8) containing 0.1% Tween-20 and 5% skim milk for blocking, and then incubated with specific antibodies: antirice α-amylase isoform AmyI-1 (rabbit serum; 1:10,000), AmyII-3 (mouse serum; 1:500), AmyII-4 (rabbit serum; 1:5000) (Mitsui et al., 1996), and AmyII-6 (rabbit serum; 1:5000) (Nanjo et al., 2004a). Horseradish peroxidase-conjugated anti-rabbit IgG (Nacalai Tesque, Japan) and antimouse IgG (MP Biomedicals, USA) were used as secondary antibodies. The immunoreactive bands were visualized using a chemiluminescence reagent (Amersham, UK), and quantified with a LAS-3000 molecular imager (Fujifilm, Japan) (Asatsuma et al. 2005) and Image Master 2D Platinum (Amersham Bioscience, UK) (Asakura et al., 2007).

Results

1. Influence of initial seed-soaking temperature on germination

At a soaking temperature of 12ºC, approximately 90% (87.0 – 95.7%) of seeds of all cultivars stored for 1.5 years, and of the seeds stored for 2.5 years, 94.6, 58.3 and 76.7% of Koshihikari, Koganemochi and Gohyakumangoku germinated normally (Table 1). At 5ºC, 96.6, 55.9, and 62.0% of Koshihikari, Koganemochi and Gohyakumangoku seeds, stored for 1.5 years germinated normally, but of those seeds stored for 2.5 years, 88.7, 12.4 and 37.0%, respectively, germinated normally.

To examine the effect of low-temperature soaking on the germination rate in more detail, we soaked seeds at 5ºC for the first 20 min to 24 hr and thereafter at 12ºC (Fig. 2).
Soaking at 5°C for 360 min decreased the germination rates of both Koganemochi and Gohyakumangoku seeds (Fig. 2A, B). The effects of soaking at 5°C for up to 360 min were comparable to those of soaking at 5°C for 5 d. In addition, the Gohyakumangoku seeds germinated in response to soaking at 5°C for 20 min.

We also tested the effect of initial soaking at 12°C for 20 min to 24 hr and thereafter at 5°C (Fig. 1B). The germination rates of Koganemochi seeds at 5°C gradually increased with the increase in the period of initial soaking at 12°C from 20 min to 240 min reaching the level of germination at continuous 12°C (control) (Fig. 3A, B). The germination rate of Gohyakumangoku seeds at 5°C was almost fully restored by initial soaking at 12°C for 24 hr (Fig. 3A, B). It is noteworthy that initial soaking at 30°C markedly improved the germination at 5°C in both Koganemochi and Gohyakumangoku; soaking 30°C for 80 min increased the germination rates at 5°C of seeds stored for 1.5 years up to 90% (Fig. 4A) and that of seeds stored for 2.5 years up to 80% (Fig. 4B). The seeds of Koshihikari...
were unaffected by initial soaking at 30°C (Fig. 4). Initial soaking at 60°C for 10 min also improved the germination of Koganemochi and Gohyakumangoku seeds stored for 1.5 years, but soaking 60°C for ≥ 40 min inhibited the germination in all three cultivars (Fig. 5A). Seeds stored for 2.5 years responded to soaking at 60°C, similarly to those stored for 1.5 years although the improvement of the germination in Koganemochi and Gohyakumangoku seeds was limited (Fig. 5B).

2. Influence of initial seed-soaking temperature on emergence from soil

The emergence tests were performed under conditions similar to those in the field in Nagaoka city. The initial soaking temperature was 5, 12, or 30°C, and the air (and water) temperature fluctuated between 4 and 12°C every 12 hr (Fig. 6). In the seeds stored for 1.5 years (Fig. 7A) initial soaking at 30°C gave an emergence rate of ≥ 90% in each cultivar, equal to or better than soaking at a constant 12°C (Fig. 7). Initial soaking at 12°C gave slightly lower emergence in Koganemochi and Gohyakumangoku. Initial soaking at 5°C reduced the emergence rates of both cultivars to 79 – 49% (Fig. 7A). In the seeds stored for 2.5 years initial soaking at 30°C improved the emergence of Koganemochi and Gohyakumangoku compared with initial soaking at 12°C and a constant soaking at 12°C (Fig. 7B). As expected, initial soaking at 5°C severely lowered the emergence of Koganemochi and Gohyakumangoku (Fig. 7B). Initial soaking at 30°C gave the same shoot length in each cultivar seed stored for 1.5 years as soaking at a constant 12°C (Fig. 8A). On the other hand, the initial 30°C treatment enhanced shoot elongation compared with the constant 12°C treatment in Koganemochi seeds stored for 2.5 years (Fig. 8B). The initial 30°C treatment also stimulated shoot elongation in Gohyakumangoku seeds stored for 2.5 years compared with the initial and continuous 12°C treatments (Fig. 8B). Initial soaking at 5°C inhibited shoot growth of Koganemochi seeds stored for 2.5 years (Fig. 8B), and it severely inhibited the shoot growth in Gohyakumangoku seeds stored for 2.5 years (Fig. 8B).

