



## Anaerobic Conditions Improve Germination of a Gibberellic Acid Deficient Rice

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**ABSTRACT** Dwarf plants are useful in research because multiple plants or plant communities can be grown in small growth chambers. We have studied a gibberellic acid (GA) mutant of rice (*Oryza sativa japonica* cv. 'Shiokari' line N-71) that is extremely dwarf (20-cm tall). Unfortunately, this GA mutation is associated with poor germination (70%) under aerobic conditions. Neither exogenous GA, nor a dormancy-breaking heat treatment improved germination. However, excellent germination (95%) was achieved by germinating the seeds anaerobically, either in a pure N<sub>2</sub> environment or submerged in unstirred tap water.

**INTRODUCTION** Extremely dwarf lines of crop plants are useful in research because plant canopies can be grown easily under electric lights on a lab bench or in small growth chambers. The short height of these dwarf lines is typically the result of mutations in hormone production or perception (Chandler and Robertson, 1999; Hanumappa et al., 1999). We have been characterizing the growth and development of a dwarf breeding line of rice. Analysis of this breeding line by Honda et al. (1996) revealed a build-up of gibberellic acid-20 (GA<sub>20</sub> inactive form) and non-detectable levels of GA<sub>1</sub> (active). This characteristic is a result of a mutation in the gene that codes for 3β-hydroxylase, the enzyme that converts GA<sub>20</sub> to GA<sub>1</sub> (Mitsunaga et al., 1994; Kinoshita and Shinbashi, 1982). Application of GA to seedlings fully restores this line to the parent phenotype. The lack of active GA results in an extremely short (20-cm tall) rice variety that we have named 'Super Dwarf'. This mutation, however, is associated with poor germination (~70%), non-uniform germination rates, and uneven stand establishment.

Germination of monocots has been extensively studied (Jones, 1926; Roberts, 1961; Cohn, 1986). Ritchie and Gilroy (1998) proposed a detailed model describing the biochemical and molecular factors preceding germination based on the barley aleurone system. In this model, GA in the seed is perceived on the plasma membrane after imbibition in aerobic conditions. The perception of GA is followed by the transcription of hydrolytic enzymes including α-amylase. α-amylase is then excreted outside the plasma membrane where it breaks down starch into simple sugars. Hanson and Jacobsen (1984) reported that α-amylase is not synthesized in barley under anaerobic conditions, even after the addition of exogenous GA, and barley seeds fail to germinate in anoxia. Conversely, rice is able to produce α-amylase under both aerobic and anaerobic conditions, although its synthesis is delayed in anaerobic conditions (Perata et al., 1992). The ability of rice to make α-amylase without O<sub>2</sub> is likely one of the reasons that it can germinate in anaerobic conditions. It is not known if this expression in anaerobic conditions is mediated by GA, is constitutively activated, or if it is stimulated by multiple signals (GA in addition to other metabolic products).

Rice and other cereal grains also exhibit post-harvest dormancy. While fresh seed of japonica varieties is usually not dormant (Ellis et al., 1983), the low germination percentage of 'Super Dwarf' rice may be the result of partial dormancy. Many methods have been used to break dormancy including light, temperature, exogenous applications of GA (Berrie, 1984), nitrate (Cohn et al., 1983), alcohols, carboxylic acids (Cohn et al., 1987), and smoke (Thomas and van Staden, 1995). Based on the findings in two papers by Roberts (1963 and 1965) wherein germination of rice was improved after three days of dry heat (50 to 55°C), the International Rice Research Institute (IRRI) heats all japonica and indica varieties regardless of their dormancy tendencies to 50°C for 3 days. This method is an easy way to treat large seed lots, but the underlying mechanism is unknown. Our objective was to improve the germination rate and uniformity of 'Super Dwarf' rice.

