Detection of Pre-Harvest Sprouting in Rice Seeds by Using $^1$H-NMR

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Pre-harvest sprouting (PHS) is a serious source of costly yield and grade losses for producers. Unreleased test lines of cereals should be screened for resistance to PHS. However, screening large numbers of test lines is relatively time-consuming or expensive. In this study, nuclear magnetic resonance (NMR) was used to characterize PHS as a nondestructive method. The water content in the rice grains of 'TC65' and 'Notched' gradually decreased until 21 days after pollination (DAP). After 21 DAP, the water content in both lines remained constant. In PHS at this time, none of the 'TC65' seeds germinated while 15% of the 'Notched' seeds had germinated.

At 14 DAP when PHS was not confirmed, NMR spin-spin relaxation time ($T_2$) in 'TC65' and 'Notched' seeds were about 40 ms and 20 ms, respectively. These results indicated that the $T_2$ is effective to screen resistance to PHS at early stage. In addition, $T_2$s in germinating seeds linearly increased to 165 ms as germination advanced during 10-day treatment period. On the contrary, $T_2$s in nongerminating seeds were about 20 ms and they remained constant during the treatment period. Thus, $^1$H-NMR relaxation times were measured as a way to screen both resistance to PHS and germination process.

Keywords: spin-lattice relaxation time ($T_1$), spin-spin relaxation time ($T_2$), pre-harvest sprouting, seed maturation, germination, rice

INTRODUCTION

Precocious germination of cereal grain is a serious problem in crop production. Pre-harvest sprouting (PHS) is closely associated with agronomic difficulties and loss in functional quality, which amount to economic losses for producers, distributors, retailers and end consumers. Sprouted grain can be difficult to thresh, resulting in harvest losses, and it is a downgrading factor that reduces economic returns for producers (Derera, 1989). The falling number and amylograph methods are applied to estimate PHS damage in wheat and rice (Imabayashi and Ogata, 1998).
However, these methods require procedures for drying and grinding seeds as a pretreatment.

Nuclear magnetic resonance (NMR) spectroscopy is a successful, nondestructive method of studying the state of water in many biological systems (Iwaya-Inoue et al., 2004; Miedziejko et al., 1997; Lenk et al., 1991). Water plays an important role not only as a solvent for biochemical reactions, but also as a stabilizer of macromolecular structure. Longitudinal and transverse relaxation behaviors of water protons can be investigated to describe the compartmentation and transport of water in tissues and seeds (Iwaya-Inoue et al., 2004; Krishnan et al., 2004). Mobile and less mobile water molecules are distinguished from each other by their different relaxation times, and their relative amounts can be calculated (Iwaya-Inoue et al., 2004). Using pulsed 'H-NMR, it has been demonstrated that the water status of woody plant seeds can be efficiently monitored by $T_1$ and $T_2$ during seed maturation (Iwaya-Inoue et al., 2001). The dynamic states of water in biological tissues were considered to reflect physiological changes. The components of $T_1$ and $T_2$ have been shown to arise from distinct populations of water in plant tissues (Gusta et al., 1979; Belton and Ratcliffe 1985; Isobe et al., 1999; Iwaya-Inoue and Nonami 2003). Thus, the NMR technique is expected to provide a novel, sensitive and direct method of characterizing water status in seeds. The objective of this study was to evaluate whether or not 'H-NMR relaxation times can be applied as a screening test of resistance to PHS and the degree of germination in PHS.

MATERIALS AND METHODS

Plant materials

Seeds of Oryza sativa L. ‘Taichung 65’ (‘TC65’) and ‘Notched’ were used for the experiments. The seeds of ‘Notched’, with a notched kernel phenotype, were obtained as BC4F2 seeds through the reciprocal crosses between O. glumaepatula and ‘TC65’ (Sobrizal et al., 1999; Sobrizal and Yoshimura, 2002). Ten individual ears of each line were harvested at 7-day intervals from 0 to 42 days after pollination (DAP).

Germination test

Five individual ears of each line were immersed in tap water for a night and then were kept in plastic pots in a growth chamber (GR-41LC, Hitachi, Tokyo, Japan) for 7 days. The ears were sprayed with distilled water (DW) at 25°C for 7 days. Germination tests were performed in quintuplicate, and the number of germinated grains of each line was counted after 7 days. There were about 60-80 grains per ear in each line.

For the germination test, ‘TC65’ seeds at 28 DAP and mature stage were treated at 25°C for 10 days. At 28 DAP, the germination rate was about 20%. The treated seeds at 28 DAP were selected as nongerminating seeds. At mature stage, the germination rate was about 100%. After the treatment, germinated seeds at mature stage the nongerminating seeds at 28 DAP were prepared for the following analysis.

