Does Pre-Germination Flooding-Tolerant Soybean Cultivar Germinate Better under Hypoxia Conditions?

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Abstract: The germination and emergence of soybean [Glycine max (L.) Merr.] can be adversely affected by the presence of excessive water as a result of continuous rainfall. The pre-germination flooding-tolerant cultivars can be tolerant to hypoxia in the flooding field. The objective of this study was to clarify the relationship between germination under hypoxia at different temperatures and pre-germination flooding tolerance. Seeds of 6 soybean cultivars were subjected to 5 oxygen concentrations and 3 temperatures. Seeds of all 6 cultivars hardly germinated under an oxygen concentration of 20 mL L\(^{-1}\), but germinated with increasing oxygen concentration regardless of temperature. The pre-germination flooding-tolerant cultivar ‘Peking’ exhibited poorer germination under hypoxia than the other cultivars. Seed coat of ‘Peking’ did not dehisce when seeds did not germinate under hypoxia regardless of temperature. The pre-germination flooding-susceptible cultivar ‘Nakasennari’ exhibited higher seed coat dehiscence, but the dehiscence mostly occurred on the seed surface rather than micropyle under hypoxia at 25ºC and 30ºC. Seven days after termination of 3-d hypoxia treatment at 30ºC, the percentage of seedlings with root hairs and/or lateral roots relative to that after ambient oxygen treatment was maintained in ‘Peking’ but decreased in the other cultivars, especially in ‘Nakasennari’. Pre-germination flooding-tolerant cultivar, especially ‘Peking’, is characterized by a delay in germination processes under hypoxia, and exhibits vigorous germination after release from hypoxia.

Key words: Flooding tolerance, Germination, Hypoxia, Oxygen, Seed coat, Soybean cultivars, Temperature.

In monsoonal areas of Asia, the climate during the sowing season of soybean is characterized by continuous rainfall for several days, which causes flooding of crop fields, especially paddy fields, which have poor drainage. Under such conditions, the germination and emergence of crops can be adversely affected, with soybean being especially vulnerable (Kokubun, 2013).

In an upland field converted from a paddy field, topsoil oxygen (O\(_2\)) during the cropping season was previously shown to drop to a partial pressure of as low as 7 kPa following rainfall (Araki, 2006). Furthermore, using pots with a ground water level maintained at 22 cm below the surface, Shiraishi (1977) reported that O\(_2\) concentrations in the soil layer 10 cm below the surface ranged from 2.7 to 5.8% during a period of 1 to 7 d after irrigation. Thus, after rainfall, seeds must initiate germination under the adverse conditions of excess water and low O\(_2\).

One of the initial processes of germination is the increase in respiratory activity, which can be detected within minutes after imbibition (Bewley, 1997). Yagasaki et al. (1994) showed that 95% of the pre-germination flooding tolerant soybean cultivar ‘Peking’ germinated in water flushed with air. Furthermore, Tian et al. (2005) reported that during a 3-d flooding period accompanied by flushing treatment, germination was observed only in water flushed with normal air, not nitrogen alone.

These findings suggest that soybean can germinate even under flooding conditions, provided the O\(_2\) supply is adequate. Al-Ani et al. (1985) revealed that soybean germination rates decreased to 50% and 0% at an O\(_2\) partial pressure of 6 kPa and 2 kPa, respectively, compared to the maximum germination rate under ambient conditions. Tian and Arihara (1998) reported that the percentage of soybean germination decreased by 25% to 75% at 5% O\(_2\) compared to 20% O\(_2\).

