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*Laboratory of Crop Science, Department of Plant Resources, Faculty of Agriculture, Kyushu University, Hakozaki, Fukuoka, Japan*

# $^{1}$H-NMR Method Enables Early Identification of Degeneration in the Quality of Sweet Potato Tubers

M. Iwaya-Inoue, R. Matsui, N. Sultana, K. Saitou, K. Sakaguchi, and M. Fukuyama

Authors's addressses: Dr M. Iwaya-Inoue (corresponding author: e-mail: mariino@agr.kyushu-u.ac.jp), Dr R. Matsui, Dr N. Sultana, Dr K. Saitou and Dr M. Fukuyama, Department of Plant Resources, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan; Dr K. Sakaguchi, Laboratory of Economic Mathematics, Department of Economic Engineering, Faculty of Economics, Kyushu University, Ropponmatsu, Fukuoka 810-8560, Japan

*With 6 figures*

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## Abstract

In sweet potato tuber, which is a tropical plant, long-term storage leads to loss of water and carbohydrate, thus water mobility was investigated using $^{1}$H-NMR spectroscopy. Electrolyte leakage indicated that tubers stored at 15 °C for 1 year were partly injured and that frozen-thawed tissues were dead. Nuclear magnetic resonance (NMR) spin–lattice relaxation time ($T_1$) and spin–spin relaxation time ($T_2$) clearly increased with the duration of storage, whereas these values decreased in the dead tissues. Furthermore, Arrhenius plots for $T_1$ and $T_2$ were determined at temperatures ranging from 20 to 0 °C in 2.5 °C steps. In the fresh tubers, a strong converse temperature dependency was shown in the $T_2$ measurement. On the contrary, there was no temperature dependency in the $T_2$ of the dead tissues. Thus, the existence of inverse temperature dependency reflected tissue viability. Additionally, any change in the $T_2$ of the fresh tubers occurred at about 14 °C, which virtually coincided with the storage temperature of 15 °C. The slope change in $T_2$ might have responded to a physiological change as a primary event. In conclusion, monitoring water status by NMR could provide early identification of changes in the quality of post-harvest crops; this method shows great promise for use in environmental-stressed crop yield research.

**Key words:** $^{1}$H-nuclear magnetic resonance — chilling stress — early identification — post-harvest — relaxation time ($T_1$,$T_2$) — sweet potato tuber

## Introduction

Sweet potato [*Ipomoea batatas* (L.) Lam.] is an important crop used as material for distilled spirits as well as for foodstuff in Asian countries. As sweet potato is a tropical crop, the critical temperature for growth ranges between 10 and 15 °C; low temperatures increase the respiratory activity of tubers after harvest, and cause injury by subsequent storage, which is also associated with their deterioration (Ravi and Aked 1996). In addition, the storage of sweet potato tubers is associated with injuries such as lignification (Stange and McDonald 1999) and ethylene production (Okumura et al. 1999) caused by long-term post-harvest before the processing of raw tubers. Increase in the respiratory activity of chill-sensitive sweet potato tubers after harvest leads to a loss of carbohydrate and water (Ravi and Aked 1996); however, no information is available on the water status under these conditions.

Water plays an important role not only as a solvent for biochemical reactions, but also as a stabilizer of macromolecular structure. Previously, it has been shown that nuclear magnetic resonance (NMR) relaxation time, indicating water status, is greatly enhanced in tumour tissues in comparison with the corresponding healthy ones (Damadian 1971, Williams et al. 1980). Recently, dynamic contrast-enhanced NMR imaging (MRI) has been widely used in the diagnosis and staging of cancer and is emerging as a promising method for monitoring the response of tumours to treatment (Podo et al. 1998, Hayes et al. 2002). The level of water binding is reflective of the degree of physiological activity in the tissue. Even in plant tissues, NMR relaxation times can be evaluated as a marker of sensitive physiological parameters (Ishida et al. 2000, Iwaya-Inoue and Nonami 2003, references therein).