**MATERIALS AND METHODS** Seeds of 'Super Dwarf' rice were obtained from Toshiro Kinoshita, Hokkaido University, Japan. This breeding line was made from a mutant selection from 'Shiokari' (Kinoshita and Shinbashi, 1982). All germination tests were performed in reach-in growth chambers set at constant 20, 23, 27, 30, 33 or 36 °C ± 0.5 °C. Seeds were counted twice daily for up to ten days, until there was no change in the number of seeds germinated over the previous 24 hours. Each treatment consisted of three replicate germination boxes each containing 25 seeds.

In the anaerobic and hypoxic treatments, 25 seeds each of 'Super Dwarf' rice, wheat (*Triticum aestivum* L. cv. 'USU Apogee'), and two types of indica rice ('29-Lu-1' and 'Ai-Nan-Tsao') were grouped in germination boxes. One layer of blotter paper was placed in the bottom and wetted with tap water by syringe through a septum in the lid

of the box after purging the head space for 24 hours with humidified N<sub>2</sub> (anaerobic) or a combination of N<sub>2</sub> and atmospheric air (hypoxic, 1%, 2% and 4% O<sub>2</sub>). Tap water was used instead of distilled water because it contains 1 mM calcium and micro-nutrients required by a germinating seed.

Many previous studies probably lacked continuous, strict anaerobic conditions for the duration of their trials. In our systems, O<sub>2</sub> concentration was continuously measured in the head space with an O<sub>2</sub> sensor (Model O2S, Apogee Instruments, Logan, UT) that was placed inside the box and monitored with a datalogger (CR10T, Campbell Scientific, Logan, UT). O<sub>2</sub> leakage into the boxes was detected by the O<sub>2</sub> sensors if the gas flow was stopped for more than 30 seconds. To prevent this leakage, boxes were maintained under a small positive pressure with the humidified gases to ensure that all leaks were from the inside out according to standard gas-exchange methods for 'open' systems. Ultra-high purity N<sub>2</sub> was used, which contains < 0.5 mol mol<sup>-1</sup> (ppm) O<sub>2</sub>. This concentration would result in a maximum of 0.6 nmol L<sup>-1</sup> O<sub>2</sub> at 20 °C to 0.42 nmol L<sup>-1</sup> O<sub>2</sub> at 33 °C (0.02 to 0.013 ppb) dissolved in solution. Because of the continuous O<sub>2</sub> measurements, we were able to verify steady-state anaerobic conditions within the boxes for the duration of each trial. For the hypoxic environments, a continuous flow of humidified gas was adjusted as needed to maintain boxes in 1%, 2%, or 4% O<sub>2</sub> according to the O<sub>2</sub> sensor.

Two visual indicators of O<sub>2</sub> concentration within the boxes were used. Without O<sub>2</sub>, the coleoptile of rice seedlings emerges and elongates without emergence of the radicle (Perata and Alpi, 1993). The radicle emerges only after the coleoptile reaches O<sub>2</sub>. This development permits the coleoptile to elongate above the water line to reach O<sub>2</sub> and allows O<sub>2</sub> diffusion to the roots via aerenchyma. Wheat seeds are often added to anaerobic germination tests as an example of a seed that fails to germinate in the absence of O<sub>2</sub>. For this reason, 'USU-Apogee' wheat was tested at all O<sub>2</sub> concentrations.

The effect of exogenously applied GA on germination was evaluated by adding GA3 to tap water at 50, 100, and 1000 M and adding the solutions to germination boxes containing 'Super Dwarf' seeds. Heat treatments were evaluated by heating seeds in a forced-air drying oven at 50 ± 1 °C for 72 hours prior to germination (Roberts, 1965).