However, there have been conflicting results concerning the relationship between pre-germination flooding tolerance and hypoxia tolerance in soybean cultivars. Tian et al. (2005) reported a cultivar difference in germination percentage at 70 mL L\(^{-1}\) O\(_2\), with flooding-tolerant cultivars showing a higher percentage of germination than susceptible cultivars. They also found that the seeds did not germinate under O\(_2\) concentrations lower than 60 mL L\(^{-1}\) regardless of the cultivar, and germination of seeds placed under an ambient O\(_2\) level after hypoxia was improved in the cultivars with flooding tolerance. In contrast, Nakajima
et al. (2010) showed that the germination percentage significantly differed with the soybean cultivar under O$_2$ concentrations varying from 2 to 6%, with flooding-susceptible cultivars showing a higher percentage of germination than more tolerant cultivars.

Temperature is another important factor affecting germination. The optimum temperature for soybean germination was between 34 and 36ºC (Inouye, 1953). Hou and Tseng (1991) proposed a screening method by which flooding tolerance of soybean seeds could be determined by soaking seeds at 25ºC. A number of germination tests have subsequently been carried out at this temperature. However, the soil temperature in temperate regions is generally below 25ºC. In Tsukuba City, Japan, average day soil temperatures varied from 18.8 to 24.7ºC in June, 2012, and from 20.6 to 25.9ºC in June, 2013 (National Institute for Agro-Environmental Sciences, 2014). Since soybean germination under flooding conditions is greatly influenced by temperature (Hou and Tseng, 1991, 1992; Wuebker et al., 2001), it is important to understand cultivar differences in tolerance to flooding and hypoxia at temperatures ranging from optimal to suboptimal. The present study attempts to clarify the relationship between germination percentage under low O$_2$ conditions at different temperatures and pre-germination flooding tolerance in 6 soybean cultivars.

### Materials and Methods

1. **Plant materials**

   Seeds of 6 soybean cultivars were used in this study (Table 1). Four cultivars, ‘Peking’, ‘Williams’, ‘Enrei’, and ‘Nakasennari’, were used in our previous study (Tian et al., 2005) to examine germination under low O$_2$ conditions, and 2 cultivars, ‘Suzuyutaka’ and ‘Hatayutaka’, were identified as tolerant to flooding (Nakayama et al., 2004; Tian et al., 2005). The seeds were harvested in 2009 from the field of the Graduate School of Agricultural Science, Tohoku University, and healthy seeds similar in size with no surface damage were selected and stored at approximately 5ºC until the experiments were conducted (2012 to 2013). The 100-grain weights were weighed and seed moisture was measured using a Grain & Seed Moisture Tester (PM-830-2; Kett Electric Laboratory Ltd., Tokyo). After sterilizing with thiamethoxam fludioxonil metalaxyl-m hydrate (Syngenta Japan K.K., Tokyo), 30 seeds per cultivar were placed in an envelope, and a set of 6 cultivars was packed in a plastic bag and stored at approximately 5ºC until use. Upon germination tests, seeds were hermetically restored at room temperature for 1 hr.

2. **Evaluation of pre-germination flooding tolerance**

   Pre-germination flooding tolerance was evaluated using 60 seeds of each cultivar. Seeds of each cultivar were placed in a plastic dish (150 × 150 × 30 mm) then flooded for 3 d by adding 600 mL of de-ionized water. The dish was placed in an incubator and the temperature inside was controlled at 20, 25, or 30ºC under dark conditions. After flooding treatment, seeds were placed on two layers of paper towels (230 × 218 mm, double-layer type; Crecia Company Ltd., Tokyo) saturated with de-ionized water, and allowed to germinate in the incubator at the respective temperatures. At 7 d after termination of flooding treatment, the percentage of seedlings with root hairs or lateral roots (Stage G3, described below) was counted as the criterion for flooding tolerance.