Measurement of $T_1$ and $T_2$ proton relaxation times

A 'H-NMR spectrometer with a magnet operating at 25 MHz for 1H (JNM Mα25A, JEOL Ltd., Tokyo, Japan) was used to measure the 'H-NMR relaxation times, the spin-lattice relaxation time ($T_1$), and the spin-spin relaxation time ($T_2$). During seed maturation, fifteen grains from each rice ear were packed into an NMR tube 10 mm in diameter. In germination treatment seeds at 28 DAP and at mature stage, $T_2$ in nongerminating and germinating seeds was measured for 10 days after the imbibition. More than five replications for $T_1$ and $T_2$ determinations were carried out.

The $T_1$ values were measured based on the saturation recovery method, using a repeated $90°-τ-90°$ pulse. The term $τ$ was the time interval between two pulses, to the equilibrium state. $M = M_0[1 - \exp(-τ/T_1)]$, where $M$ is the proton signal intensity and $M_0$ is the magnetization amplitude of the proton signal. $T_2$ was measured by the Carr-Purcell-Meiboom-Gill (CPMG) method. $T_2$ is determined from $M_{sw} = M_0 \exp(-2τ/T_2)$, where $M_0$ is the magnetization amplitude of the proton.
DETECTION OF PREHARVEST SPROUTING

signal occurring at time 2τ after the initial 90° pulse in the CPMG (90°x -τ -180°y -2τ -180°y -2τ⋯) pulse sequence. The T2s were calculated based on 500 echo signals acquired by the accumulation of 32 scans. The probe temperature was 30°C controlled by a thermostat connected to the spectrometer's sample chamber.

Water content
The fresh weights of the rice seeds used in the NMR determinations were measured. The samples applied by NMR spectroscopy were dried for 20 h at 90°C. Water content was expressed as a percentage of fresh weight.

RESULTS AND DISCUSSION

Germination rate during seed maturation
The PHS percentages in the ears of each rice line during maturation are shown in Fig. 1. At 21 DAP, none of the 'TC65' seeds and 15% of the 'Notched' seeds had germinated. At 28 DAP, the germination rate was about 20% in 'TC65' and 70% in 'Notched'. In both lines, nearly 100% of seeds had germinated by 42 DAP under the high-humidity condition. These results indicated that these rice lines have different PHS characteristics (Sobrizal et al., 1999; Sobrizal and Yoshimura, 2002).

Changes in water content during seed development
The water content in the rice grains of both lines was highest at 7 DAP and decreased markedly until 21 DAP (Fig. 2). After 21 DAP, the water content in both lines remained at about 30%. From 3 to 35 DAP, water content did not differ significantly between the two lines. These results indicated that water content during maturation is apparently unrelated to PHS characteristics.

Possibility of screening for PHS by NMR relaxation times (T1, T2)
In general, cellular water exists in two or three components in plant tissues; these water com-

![Fig. 1](image1.png)

**Fig. 1** Ratio of pre-harvest sprouting (PHS) of ears in the 'TC65' and 'Notched' rice lines during the development and maturation processes. n = 5 (80 seeds/car).

![Fig. 2](image2.png)

**Fig. 2** Water content of 'TC65' and 'Notched' seeds during development and maturation processes. n = 5.
ponents consist of three water statuses: free, loosely bound, and tightly bound (Iwaya-Inoue et al., 2004). The $T_1$ and $T_2$ of water protons in biological systems provide important clinical information that can distinguish free, loosely bound, and tightly bound water by virtue of the fact that the relaxation mechanism depends on the intrinsic state of water in cells and tissues (Danadian, 1971).

In both of the grain lines studied here, the $T_1$s decreased markedly until 21 DAP and were constant thereafter. At 14 DAP, the $T_1$s were about 140 ms in ‘TC65’ grains and about 90 ms in ‘Notched’ grains (Fig. 3A). The $T_2$s of the long fraction in the grains changed from about 500 to 70 ms in both lines from 7 to 42 DAP. At 14 DAP, the $T_2$s of the long fraction were about 220 ms in ‘TC65’ grains and about 120 ms in ‘Notched’ grains. The $T_2$s of the short fraction changed from about 80 to 10 ms in both lines during maturation. At 14 DAP, the $T_2$s of the short fraction were about 40 ms in ‘TC65’ grains and about 30 ms in ‘Notched’ grains. Therefore, there were significant differences between the two seed lines in the long fraction of $T_1$ at 14 DAP.

