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Abbreviations: AG, anaerobic germination; ANOVA, analysis of variance; DAS, days after seeding; HSD, honestly significant difference; IRRI, International Rice Research Institute; MGD, mean germination day; SE, standard error.

Seed Germination and Coleoptile Growth of New Rice Lines Adapted to Hypoxic Conditions

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Abstract: We investigated the morpho-physiological traits of rice (Oryza sativa L.) during the germination and post-germination phases to explore avoidance of hypoxic conditions. We compared four lines selected for anaerobic germination (AG lines) with the variety IR42. The germination capacity of AG lines was higher than that of IR42. The germination percentages and coleoptile elongation differed among the four AG lines; IR06F459 showed the fastest germination and rapid coleoptile elongation. The coleoptiles of IR06F459 were significantly longer than those of IR42. The α-amylase activity in germinating seeds was significantly higher in IR06F459 than in IR42. At 2 days after sowing, the sucrose and glucose concentrations in germinating seeds were higher in IR06F459 than in IR42. These results show that IR06F459, an AG line with a long coleoptile, has high α-amylase activity and high sucrose and glucose concentrations in germinating seeds. These attributes partly explain its vigorous germination and coleoptile growth under hypoxic conditions.

Key words: Coleoptile, Direct sowing, Flooding avoidance, Germination, Hypoxic conditions, Rice.
coleoptile elongation of pre-germinated seeds under anoxia have been investigated (Setter and Ella, 1994), but the mechanism of low-oxygen stress avoidance during germination and subsequent growth (post-germination growth and seedling emergence) of AG line seeds is not yet fully understood. Therefore, this study aimed to evaluate the morpho-physiological traits associated with hypoxic germination and coleoptile growth of AG lines, with a focus on developing seedling shoots and the energy source for germinating seeds.

Materials and Methods

1. Rice genotypes and culture methods

We used five rice genotypes; four adapted to anaerobic germination (AG lines) and one control cultivar, IR42. The four AG lines IR07F323, IR06F434, IR06F459, and IR06F463 (El-Hendawy et al., 2011, 2012) were used; these are distinguished lines for seed germination performance. Uniformly sized rice seeds were sterilized with 3.3% (w/v) benomyl solution (Sumitomo Chemical Co., Tokyo, Japan) for 10 min according to the manufacturer’s instructions. Then, seeds of each genotype (10 seeds/tube) were placed in test tubes (4 cm diameter × 10.5 cm height) with screw caps filled with 97 mL of distilled water to adjust the water surface 1.5 cm below the top of the tube; the seeds were submerged in 9 cm distilled water in test tubes. All test tubes were placed in a clear acrylic case (40.0 cm × 32.0 cm × 35.0 cm) and covered with a clear acrylic lid. The tubes were incubated at 25ºC, under a 12-h light/12-h dark photoperiod. The seeds were grown for 10 days in a growth chamber (EZ-022, Nippon Medical Chemical Instruments, Osaka, Japan). Fluorescent lamps supplied light at a photosynthetic photon flux density of 120 µmol m⁻² s⁻¹.

2. Seed germination and coleoptile elongation

The germination percentage of each genotype was recorded and the length of the coleoptile was measured with a ruler. The mean germination per day (MGD) was calculated for each lot using the modified formula cited by Mavi et al. (2010), as follows:

\[ \text{MGD} = \frac{\sum (n D)}{\Sigma n} \]

where, \( n \) = number of seeds newly germinated (plumule or radicle appearance) at day D at 25ºC; D = days from the beginning of the germination test, and \( \Sigma n \) = total number of germinated seeds.

3. α-amylase activity and sugar concentrations in germinating seeds

To determine α-amylase activity and soluble sugar concentrations in the germinating seeds of AG lines and control rice, we incubated the seeds in test tubes at 25ºC, as described above. The seeds were collected at 0, 2, 4, and 8 days after sowing (DAS), and the roots and hulls were removed with a razor blade. Samples collected at each time point were separated into the brown rice and plumule, immediately frozen in liquid N, and then stored at –80ºC until analysis. The brown rice samples were used in the present study. The α-amylase activity and sugar content were assayed following the methods described by Fukuda et al. (2008), with slight modifications. The protein concentration in the extracts was measured using Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA). A Ceralpha kit (Megazyme Co., Ltd., Sydney, Australia) was used to assay α-amylase activity. The concentrations of soluble sugars (sucrose, glucose, and fructose) were determined using F-kits (J.K. International Co., Ltd., Tokyo, Japan).