3. **Combined effects of oxygen and temperature on germination**

   Seeds were subjected to 5 O$_2$ concentrations (20, 40, 60, 80, and 210 mL L$^{-1}$) and 3 temperatures (20, 25, and 30ºC). Seeds were folded in two layers of paper towels saturated with de-ionized water in a plastic dish (150 × 150 × 30 mm) then placed in a germination chamber (39 × 58 × 23 cm) for 3 d under dark conditions. The O$_2$ concentration inside the germination chamber was controlled at the respective levels by mixing N$_2$ gas with ambient air supplied through an air compressor. The flow rate of the O$_2$ concentration-controlled air into the chamber was regulated at 300 mL min$^{-1}$. The O$_2$ concentrations were monitored using O$_2$ sensors installed

### Table 1. Characteristics of the study cultivars.

| Cultivar   | 100-seed weight (g) | Seed moisture (%) | Criterion for pre-germination flooding tolerance |
|------------|---------------------|-------------------|-----------------------------------------------|
| Peking     | 10.8                | 11.4              | 85 85 97                                       |
| Williams   | 22.8                | 10.1              | 48 30 0                                        |
| Suzuyutaka | 27.8                | 10.0              | 52 50 0                                        |
| Hatayutaka | 36.4                | 10.1              | 32 27 0                                        |
| Enrei      | 35.9                | 9.8               | 73 23 0                                        |
| Nakasennari| 33.2                | 10.3              | 42 15 0                                        |

The criterion for pre-germination flooding tolerance was the percentage of the seeds having a hypocotyl with root hairs or lateral roots after 7 d of optimum moisture conditions after the termination of 3-d flooding treatment.
inside the chamber, and concentrations adjusted every minute. The temperature inside the chamber was controlled by placing the chamber in an air-conditioned room. The relative humidity inside the chamber was maintained at 100%. After O₂ and temperature treatment, germinating stage was observed according to the method of Muthiah et al. (1994) with slight modifications. Stages were as follows: SI, seed imbibition; SS, splitting of the seed coat and radicle growth up to 2 mm; G1, emergence of the hypocotyl and elongation from 2 to 10 mm; G2, elongation of the hypocotyl to more than 10 mm; and G3, root hair development and/or lateral root primordia development. Various definitions of seed germination have been proposed (Copeland and McDonald, 1995); Bewley and Black (1983) described the process as emergence of the radicle through the seed coat, while the ISTA (1985) refers to the stage at which the seedling with its essential structures is likely to develop further into a morphologically normal plant under favorable conditions in soil. In the present study, seeds were considered to have germinated when they reached Stage G1, at which point a burst of respiratory activity occurs (Bewley, 1997). At Stage G3, seedlings were considered to have essential structures. The number of abnormal (e.g. folded or broken hypocotyl axis) seeds, infected seeds, and seeds with abnormally dehisced seed coat (Fig. 1) was also counted. After combined treatment, seeds were subjected to ambient O₂ conditions under the three temperature regimes, and germinating stage was observed 1 d (data not shown) and 7 d after termination of treatment. All experiments were repeated 3 times, each using 30 seeds per treatment and cultivar. The relative germination percentage was calculated as the number of germinated seeds in sub-ambient O₂ treatments divided by that in an ambient O₂ concentration regime.

4. Statistical analysis
Because the germination stage distribution pattern was binomial, not normal, statistical analyses were based on binomial distribution using 90 seeds per cultivar per treatment. The differences in the means of germination percentage were analyzed by likelihood-ratio tests with Nominal Logistic Regression using JMP statistical software (ver. 9.0.3; SAS Institute Japan, Tokyo).

Results

1. Cultivar differences in pre-germination flooding tolerance
‘Peking’ was the most tolerant of the 6 cultivars regardless of temperature (Table 1). In the other 5 cultivars, the germination percentage was zero at 30°C, and although responses to 20°C and 25°C differed, some cultivars (‘Suzuyutaka’ and ‘Hatayutaka’) exhibited similar tolerance in both temperature regimes while others (‘Williams’, ‘Enrei’, and ‘Nakasennari’) exhibited higher values at 20°C than at 25°C.