Moreover, in temperature-stressed studies, a variety of biological reaction rates has been shown in

Arrhenius plots, and non-linear temperature dependency or the presence of discontinuity has been seen as diagnostic of membrane lipid-phase transitions and as indicative of chilling injury in plants (Lyons and Raison 1970, Lyons 1973, Steponkus 1981, Martin 1986, Wilson 1987). Previously, we indicated that changing points of Arrhenius plots of water mobility in chilling-sensitive seedlings of *Vigna radiata* and *V. mungo* clearly corresponded to the sensitivity to cold storage temperatures (Iwaya-Inoue et al. 1989). On the contrary, similar values determined for the seedlings of the comparatively insensitive *Pisum sativum* linearly dropped from 20 to 0 °C, indicating that there were no break points. This finding suggested that water mobility, indicated by the spin–lattice relaxation time ($T_1$), would be a valuable parameter for estimating specific responses in individual species.

This study has two objectives: (1) to compare changes in NMR relaxation times ($T_1$ and $T_2$) of injured and dead tuber tissues, (2) to determine the temperature dependency of $T_1$ and $T_2$ in Arrhenius plots for these tissues for the purpose of plant biological research. Furthermore, we use $^1$H-NMR to address the possibility of the early identification of degeneration changes in the quality of agricultural products subjected to post-harvest conditions.

## Materials and Methods

### Plant materials

Sweet potato (*I. batatas* Lam. cv. Koganesengan) tubers were used as materials. Koganesengan, a recently improved high-yield cultivar, is currently grown at great expense in southern Japan. According to a productivity test conducted by the Kyushu National Agricultural Experimental Station, this cultivar was grown in an experimental field of Kyushu University according to normal practice (Kadowaki et al. 2001). Young shoots were transplanted in the field (sandy soil) in mid-June, and were watered and fertilized in 2001 and 2002. Tuberous roots (*c.* 10–50 mm in diameter at the largest section) were chosen from among the roots harvested at September in 2001. These roots were washed with tap water and were stored at 15 °C for 1 year. Other similar tubers grown in 2002 were immediately used as fresh or frozen-thawed samples. These materials were used for the following experiments.

### Measurement of $T_1$and $T_2$ proton relaxation times – $^1$H-NMR analysis

A $^1$H-NMR spectrometer with a magnet operating at 25 MHz for $^1$H (JNM Mμ25A, Jeol Ltd, Tokyo, Japan) was used for the measurement of $^1$H-NMR spin–lattice relaxation time ($T_1$) and spin–spin relaxation time ($T_2$).

For the $T_1$ measurements, the saturation recovery method (90-τ-90 pulse sequence) was used. By this method, $T_1$ was determined from $M_\tau = M_0[1 - \exp(-\tau/T_1)]$, where $M_\tau$ is the magnetization amplitude of a proton at interval time τ, and $M_0$ is the magnetization amplitude of a proton in the equilibrium state. In this experiment, the free induction decay (FID) signal at every interval time τ was obtained by the accumulation of four scans. The sequence repetition time was constantly maintained at more than five times of $T_1$. $T_2$ was measured by the Carr-Purcell-Meiboon-Gill (CPMG) method. Briefly, $T_2$ was determined from $M_{2n\tau} = M_0 \exp(-2n\tau/T_2)$, where $M_0$ in the magnetization amplitude of the proton signal occurr(ed) at time 2τ after an initial 90° pulse in the CPMG (90°x – τ – 180°y – 2τ – 180°y – 2τ…) pulse sequence. $T_2$s were calculated based on 500 echo signals acquired by the accumulation of 16 scans. The probe temperature was controlled by a thermostat connected to the sample chamber of the spectrometer using $LN_2$. The measurements were carried out after the NMR tubes were held at the individual temperatures for 5 min.