4. Statistical analysis

The experiments had a randomized complete block design with four replications. Data were subjected to analysis of variance (ANOVA). Statistical analyses were carried out using JMP for Windows, version 4.0 (SAS Institute Inc., Cary, NC, USA).

Results

1. Seed germination and coleoptile elongation

We monitored the seed germination percentages of AG lines and the control (IR42) over time (Fig. 1). The seeds of AG lines germinated more rapidly than did those of IR42. At 2 DAS, the percentage germination of IR06F459, IR06F463, IR06F434, IR07F323, and IR42 was 75.0%, 50.0%, 40.0%, 20.0%, and 0%, respectively. Compared with the AG lines, IR42 showed delayed, but not reduced, seed germination; there was almost full germination at 3

![Fig. 1. Seed germination of AG lines and IR 42. Seeds were allowed to imbibe in water and germination was monitored for 5 days. Values are means ± standard error (n = 3). Different letters indicate significant differences among rice genotypes by Tukey’s HSD test (p < 0.05).](image-url)
DAS in AG lines, but at 4 DAS in IR42. Among the AG lines, IR06F459 showed the fastest seed germination (Fig. 1). The MGD of IR06F459 was significantly shorter than that of the other AG lines and IR42 (Fig. 2).

The coleoptile lengths varied markedly among AG lines and IR42 growing in hypoxic conditions (Fig. 3). Compared with the coleoptile of IR42, those of IR06F434 and IR06F459 were 77.0% and 53.7% longer, respectively. The shoot growth of IR06F459 was also better than that of IR42; that is, the first and second leaves of IR06F459 emerged and elongated, whereas the foliage leaves did not appear by 10 DAS in IR42 (Fig. 4). We monitored the change in the length of coleoptiles in IR06F459 and IR42 over time (Fig. 5). The coleoptiles of IR06F459 and IR42 continued to elongate until 8 DAS, and stopped elongating at 10 DAS. The coleoptiles of IR06F459 were significantly longer than those of IR42 at 4 DAS. Also, from 2 to 4 DAS, the coleoptiles of IR06F459 elongated faster than did those of IR42. This may explain why the final coleoptile length was longer in IR06F459 than in IR42.

2. α-amylase activity and sugar concentrations in germinating seeds

We determined α-amylase activity during seed germination in IR06F459 and IR42 (Fig. 6). The amylase
activity increased over time in germinating seeds of IR06F459 and IR42 (from 1 to 4 DAS), but the increase was slower in IR42 than in IR06F459. The $\alpha$-amylase activity in IR06F459 was 5.1-, 3.2-, and 1.9-times higher than that in IR42 at 1, 2 and 4 DAS, respectively.

We determined the concentrations of soluble sugars in germinating seeds of IR06F459 and IR42 at 2 DAS (Fig. 7). The germinating seeds of both lines had relatively high sucrose and glucose contents, but only trace amounts of fructose. The sucrose and glucose contents were 1.6- and 2.0-times higher, respectively, in IR06F459 than in IR42.

**Discussion**

This study focused on the evaluation of morpho-physiological traits associated with both seed germination and coleoptile growth in AG lines, which were selected based on their ability to germinate under anaerobic conditions at the IRRI.

First, we found that the AG lines germinated more rapidly than the control cultivar IR 42 (Figs. 1 and 2). Most AG lines were placed in the same cluster in terms of their germination characteristics (El-Hendawy et al., 2011). Our results were consistent with those of El-Hendawy et al. (2011) concerning the fast germination traits of AG lines. However, our results showed that there were genotypic differences among the four AG lines regarding the time course of germination (Figs. 1 and 3). Considering the results of El-Hendawy et al. (2011) and the present study together, the AG lines show variations in germination performance.

The fast germination of AG lines was attributed to rapid water uptake during seed imbibition (El-Hendawy et al., 2011). However, it is possible that other physiological factors affect the rapid germination of AG lines. There are several reports on the relationship between seed germination and physiological processes such as $\alpha$-amylase activity and starch mobilization. Starch is a major energy source for germinating seeds and $\alpha$-amylases play an essential role in starch degradation under low-oxygen conditions (Guglielminetti et al., 1995; Perata et al., 1997; Hwang et al., 1999; Ismail et al., 2009). For instance, under low oxygen stress, tolerant rice genotypes germinate and grow faster, and more seedlings survive. They maintain their ability to use stored starch reserves through higher amylase activity and anaerobic respiration (Ismail et al., 2009). However, it is unknown whether similar physiological factors to those described above are involved in the fast germination in the AG lines.