2. Combined effects of low O₂ and the three temperature regimes on germination
Figure 2 shows the relative germination percentage of seeds allowed to germinate under combined O₂ and temperature treatment for 3 d. Seeds were considered to have germinated when the hypocotyl emerged from the seed coat and elongated to more than 2 mm. Relative germination percentage was almost zero at 20 mL L⁻¹ O₂ in all cultivars, but increased with increasing O₂ concentration regardless of temperature. ‘Peking’ showed the lowest relative germination percentage among the cultivars regardless of the treatment regime. In contrast, ‘Nakasennari’, which was the most susceptible to flooding, showed a higher relative percentage of germination. Effects of temperature varied with the cultivar, the relative germination percentage declining with the rise in temperature in ‘Peking’ and remaining lower at 30°C than at 20°C and 25°C in the other cultivars. Thus, the pre-germination flooding-tolerant cultivar ‘Peking’ exhibited poorer germination under hypoxia, becoming more obvious under warmer conditions, than the other cultivars examined.

Under hypoxia conditions, a substantial number of seeds did not germinate, differing with the temperature regime. Figure 3 shows the percentage of seeds in which the seed coat did not dehisce after imbibition (Stage SI) under each treatment regime. Generally, the percentage of seeds remaining at Stage SI was higher at a lower temperature regardless of cultivar. At 20°C, 64 to 83% of seeds, depending on the cultivar, did not dehisce their
seed coat under 20 mL L⁻¹ O₂, but the percentage dropped sharply with increasing O₂ concentration regardless of cultivar. At 25°C and 30°C, the percentage of seeds remaining at stage SI was significantly higher in ‘Peking’ than in any other cultivar. These findings suggest that in the pre-germination flooding-tolerant cultivar ‘Peking’, seed coat dehiscence was less likely to occur under hypoxia over the tested temperature range of 20 – 30°C, whereas it was more likely to occur and was further accentuated at higher temperatures in more susceptible cultivars, especially ‘Nakasennari’, even under hypoxia.

Hypoxia treatment results in morphologically abnormal germinating seeds showing a fold or break in the hypocotyl, disease, abnormal seed coat dehiscence, and so on. Among these abnormal seeds, more seeds had abnormal seed coat dehiscence (dehiscence occurring on the seed surface rather than micropyle, Fig. 1) than any other type of abnormality. At 20°C, abnormal seed coat dehiscence was rare, regardless of cultivar and O₂ concentration (Fig. 4). At the higher temperature regimes
root hairs or lateral roots and void of disease or deformity (Stage G3) were considered to have reached germination, according to the definition of the ISTA (1985), and their number was counted. Relative seedling percentage was calculated as the number of seedlings under hypoxia divided by the number under ambient O₂. No effects of hypoxia were observed at 20ºC, with most seeds reaching Stage G3 regardless of cultivar, except ‘Peking’ at 80 mL L⁻¹ O₂ (Fig. 5). At 25ºC, most cultivars exhibited a high (25ºC and 30ºC), however, abnormal dehiscence was substantial, being most frequent at warmer temperatures (30ºC > 25ºC) and low O₂. Abnormal dehiscence rarely occurred in ‘Peking’ and frequently occurred in ‘Nakasennari’ at any temperature or O₂ concentration, but varied with the treatment in the other cultivars.

3. Germination after release from hypoxia

After release from hypoxia and temperature treatment, seeds were allowed to germinate for 7 d under ambient O₂ conditions in each temperature regime. Seedlings with

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**Fig. 4.** Percentage of seeds showing abnormal seed coat dehiscence under combined low O₂ and temperature treatment for 3 d with adequate water. ** and *** indicate a significant difference between ‘Peking’ and ‘Nakasennari’ (P < 0.01, 0.001).