On the other hand, $T_1$ reflects the compartment size of crosslinked polymer gel and is strongly affected by the concentration of crystalline water (Murase and Watanabe, 1989). The $T_1$ values in the dry seeds of both lines indicated that the cellular water consisted mainly of loosely bound and tightly bound water. $T_2$ reflects the dynamic states of water, such as bound water binding to macromolecules (Iwaya-Inoue et al., 2003). Here, the $T_2$s decreased markedly until 21 DAP and remained constant thereafter (Fig. 3B). At 14 DAP, although water content was the same between the rice lines, the $T_2$s were about 40 ms in ‘TC65’ grains and about 20 ms in ‘Notched’ grains. The $T_2$s of the long fraction changed from about 120 to 30 ms in the grains of both lines from 7 to 42 DAP. At 14 DAP, the $T_2$s of the long fraction were about 65 ms in ‘TC65’ grains and about 40 ms in ‘Notched’ grains. At 14 DAP, the $T_2$s of the short fraction were about 10 ms in ‘TC65’ grains and about 5 ms in ‘Notched’ grains. The long and short fraction of $T_2$ in seeds of both lines during maturation also differed significantly from the values at 14 DAP. These results indicated that $^1$H-NMR relaxation times ($T_1$, $T_2$) can be applied as a test to screen PHS.

![Fig. 3](image_url)  

**Fig. 3** $T_1$ (A) and $T_2$ (B) values of ‘TC65’ and ‘Notched’ seeds during development and maturation processes. $n=5$. 

134 (64) Environ. Control Biol.
DETECTION OF PREHARVEST SPROUTING

Applicability of T2 as a PHS screening test

In general, the T1 value decreases linearly as the water content of the leaf tissues of evergreen woody plants decreases (Kaku et al., 1992). It is known that T1 of gladiolus petals is strongly affected by the concentration of free water (Iwaya-Inoue et al., 1999; Iwaya-Inoue and Nonami, 2003). Figure 4A shows changes in the relative value of the T1 signal intensity of rice grains. For both rice lines, the ratio of the long fraction gradually decreased from 3 to 14 DAP and slightly increased from 21 to 42 DAP. The value of T2 is often examined in seed studies (Iwaya-Inoue et al., 2003; Krishnan et al., 2004). In the present study, the ratio of the long fraction of T2 markedly decreased from 7 to 21 DAP and remained constant thereafter in both lines (Fig. 4B). At 14 DAP, a significant difference was observed between the rice lines.

In both lines, the water content of the rice grains during maturation gradually decreased (Fig. 2), while the ratio of the long fraction of T1 indicated an almost constant value. On the other hand, the change in T2 strongly corresponded with the change in water content. These results indicated that 1H-NMR relaxation times, especially T2s, can be applied as a PHS screening test in seed studies.

Relation of T2 and characteristics of germination

Figure 5 shows T2 and water content in ‘TC65’ after the germination treatment. At 28 DAP, the germination rate was low, about 20% (Fig. 1). T2s and water content in nongerminating seeds at 28 DAP were about 20 ms and about 30%, respectively, and they remained constant during the 10-day germination period that began at 28 DAP. On the other hand, T2s and water content in germinating seeds in the mature stage, when the germination rate was about 100%, linearly increased to 165 ms as germination advanced. After imbibition, the starch in seeds decomposes into soluble sugars, following germination should be used (Guglielminetti et al., 1995). In the first 6 days of barley seed germination, sugars were detected in imbibed seeds by 13C-NMR and the maltose content increased (Ishida et al., 1996). They indicated that sugars were located by 1H-NMR images.
and ¹H-NMR localized spectra in the vascular bundles of the barley seeds as well as in the solubilized endosperm. Our results suggested that endosperm in nongerminating seeds barely decomposed and that germinating seeds decomposed gradually. Therefore, $T_2$s in germinating seeds indicate the degree of endosperm degradation. Hence, $T_2$ can be applied to determine the germination process.

CONCLUSION

NMR relaxation times ($T_1$, $T_2$) were correlated with PHS characteristics. Especially, $T_2$s indicated PHS characteristics during seed maturation. $T_2$s for nongerminating seeds at 28 DAP remained constant during the germination treatment that followed, while $T_2$s for germinating seeds increased linearly as the germination process advanced. Therefore, $T_2$ reflected the development, maturation, and germination processes, and the measurement of $T_2$ was suitable for use as a test to screen seeds for their resistance to the pre-harvest sprouting phenomenon.

REFERENCES

DETECTION OF PREHARVEST SPROUTING