### Statistical methods

To confirm whether a 'break' (discontinuity) existed in terms of the thermal dependency of NMR relaxation times in the Arrhenius plots, a one-phase or a two-phase regression model was statistically determined as an adequate model. The basic model for the one-phase regression model attempted to fit the observed theoretical line $Y = ax + b$.

The two-phase regression model attempted to fit the observed values to two half-lines: one before and one after the break point. Two theoretical lines, $Y = a_1x + b_1$ and $Y = a_2x + b_2$ should meet at break point $x_0$ and the following relation must be satisfied:

$$a_1x_0 + b_1 = a_2x_0 + b_2 \quad (1)$$

We assumed that the errors are independently and normally distributed with a mean of 0 and a variance of $\sigma^2$, with the least-square estimates equal to the maximum likelihood estimates. The parameters for the one-phase and two-phase regression models were estimated by a least-square estimate, where *Se* is the sum of the squares of bias between the observed values $Y_i$ and the expected values $f_i$:

$$n_{i=1}\, Se = \sum (y_i - f_i)^2 \quad (2)$$

### Water content and leakage of electrolytes

The fresh weights of the sweet potato tubers used for the NMR determinations were measured. The samples examined by NMR spectroscopy were dried for 20 h at 90 °C. Water content was expressed as the ratio of the amount of water vs. fresh weight basis.

The sweet potato pieces were immersed in distilled water (50 ml) and shaken at 180 reciprocates $min^{-1}$. The extent of leakage of the electrolytes was determined with an electrolyte conductivity meter (Toa conductivity meter, Model

CM-20E, Toa Electronics Ltd, Tokyo, Japan) and was expressed as the percentage of the total electrolytes in each sample, measured after the samples were killed by a cycle of freezing and thawing (Kaku and Iwaya-Inoue 1990).

## Results

### Influences of storage on NMR relaxation times ($T_1$,$T_2$) in sweet potato tubers

The NMR spin–lattice relaxation time ($T_1$) and the spin–spin relaxation time ($T_2$), indicating dynamic states of water, were determined in newly harvested sweet potato tubers, frozen-thawed tubers, and tubers stored at 15 °C for 1 year. Initial $T_1$ values for the fresh tissues examined at 30 °C ranged between 300 and 500 ms, whereas those for the stored tuber tissues ranged between 600 and 800 ms (Fig. 1). On the contrary, the initial $T_1$ values for the frozen-thawed tuber tissues were around 200 ms. Moreover, the initial $T_2$ values of the fresh tissues were around 100 ms, whereas those of the stored tubers ranged between 200 and 300 ms, and those for the frozen-thawed tissues ranged between 40 and 80 ms (Fig. 2). From these results, the $T_1$ and $T_2$ values of the sweet potato tubers increased with long-term storage, and they decreased after freeze-thaw treatment.

Cellular water exists in two to three components, which are shown by NMR relaxation times; in plant tissues, these water components consist of three states of water, i.e. free water, loosely bound water and tightly bound water (Iwaya-Inoue and Nonami 2003, references therein). The three compartmentation of water originally identified from the vacuole, cytoplasm and cell wall/extracellular space (apoplast), is reflected by different relaxation times in the parenchyma tissue of apples (Snaar and Van As 1992, Hills and Remigereau 1997). Thus, differences in the relaxation times ($T_1$,$T_2$) of biological tissues can be interpreted as differences between the ratio of 'free water' to 'bound water'. Several components of $T_1$ and $T_2$ for the tuber tissues were calculated from semi-log plots of $^1$H-NMR signal intensities using a curve fitting. The present method revealed that water in the tissues consisted of at least two water components. An initial $T_2$ value of the long fraction was about 200 ms, whereas that of the short fraction was about 60 ms (Fig. 3a). In the stored tissues, although the $T_2$ value of the long fraction was slightly higher than that of the fresh tissues, the short fraction of $T_2$ was lower than that of the fresh tissues (Fig. 3b). On the contrary, the long fraction,