**Fig. 5.** Relative percentage of seedlings with root hairs and/or lateral roots (Stage G3) at 7 d after combined low O₂ and temperature treatment for 3 d with adequate water. Relative seedling percentage was calculated as the number of seedlings after sub-ambient O₂ treatment divided by that in ambient O₂. ** and *** indicate a significant difference between ‘Peking’ and ‘Nakasennari’ (P < 0.01, 0.001).
relative seedling percentage, although ‘Nakasennari’ showed a decrease below 80% depending on the preceding O\textsubscript{2} treatment. At 30ºC, the cultivar difference was obvious, with ‘Peking’ maintaining a high relative percentage regardless of the preceding O\textsubscript{2} treatment. All other cultivars showed a substantial reduction in the relative seedling percentage, with ‘Nakasennari’ exhibiting the lowest relative percentage at 20 mL L\textsuperscript{-1} O\textsubscript{2} and ‘Williams’ maintaining a fairly high relative percentage following ‘Peking’. The pre-germination flooding-tolerant cultivar ‘Peking’ therefore exhibited the highest relative seedling percentage after release from hypoxia.

Discussion

The present study revealed that the pre-germination flooding-tolerant cultivar ‘Peking’, compared with more susceptible cultivars, delays the germination process under low O\textsubscript{2} conditions (Figs. 2 and 3). However, ‘Peking’ also exhibited vigorous germination, becoming more obvious at higher temperatures, after release from hypoxia (Figs. 2 and 5).

Germination processes can be divided into phase 1 and phase 2. Phase 1 consists of rapid water absorption and a subsequent rapid increase in O\textsubscript{2} consumption, and in phase 2, water content and respiration rate are stabilized (Bewley and Black, 1983). Phase 1 is characterized by a sharp rise in respiration, increasing linearly with swelling of the cotyledon, and in phase 2, the respiration rate stabilizes after hydration of the cotyledons is completed. Respiration in phase 1 requires an O\textsubscript{2} concentration as high as that of the surrounding atmosphere, while in phase 2 the requirement drops to below the atmospheric level (Yentur and Leopold, 1976). The limiting factor for respiration at this stage of germination is O\textsubscript{2} availability, with the intact seed coat preventing an adequate supply (Bewley, 1997). Spragg and Leopold (1959) reported evidence to support this hypothesis in pea seeds, whereby removal of the seed coat increased both the rate of water uptake and swelling, and shortened the duration of phase 2.

The outer portion of the seed including the seed coat also controls the speed of imbibition. Matsu et al. (1996) revealed that the existence of an aleurone layer mitigated damage caused by rapid imbibition, and moreover, that Casparian strip-like tissue was present in this layer. They also observed leakage of sugar and amino acids, and the cracking ratio of cotyledons on water uptake in embryos without a seed coat and aleurone layer compared to intact seeds and those with an aleurone layer only. Muramatsu et al. (2008) also found that both the seed coat and the aleurone layer play a role in reducing the speed of water absorption in ‘Peking’. Soybean seeds are severely damaged by water with a high osmotic potential (Woodstock and Tao, 1981; Woodstock and Taylorson, 1981; Nakayama et al., 2005). At higher temperatures, seed germination was reduced significantly by increasing duration of soaking (Hou and Thseng, 1991); the results of the present study agreed with their finding (Table 1), but further study is needed to clarify the mechanism underlying the difference in the responses to 20ºC and 25ºC among cultivars.

Permeability of the seed coat or aleurone layer or both probably regulates the inflow of O\textsubscript{2}. In most plant seeds, an internal deficiency in O\textsubscript{2} is caused by a reduction in gaseous diffusion due to structures of the outer portion of the seeds (Bewley, 1997). Brown (1940) revealed that the seed coat of Cucurbita consists of two membranes (an inner and an outer), of which the outer is much less permeable to gases. They further showed that moderate absorption of water by the inner membrane increased permeability to gases, whereas a water-saturated membrane was less permeable. In the present study, the relative germination percentage of the pre-germination flooding-tolerant cultivar ‘Peking’ was lower under hypoxia conditions compared to the remaining cultivars (Fig. 2). Our unpublished data showed that ‘Peking’ seeds with partially scraped seed coat germinated better than intact seeds at 40 mL L\textsuperscript{-1} O\textsubscript{2} at 25ºC. The seed coat or aleurone layer or both of flooding-tolerant cultivars may be less permeable to O\textsubscript{2} than that of susceptible cultivars, but this needs to be verified.