3. Western blotting analysis

Seeds stored for 2.8 years were germinated, and α-amylases were analyzed by western blotting with specific antibodies (Mitsui et al., 1996; Nanjo et al., 2004a). The amounts of α-amylase proteins in dry seeds were below the detection limit (Fig. 9A,B) or very low (Fig. 9C,D) compared with that in the germinated and non-germinated seeds (soaked seeds) (Fig. 10). The expression of Amyl-1 was high in the germinated seeds of all three cultivars, but low in the non-germinated seeds (Fig. 10A). The pattern of Amyl-1 expression in these seeds was similar to that of Amyl-1 expression, although the reduction of Amyl-3 expression in the non-germinated
Fig. 7. Influence of initial soaking temperature on seedling emergence of seeds stored for 1.5 years (A) and 2.5 years (B). ■, normal germination; □, delayed germination (< 6 mm shoot length); □□, non-germinated seeds. Values are means ± SEM (n = 2) of data in 2012–2013 (†2013 only). Means followed by the same letter are not significantly different at the 5% level as determined by Tukey’s test. “c.t.”, continuous temperature.

Fig. 8. Influence of initial soaking temperature on seedling growth of seeds stored for 1.5 years (A) and 2.5 years (B). Values are means ± SEM (n = 2) of data in 2012–2013 (†2013 only). Means followed by the same letter are not significantly different at the 5% level as determined by Tukey’s test. “c.t.”, continuous temperature.
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seeds was relatively weak (Fig. 10B). It is noteworthy that AmyI-1 expression level in non-germinated Koshihikari seeds was relatively higher than those in Gohyakumangoku and Koganemochi (Fig. 10A, Table 2). AmyII-4 expression was lower in the germinated seeds than in the non-germinated seeds (Fig. 10C, Table 2). AmyII-6 expression was higher in the germinated seeds than in the non-germinated seeds of Koshihikari, but lower in the germinated seeds than in the non-germinated seeds of Gohyakumangoku and Koganemochi (Fig. 10D, Table 2).

Fig. 9. Western blotting analyses of α-amylase isoforms in dry and germinated seeds. Preparation of sample seeds and protein extraction were described in Materials and Methods. Proteins extracted from dry and germinated seeds were separated by SDS-PAGE, followed by Western blotting with anti-AmyI-1 (A), AmyII-3 (B), AmyII-4 (C), and AmyII-6 (D) antibodies and horseradish peroxidase-conjugated secondary antibodies.

Fig. 10. Western blotting analyses of α-amylase isoforms in germinating and non-germinating seeds. Preparation of sample seeds and protein extraction were described in Materials and Methods. Proteins extracted from germinated and non-germinated seeds were separated by SDS-PAGE, followed by Western blotting with anti-AmyI-1 (A), AmyII-3 (B), AmyII-4 (C), and AmyII-6 (D) antibodies and horseradish peroxidase-conjugated secondary antibodies.

Table 2. Quantitation of α-amylase isoform proteins in germination and non-germination seeds.

|                | AmyI-1 | AmyII-3 | AmyII-4 | AmyII-6 |
|----------------|--------|---------|---------|---------|
| Germination    |        |         |         |         |
| Koshihikari    | 1      | 1       | 1       | 1       |
| Koganemochi    | 0.42 ± 0.08 | 0.54 ± 0.15 | 1.03 ± 0.20 | 0.49 ± 0.11 |
| Gohyakumangoku | 1.45 ± 0.46 | 0.82 ± 0.15 | 1.52 ± 0.27 | 0.60 ± 0.11 |
| Non-germination|        |         |         |         |
| Koshihikari    | 0.74 ± 0.30 | 0.92 ± 0.14 | 2.12 ± 0.36 | 0.66 ± 0.03 |
| Koganemochi    | 0.24 ± 0.15 | 0.36 ± 0.16 | 2.81 ± 0.95 | 0.87 ± 0.30 |
| Gohyakumangoku | 0.46 ± 0.27 | 0.39 ± 0.16 | 2.11 ± 0.23 | 1.38 ± 0.50 |

Western blotting experiments were carried out as described in Fig. 10. Quantitation of α-amylase protein bands was performed as described in Materials and Methods. Each amount of α-amylase isoform protein in germinated Koshihikari seeds was normalized to 1. Values are means ± SEM. (n = 3).
Discussion

Low-temperature (5°C) soaking within the first 24 hr (Fig. 2) inhibited germination of certain cultivars. The common problem of poor germination even after soaking at 12°C for most of the soaking period is therefore due to low temperature at the start. Even where the outside air temperature falls to < 10°C, if the water temperature can be kept at ≥ 12°C during the first day, following lower temperatures will be of no concern (Fig. 3). Although incubation at 30°C is usually favorable enough for rice seed germination, it takes at least 24 hr for seeds to absorb water and begin germination. Seeds are commonly soaked at < 15°C before sprouting at 30°C to promote uniform germination in rice production. In contrast, germination tests are carried out at 25 to 30°C, to avoid the adverse effect of cold temperature on germination. Thus, germination in the field is a difficult task can be even for experienced growers. We found that initial soaking at 30°C for 80 min induced normal germination even if the soaking temperature thereafter dropped to 5°C (Fig. 4). It is noteworthy that 30°C was more effective than 12°C; emergence tests performed in conditions close to those in the field further support the advantage of initial soaking at 30°C (Figs. 7, 8). We consider that this treatment should be promoted as a method for improving the germination of long-term-stored seeds, although field testing is still needed.