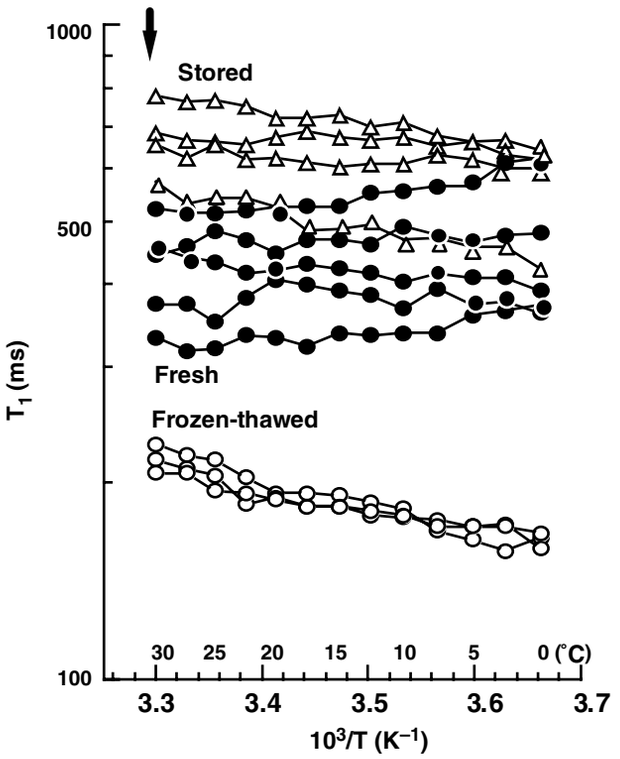


Fig. 1: Temperature dependency in Arrhenius plots of nuclear magnetic resonance (NMR) spin–lattice relaxation time ($T_1$) in sweet potato tuber tissues with temperature ranging from 30 to 0 °C. Fresh tubers (●), tubers stored at 15 °C for 1 year (△), frozen-thawed tubers (○). An arrow shows initial $T_1$ values determined at 30 °C

indicating about 200 ms, disappeared in the freeze-thaw-treated samples (Fig. 3c).

### Influences of storage on the temperature dependency of NMR relaxation times ($T_1$,$T_2$) in sweet potato tubers

The temperature dependency of $T_1$ and $T_2$ for these tissues was determined at temperatures ranging from 30 to 0 °C (Figs 1 and 2). The temperature dependency of the fresh tissues was not clear in the $T_1$ determination, while an inverse temperature dependency of the same tissues was manifested in $T_2$ with a drop in temperature. On the contrary, in the frozen-thawed tuber tissues, a positive temperature dependency of the Arrhenius plots was clear in $T_1$ whereas no temperature dependency was observed in $T_2$. In the stored tubers, although the initial $T_1$ and $T_2$ values were significantly higher than in the frozen-thawed tissues, the thermal dependency of $T_1$ and $T_2$ indicated a tendency similar to that observed in the frozen-thawed tissues. The $T_2$ values of both the long and the