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**RESULTS AND DISCUSSION** 'Super Dwarf' rice germinated best at 33 °C (Fig 1). The indica varieties '29-Lu-1' and 'Ai-Nan-Tsao' were much less sensitive to temperature than 'Super Dwarf' and germinated above 90% at all temperatures. In anaerobic conditions, 'Super Dwarf' had higher germination percentages at all temperatures, and reached 95% germination at 33 °C. At 1% O<sub>2</sub>, germination decreased to 76%, and was 68% at 4% and 20.9% O<sub>2</sub> (fully aerobic conditions) (Fig 2). There was no effect of O<sub>2</sub> on final germination percentages of indica varieties. Perata et al. (1992) suggested that indica varieties have lower rates of germination in anoxia than japonica varieties, but reach similar final germination percentages after four days in anoxia. We observed a one-day delay in the start of germination in anoxia when compared to aerobic germination, but no difference between indica or japonica varieties in the start of germination. 'USU-Apogee' wheat did not germinate until the O<sub>2</sub> concentration was above 1% O<sub>2</sub>. The O<sub>2</sub> requirement was likely due to the warm temperatures, as Alpi and Beevers (1983) reported 50% wheat germination in 1% O<sub>2</sub> at 20 °C. In our studies, wheat germination was only 65% at 33 °C because this temperature is well above optimum for germination. At 17 °C, germination of this seed lot was 92% in fully aerobic conditions. Warm temperatures increase respiration rates (Q<sub>10</sub> of ~2.0) and decrease O<sub>2</sub> solubility in solution (~30% from 20 °C to 35 °C). This could make seeds more sensitive to O<sub>2</sub> at warmer temperatures.

We hypothesized that exogenous application of GA would compensate for the lack of active GA in this mutant line. The difference between the highest rate of GA application (1000 M) and no GA treatment was not statistically significant (Fig 3). While high application rates of GA may have slightly improved germination, it was still less than that of anaerobically germinated seeds. This indicates that GA was either not entering the seeds or that GA by itself is not sufficient to fully break dormancy in this breeding line. The former of the two options is not likely because the seedlings had significant elongation of the coleoptile for up to 10 days after final application of GA when compared to lower GA additions or no GA applications. Seed germination models based on the barley aleurone system include GA signaling as one of the first steps in the biochemical mechanism of seed germination. The germination mechanism in rice has not been studied in such detail. The fact that  $\alpha$ -amylase is synthesized under anaerobic conditions in rice but not in barley and wheat suggest that the biochemical mechanism for rice germination differs from other cereals. Also, since GA remains at undetectable levels in this breeding line (Honda et al., 1996) yet is able to germinate to 95% anaerobically suggests that  $\alpha$ -amylase is not activated by GA under anaerobic conditions.

The heat treatment did not improve germination percentages in this rice in aerobic conditions and reduced

germination in the anaerobic treatment (Table 1). Many forest species require fire to break dormancy while other seeds in the same ecosystems only require exposure to smoke (de Lange and Boucher, 1990). Mechanical disruption of the seed structure (i.e. seed coat, perisperm) may occur at high temps, as in priming (Oluoch and Welbaum, 1996).

Fermentation products can break dormancy. Small alcohols and carboxylic acids such as ethanol, pyruvate, and lactate are produced anaerobically and these types of compounds are known to have dormancy-breaking activity (Cohn et al., 1989). However, the concentrations required for such activity are as much as five orders of magnitude larger than for compounds such as GA when applied exogenously (Cohn et al., 1989).

Our results indicate that 'Super Dwarf' rice has a seed dormancy that can be overcome by germination in anaerobic conditions. The fermentation products that are formed in the anaerobic conditions may break dormancy. Fermentation products or intermediates of glycolysis may activate  $\alpha$ -amylase under anaerobic conditions. Because 'Super Dwarf' does not contain measurable levels of active GA, it would indicate that GA is not an essential hormone for activating  $\alpha$ -amylase as it is in the barley aleurone system.

It is not always practical to purge the head space of imbibed seeds with N<sub>2</sub> to achieve 95% germination. Good germination can be achieved, however, by germinating seeds under at least 5 cm of unstirred water. While not strictly anaerobic, the amount of O<sub>2</sub> dissolved in the water and subsequent diffusion to the seeds is small enough to allow good germination.

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