Initial soaking at 60°C is similar to the warm-water sterilization method commonly used for disinfection without pesticides (Okabe et al., 2009). Although Tsujimoto and Izumi (2009) examined the effect of warm-water sterilization on rice after 1-year storage, its relevance to seed germination was limited, since warm-water sterilization is rarely used in seed production. However, we found that initial soaking at 60°C for 10 min improved germination (Fig. 5). Since warm-water treatment has been found to stimulate the germination of deeply dormant *indica* seeds and hasten the germination of weakly dormant *japonica* seeds (Fukuda et al., 2013), the effect of the 60°C treatment on germination might be related to dormancy breaking.

Like seeds of Koganemochi and Gohyakumangoku, after a long-term storage seeds of Koshihikari (a nonglutinous rice widely planted in Niigata) showed a low germination rate after low-temperature soaking (unpublished data). Many *japonica* cultivars grown in other prefectures also germinate poorly when soaked at a low temperature (Hirai et al., 2008; Kitano et al., 2010). Therefore, it is necessary to determine the optimum seed-soaking conditions for individual cultivars. Seeds of the *indica* cultivar Kasalath harvested within the previous year showed a germination rate of 99% when soaked first at 5°C for 14 d followed by incubation at 25°C for 7 d. However, initial soaking at 8 to 19°C for 14 d (during which soaking at ≥ 18°C caused partial germination) caused secondary dormancy. Seven other cultivars originating from India showed a similar phenomenon (Miura and Araki, 1996). It is still unclear whether the failure to germinate caused by low-temperature soaking represents an irreversible loss of germinability or a reversible secondary dormancy.

Cereal α-amylases are encoded by a multigene family (Huang et al., 1992; Yu et al., 1996; Mitsui and Itoh, 1997). The several α-amylase isoforms identified in rice cells are grouped into two major classes, AmyI and AmyII, by their enzyme properties (Mitsui et al., 1996; Nanjo et al., 2004a; Kitajima et al., 2009). AmyI-1, II-3, II-4, II-5, and II-6 were the major isoforms in rice seedlings and suspension-cultured cells derived from embryos (Mitsui et al., 1996; Nanjo et al., 2004a). AmyI-1 expression levels were much greater in germinated seeds of all three cultivars than in non-germinating seeds (Fig. 10A, Table 2). In contrast, AmyII-4 expression was lower in germinated seeds of all cultivars than in non-germinated seeds (Fig. 10C, Table 2). The mode of AmyII-6 expression in Koshihikari was similar to that of AmyI-1, whereas that in Koganemochi and Gohyakumangoku resembled AmyII-4 expression (Fig. 10).

AmyI-1 is the main amylolytic enzyme in germinating rice seeds (Mitsunaga et al., 2001), and its suppression markedly delayed germination and seedling growth of rice (Asatsuma et al., 2005). Indeed, the non-germinated seeds of Koganemochi and Gohyakumangoku exhibited severe reduction of AmyI-1 expression (Table 2). This decrease strongly suggests that long-term storage damaged the AmyI-1 expression in seeds of Koganemochi and Gohyakumangoku. On the other hand, the non-germinated seeds of Koshihikari expressed AmyI-1 at significant levels (Table 2). We consider that the loss of germinability of Koshihikari seeds might be caused by some factors not related to the expression of AmyI-1. In cold-soaking-tolerant cultivars, a certain level of AmyI-1 expression might remain regardless of germination status. The expression of both AmyII-3 and AmyII-4 is precisely regulated by sugars (Huang et al., 1990; Chan and Yu, 1998; Mitsui et al., 1999; Nanjo et al., 2004b; Chen et al., 2006). However, in the present study, these expression patterns in non-germinated seeds were different (Table 2). We infer that marked accumulation of AmyII-4 in non-germinated seeds caused an uncharacterized stress-induced event rather than a sugar-starvation response.

In summary, we investigated the physiological and biochemical influence of cold-water soaking on seed germination. The soaking temperature during the first 24 hr was extremely important for the germination of long-term-stored rice seeds. Notably, initial soaking at 30°C improved germination in soil under conditions similar to field conditions. We also found differences in expression patterns of AmyI-1, AmyII-3, AmyII-4 and AmyII-6 in imbibed seeds between cold-soaking-tolerant and -susceptible cultivars. Further characterization of low-
temperature-induced poor germination in long-term-stored rice seeds is in progress.

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