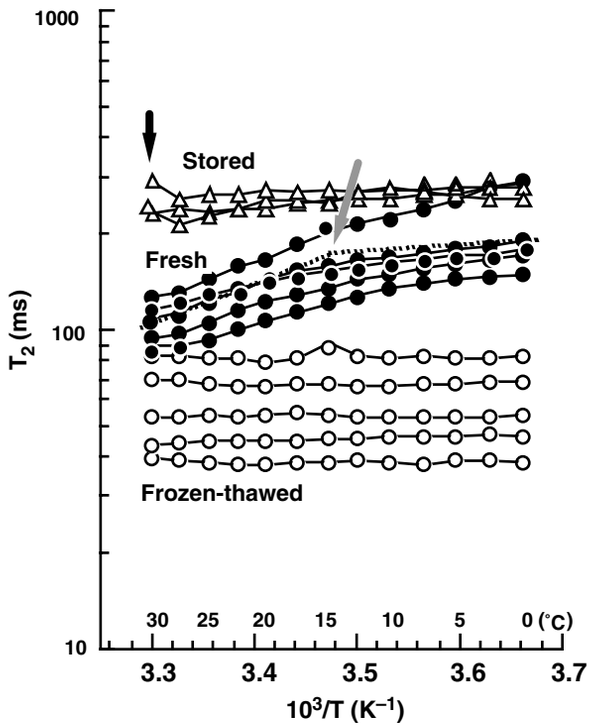


Fig. 2: Temperature dependency in Arrhenius plots of nuclear magnetic resonance (NMR) spin–spin relaxation time ($T_2$) in sweet potato tuber tissues with temperature ranging from 30 to 0 °C. Symbols and an arrow are as in Figure 1. In the plots of the fresh tuber tissues, a broken line of mean of five determinations show two half lines connecting at a break point. An angled arrow shows the break point, $x = 3.490 \times 10^{-3}$ ($K^{-1}$), i.e. 13.53 °C

short fractions in the fresh tuber tissues increased with decreases in temperature (Fig. 3a). On the contrary, the inverse thermal dependency of both fractions disappeared in both the stored (Fig. 3b) and the frozen-thawed tissues (Fig. 3c).

In addition, an incline change in the Arrhenius plots of the $T_1$ and $T_2$ values was statistically investigated. The $T_2$ temperature dependency in the fresh tissues consisted of two straight lines from 30 to 0 °C; however, there were no break points in $T_1$. As a result, a gradient change in the $T_2$ of the fresh tissues was observed at about 14 °C, although there were no break points in the Arrhenius plots of $T_2$ in the frozen-thawed tissues. Based on these results, it is possible to conclude that the gradient change in $T_2$ of the Arrhenius plots of fresh sweet potato tissues might be reflective of the critical cold-storage temperature.

## Discussion

### Shortening of the NMR relaxation times ($T_1$ and $T_2$) of dead sweet potato tubers

Changes in the water status in various organs and tissues is thought to be reflective of physiological changes in those tissues. In previous reports, a shortening of the $T_1$ or $T_2$ value of water protons has been observed in cold-acclimated red osier dogwood stems (Burke et al. 1974), azalea flower buds (Kaku et al. 1984), winter wheat (Yoshida et al. 1997) and wilting flowers (Iwaya-Inoue and Nonami 2003). These phenomena are accompanied by a considerable decrease in the water content. The $T_1$ and $T_2$ of sweet potato tuber tissues markedly decreased by treatment involving both freezing and thawing (Figs 1 and 2). The decrease in $T_2$ values depended on a decrease in the long

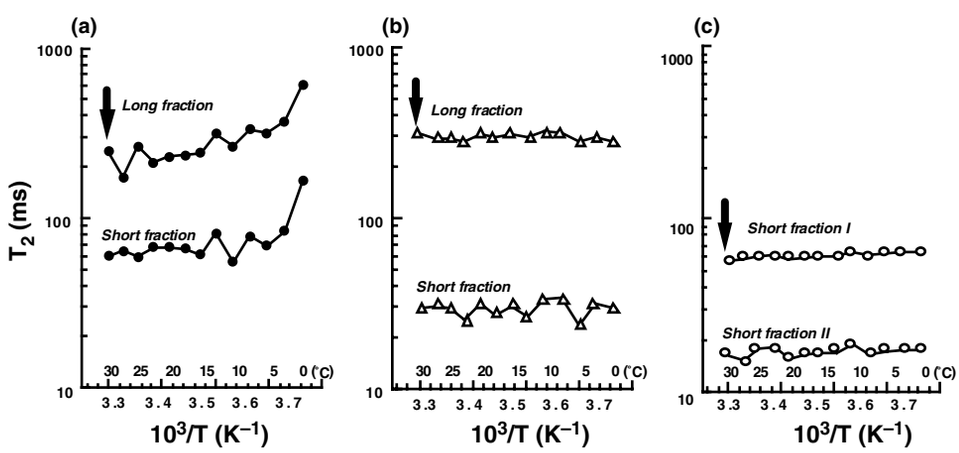


Fig. 3: Temperature dependency in Arrhenius plots of two fractions of $T_2$ in sweet potato tubers with temperature ranging from 30 to 0 °C. (a) $T_2$ in fresh tuber tissues (●), (b) $T_2$ in stored tuber tissues (△), (c) $T_2$ in frozen-thawed tuber tissues (○). Arrows are as in Figure 1. Each Figure represents a typical profile among three to five replications