Higher $\alpha$-amylase activity in germinating seeds contributes to the rapid germination of AG lines in addition to the increased water uptake noted by El-Hendawy et al. (2011). In fact, under low-oxygen conditions, $\alpha$-amylase activity in the germinating seeds was higher in IR06F459 than in IR42 from 1 to 4 DAS (Fig. 6). Higher $\alpha$-amylase activity was also reflected by higher soluble sugar concentrations in IR06F459 seeds than in IR42 seeds during germination under hypoxic conditions (Fig. 7). Our results are consistent with prior studies, where the activity of $\alpha$-amylases needed to break down starches was higher in tolerant cultivars under low-oxygen conditions than in intolerant ones (Ismail et al., 2009).

Coleoptile elongation is a prerequisite for the seedling establishment of direct-sown rice in submerged or hypoxic conditions. Differences in coleoptile elongation among various rice genotypes have been reported (Ogiwara and Terashima, 2001; Bosetti et al., 2012). However, no information is available about the coleoptile growth of AG lines. In this study, there were phenotypic differences in coleoptile length among the four AG lines (Fig. 3),
indicating that the gene controlling coleoptile growth might not be relevant for AG traits. Also, the first and second leaves of IR06F459 grew under hypoxic conditions, while those of the control cultivar IR42 did not (Fig. 4), suggesting that the better growth traits of the first and second leaves of IR06F459 are also responsible for seedling establishment in direct seeding.

Knowledge of physiological factors affecting coleoptile growth in AG lines is still lacking. There are some reports on the relationship between α-amylase activity and coleoptile growth. For instance, a weak correlation has been observed between α-amylase activity and coleoptile growth under low temperature conditions (Fukuda et al., 2008; Ogiwara and Terashima 2010). Our results concerning the relationship between α-amylase activity and coleoptile growth are in poor agreement with the reports of Fukuda et al. (2008) and Ogiwara and Terashima (2010). The differing results between previous studies and ours may be partly due to the different cultivars used in the experiments. Alternatively, differences in growing temperatures may have affected the α-amylase activity and coleoptile growth. The temperatures used in the experiments were 16°C in their studies (Fukuda et al., 2008; Ogiwara and Terashima 2010) and 25°C in our study. It is also possible that other enzymes affected coleoptile elongation under low temperature more than α-amylase.

In contrast, some studies showed that higher α-amylase activity promotes coleoptile growth in hypoxic conditions. Both faster coleoptile growth and higher amylase activity were found in primed rice seeds compared with unprimed seeds under submergence (Mori et al., 2012). Similarly, correlations between seedling survival and α-amylase activities, as well as with shoot length, were higher in primed rice seeds than unprimed ones (El-Hendawy et al., 2011). Coleoptile elongation was positively correlated with α-amylase activity in embryos (Pompeiano et al., 2013). Furthermore, α-amylase activity correlated positively with elongation of shoots, including the coleoptile, during flooded conditions (Ismail et al., 2009). The results of the present study coincide with the previous studies described above, where higher α-amylase activity was reflected in promotion of coleoptile growth.

High α-amylase activity is also responsible for faster of starch breakdown and higher soluble sugar concentrations in germinating seeds under flooding (Ismail et al., 2009), which is presumably necessary for coleoptile growth (Guglielminetti et al., 1995; Perata et al., 1997; Hwang et al., 1999; Ismail et al., 2009; Mori et al., 2012). In fact, the sucrose and glucose contents were 1.6- and 2.0-times higher, respectively, in IR06F459 than in IR42 (Fig. 7). These results show that IR06F459, an AG line with a long coleoptile, has high α-amylase activity and high sucrose and glucose concentrations in germinating seeds. These attributes partly explain its vigorous germination and coleoptile growth under hypoxic conditions.

From an agronomic viewpoint, rapid germination and longer coleoptiles are crucial for a high seedling establishment rate. Additionally, coleoptile elongation is often targeted when selecting rice genotypes that can germinate under hypoxic conditions. Therefore, the rapid germination and longer coleoptile traits of IR06F459 are useful criteria for breeding cultivars with strong early growth in hypoxic conditions. These traits could be introduced into elite cultivars to increase their establishment rates in direct seeding cultivation. Although the present experiment was carried out in the test tubes under environmentally-controlled conditions, the study’s implications may be of particular importance in situations where rice seeds were direct seeded in flooded conditions.

Tolerance to hypoxia during germination is thought to be controlled by starch breakdown and other essential processes, including glycolysis and fermentation (Bailey-Serres and Chang, 2005; Ismail et al., 2009, 2012). This study is a first step towards elucidating the morphophysiological mechanisms underlying the vigorous growth of AG lines in hypoxic conditions, therefore further research efforts are needed to address these issues.

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