Appearance of the radicle from the seed coat is an essential process in germination. Elongation of the radicle is a turgor-driven process that requires cell wall expansion resulting from the synthesis and secretion of polymers and/or from biochemical loosening of the cell wall (Cosgrove, 1997). There are three possible factors necessary for the commencement of radicle growth: a decline in osmotic potential inside the cells themselves, an increase in extensibility of the cell wall, and an increase in softness of seed tissues surrounding the radicle tip (Bewley, 1997). In the present study, seeds did not germinate at 20 mL L\textsuperscript{-1} O\textsubscript{2} regardless of cultivar (Fig. 2), although the radicle was fully swollen, suggesting that a higher level of O\textsubscript{2} is needed for the commencement of radicle growth. Further study is needed to clarify the effect of hypoxia on the possible factors described by Bewley (1997).

The mechanical strength of the seed coat of flooding-susceptible cultivars did not appear to tolerate the turgor pressure of embryo expansion under hypoxia conditions at 25ºC and 30ºC (Fig. 3). Five cultivars excluding ‘Peking’ dehisced their seed coat without elongation of the radicle under hypoxia conditions at 25ºC and 30ºC, and many of them dehisced the seed coat abnormally (Fig. 4). Ma et al. (2004) found that the cuticle of permeable cultivars is prone to breakage, and concluded that the cuticle of a permeable seed coat is mechanically weak, resulting in small cracks through which water can pass. In ‘Peking’, both the seed coat and aleurone layer were previously
found to be relatively impermeable to water (Muramatsu et al., 2008). The seed coat of the flooding-tolerant cultivar ‘Peking’ therefore appears to be mechanically strong, possibly helping mitigate dehiscence of the seed coat, while that of the remaining 5 cultivars, especially the most susceptible ‘Nakasennari’, seems weak, resulting in more frequent and abnormal dehiscence of the seed coat (Figs. 3 and 4).

When seeds are physically injured, microbial infection can occur more easily, with infection by microbiological organisms being partly responsible for pre-emergence seedling rot under flooded conditions (Kato et al., 2013). In 5 cultivars, excluding ‘Peking’, the number of seedlings that did not produce root hairs and/or lateral roots increased under hypoxia and higher temperatures (Fig. 5). Furthermore, many of these seedlings were infected with fungi in spite of sterilization (data not shown).

The results of the present study were inconsistent with the results shown by Tian et al. (2005) who used similar materials and methods. Since the flooding tolerance was reported to vary with the site of harvest (Yamamoto and Ishikawa, 1959; Thseng et al., 1996), this disagreement might be due to the different harvest site, i.e., Crop Institute (Tian et al., 2005) and Tohoku University. The results of another experiment we conducted at 25°C in 2012 using the seeds harvested in 2011, were similar to those of the present study (Nakajima and Kokubun, 2012). Therefore, the seeds used in this study appeared to have maintained their vigor under hypoxia.

In conclusion, the present results show that the pre-germination flooding-tolerant cultivar ‘Peking’ is characterized by a delay in germination processes under low O₂ conditions. Furthermore, the O₂ supply to the ‘Peking’ embryo appeared to be lower than in the more susceptible cultivars. The seed coat of ‘Peking’ appears tolerant to dehiscence when the radicle and cotyledon imbibe water and swell. In more susceptible cultivars, however, the radicle readily elongates under low O₂ concentrations, and the seed coat is likely to dehisce abnormally with swelling of the embryo under hypoxia like 20 mL L⁻¹ O₂ concentration during which the radicle does not elongate more than 2mm. It is likely that differences in germination behavior under hypoxia between tolerant and susceptible cultivars might be due to the reduction in O₂ permeability of the seed coat, but further experiments are needed to verify this estimation.

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