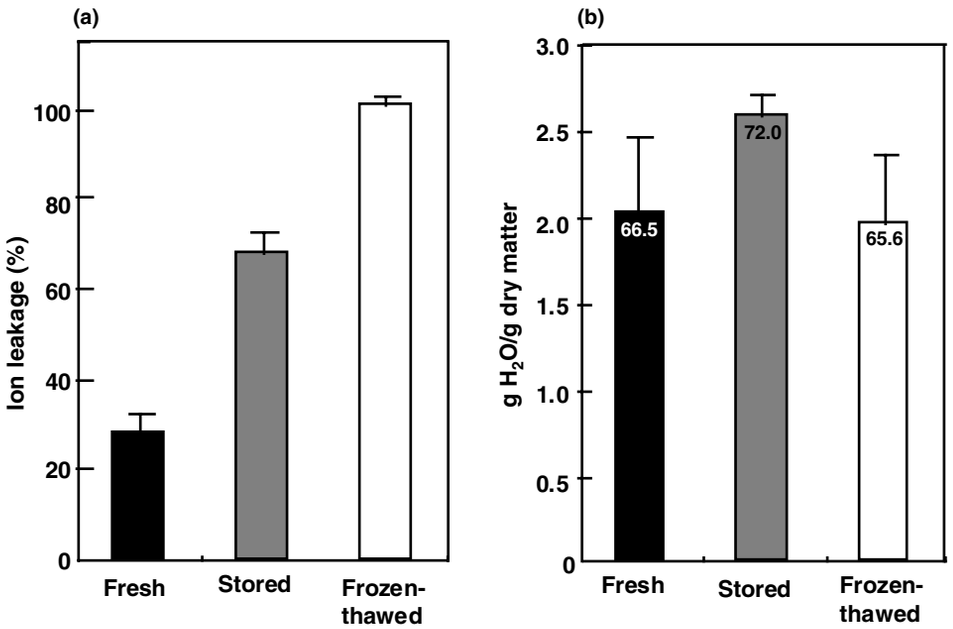


Fig. 4: Change in water content and electrolyte leakages. (a) Water content, (b) electrolytes leakages. Each bar indicates mean and S.D. of five to six replications

fraction of $T_2$ (Fig. 3c). However, the tissue water content was about 65 % per fresh weight and there was no such decrease compared with the results associated with the water content of the fresh tubers (Fig. 4a). Thus, relaxation times in tuber tissues containing relatively low water content are likely to be sensitive to changes in other factors.

Additionally, a 100 % leakage of electrolytes in the frozen-thawed tuber tissues was determined, whereas in the fresh tissues, this leakage amounted to *c*. 30 % (Fig. 4b). Injury related to ionic conductance from freeze-stressed cabbage (*Brassica oleracea* L. cv. Capitata ) (Manley and Hummel 1996) and nitric oxide-stressed wheat leaves (Mata and Lamattina 2001) has been reported. Judging from the electrolyte leakage in the frozen-thawed sweet potato tubers, it can be concluded that these samples were entirely damaged. Marked shortening of the $T_1$ and $T_2$ values have been shown in denatured intact *V. radiata* hypocotyl tissues (Iwaya-Inoue et al. 2000) and in denatured frog lens tissues (Neville et al. 1974). These findings indicate that irreversible changes are accompanied by a shortening of $T_1$ and $T_2$ values.

## Prolongation of NMR relaxation times ($T_1$ and $T_2$) of partly injured sweet potato tubers

Long-term storage led to the prolongation of $T_1$ and $T_2$ values of sweet potato tubers (Figs 1 and 2). Ion leakages from the stored tissues were at *c*. 70 % (Fig. 4b); thus, the stored tissues were considered to be partly damaged. In woody plants (i.e. galled-leaves invaded by insects), a higher ratio of ion leakage was highly associated with a prolongation of the NMR relaxation times (Kaku and Iwaya-Inoue 1990). In addition, we statistically determined the $T_2$ relaxation behaviour in relation to the temperature dependency of the fresh tuber tissues. The *Se* of the two-phase regression model, where the break point occurred within a range of 12.5–15 °C, was 1.2044. On the contrary, the *Se* of the one-phase regression model, where there was no break point, was 3.4745 (see Materials and Methods). Therefore, the two-phase regression model more than fitted the one-phase regression model. In the plots of the fresh tuber tissues, the broken line shows two half-lines connecting at a break point indicated by (the) angled arrow, where $x = 3.490 \times 10^{-3}$ ($K^{-1}$), i.e. 13.53 °C (Fig. 2). It is known that the critical cold-storage temperature ranges between 10 and 15 °C for sweet potato tubers (Ravi and Aked 1996, references therein). The 15 °C-stored tubers were partly injured (Fig. 4b) by this method of evaluation, and the gradient change occurred at about 14 °C in the $T_2$ of the fresh tissues, which was consistent with the storage temperature. On the contrary, there were no break points among the $T_2$ values of the dead tubers (Figs 2 and 3c). Therefore, the findings suggest that the change in the temperature dependency of $T_2$ is reflective of a primary event during chilling stress in the case of the fresh sweet potato tubers.

### Characteristic temperature dependency in the Arrhenius plots of $T_1$ and $T_2$ in sweet potato tubers

There was not a clear thermal dependency on $T_1$ in the fresh tissues as regards temperatures ranging from 0 to 30 °C, whereas a linear thermal dependency was observed in $T_1$ in the dead tuber tissues (Fig. 1). In contrast, a strong inverse relation between temperature and $T_2$ was observed in the fresh tissues, whereas there was no such temperature dependency of $T_2$ in the dead tissues (Fig. 2). $T_2$ changes related to chilling temperature have been discussed in animal cells; for example, when chicken eggs were stored between 5 and 8 °C over a period of 2 weeks, an increase in $T_2$ was observed in comparison with that of unchilled eggs (Schwagele et al. 2001). In a previous report, the $T_1$ values of the epicotyl of *P. sativum*, a chill-insensitive plant, linearly depended on temperatures ranging from 0 to 20 °C (Iwaya-Inoue et al. 1989). The relaxation behaviours of $T_1$ and $T_2$ in the sweet potato fresh tubers were markedly different from those in the pea seedlings. The water content of the fresh tuber tissues was about 65 % (Fig. 4a), whereas that of the pea seedlings exceeded 95 %. The discrepancy between the NMR relaxation behaviours suggested that relaxation behaviour was attributable to water content, cell structure, cellular constitution, chilling sensitivity and other factors.

Therefore, the temperature dependency of $T_1$ or $T_2$ *in vitro* was discussed. Sugar solutions adjusted to the sugar content in fresh sweet potato tubers were prepared. In distilled water (control), $T_1$ and $T_2$ linearly decreased with decreases in temperature. On the contrary, the $T_1$ of 0.1 M sucrose linearly decreased with decreases in temperature (Fig. 5), whereas the $T_2$ of the solution depended less on temperature within the range of 0–30 °C (Fig. 6). A similar tendency was observed using a maltose solution. The difference between $T_1$ and $T_2$ observed in these sugar solutions was similar to those in dead tuber tissues with corresponding sugar contents (Figs 1 and 2). In other words, the $T_2$s of the sugar solution and the dead tissues appeared to respond to viscosity. The suppression of water mobility was more intensified at higher temperatures, i.e. at temperatures between 0 and 30 °C.

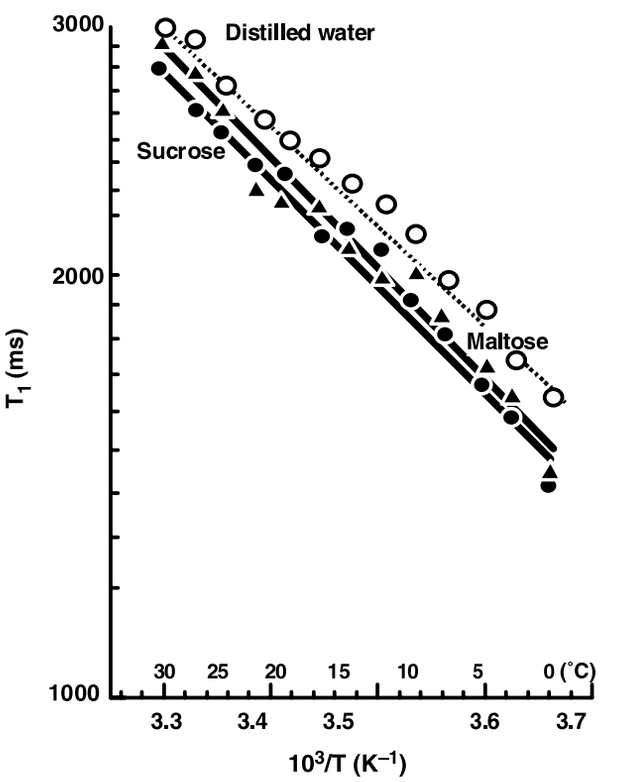


Fig. 5: Temperature dependency in Arrhenius plots of $T_1$ in sugar solutions. 0.1 M sucrose (●), 0.1 M maltose (▲), distilled water (○). Each plot indicates mean of two replications

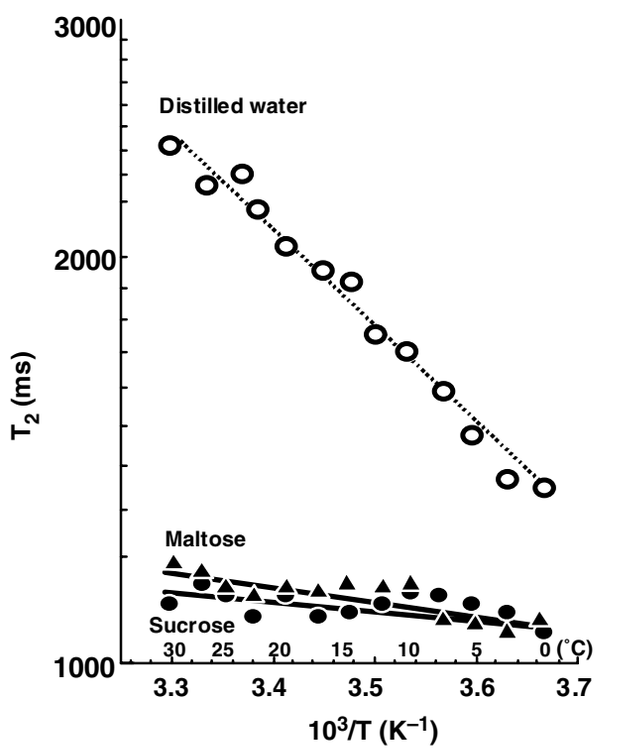


Fig. 6: Temperature dependency in Arrhenius plots of $T_2$ in sugar solutions. 0.1 M sucrose (●), 0.1 M maltose (▲), distilled water (○). Each plot indicates mean of two replications

In conclusion, monitoring NMR relaxation times ($T_1$, $T_2$) in sweet potato tuber tissues provided sensitive and non-invasive information for evaluating the effects of temperature stress in these plants. This method can be widely applied for the study of major crops such as wheat grain, various types of potato tubers and other types of plants as well.

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