Time domain (TD)-NMR relaxometry as a tool to investigate the cell integrity of tomato seeds exposed to osmotic stress (OS), ultrasonication (US) and high hydrostatic pressure (HHP)

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Abstract NMR relaxometry was used to investigate the proton relaxation distribution of the tomato seeds and analyze the damages of the three different processes on the cell membrane integrity of the tomato seed. Tomato seeds were subjected to osmotic stress (OS) (10, 20, 30% NaCl solutions), ultrasonication (US) (5, 10 and 20 min) and high hydrostatic pressure (HHP) (300, 400 and 500 MPa for 15 min at 20 °C). Four peaks were observed in the NMR relaxation spectra of tomato seeds due to multiexponential relation behavior of the plant cell. Each peak corresponds to different water proton compartment within the cell. According to the results, all the three treatments resulted in cell permeabilization and disruption of cellular compartmentalization. Among the treatments, HHP at 500 MPa for 15 min at 20 °C resulted in the most detrimental effect in the cell structure and OS treatment with 10% NaCl solution caused the least changes in the cell structure. In order to further analyze the extent of damage to the cell, tomato seeds exposed to OS, US and HHP were also analyzed by scanning electron microscopy (SEM). These results have demonstrated that NMR relaxometry is a useful tool to investigate the cell integrity of tomato seeds subjected to different treatments.

Keywords Plant seed · NMR relaxometry · T₂ relaxation time · Osmotic stress (OS) · Ultrasonication (US) · High hydrostatic pressure (HHP)

Introduction

Seeds play a very important role in agriculture by providing most of the world's food requirement. They are responsible of propagation and determination of the genetic potential of the new crops. The ability to germinate living and resistant seedlings depends on the parameters of the seed quality for propagation (Bewley 2002). Plant seeds contain an embryo surrounded by a relatively hard and brittle endosperm and a thin testa. Cells that compose the plant seed tissue have a basic eukaryotic cell structure and consist of a nucleus, cytoplasm and subcellular organelles which are all bounded by the cell membrane and cell wall. The cell membrane is a thin semi-permeable membrane that covers the cytoplasm of a cell. Mature viable plant cells involve a large central water-filled vacuole capable of occupying 80–90% of the cell's total volume and enveloped by the tonoplast, a vacuolar membrane. In plant cells, the membrane-bounded compartments or organelles provide the occurrence of the biochemical reactions that are essential to life (Taiz and Zeiger 2006). The quality of seeds and eventually the agricultural produce is affected by environmental stresses, whether natural or artificial. In plants, one of the first targets of stresses are cell membranes and changes in membrane structure may result in loss of semipermeability of the membrane. The cell membrane acts as permeability barriers to proton exchange and if semipermeability of the membrane is lost, integrity of the cell is also lost, causing to the alteration in the cellular compartmentalization (Taiz and Zeiger 2006;

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Vasquez-tello et al. 1990). Quantifying the cellular degradation in plant tissues may provide a correlation between changes in cell structure at the molecular and microscopic level and its effect on functionality and quality of the plant cells since the degree of deterioration of cellular structure affects quality of the material. Therefore, quality assessment of seeds is an essential research area for the food and agricultural industry (Angersbach et al. 1999; Gonzalez et al. 2010; Knorr 1994). Methods that have been used to measure the permeability or integrity of cell membrane in plant tissues are mostly based on microscopy and molecular biology that is insufficient to provide certain information about the flow of water between the compartments (Brizi et al. 2015).

In the study of plants and plant based foods, the application of proton nuclear magnetic resonance (H-NMR) imaging and relaxometry has been demonstrated to be a valuable technique with the features of being fast, non-invasive and non-destructive. In plant tissues, the proton signals are dominated by water protons and the proton density of the tissue is proportional to the NMR signal intensity, and accordingly H-NMR relaxation signals coming from plant tissues provides information about water environments (Van der Weerd et al. 2001; Westbrook 1993). It provides the study of physiological changes in plant tissues exposed to natural or artificial stresses by displaying anatomic information of the whole tissue, spatial distribution and physical properties of water. Physiological changes in plant tissues results variations in cell compartmentalization and water distribution. These variations are investigated by obtaining the relaxation time parameters, T_1 (spin–lattice or longitudinal relaxation) and T_2 (spin–spin or transverse relaxation) (Snaar and Van As 1992; Van der Weerd et al. 2001; Van et al. 2002). In compartmentalized systems such as plant cells, proton relaxation is usually a multiexponential process that indicates the existence of numerous water compartments with different relaxation times in a plant tissue (Belton and Ratcliffe 1985; Hills and Clark 2003). This multiexponential behavior of the relaxation provides investigation of cellular compartmentalization and water distribution within the plant tissue (Ersus and Barrett 2010; Hills and Remigereau 1997; Marigheto et al. 2004; Raffo et al. 2005). In the plant tissues, researchers have been identified mostly three or four proton relaxation peaks previously. The vacuole, the cytoplasm, and the cell wall were attributed to the first three peaks; respectively. The last peak was generally assigned to the protons of starch or non-exchangeable macromolecular protons or extracellular water in plant cells (Musse et al. 2009; Raffo et al. 2005; Sibgatullin 2005). When the cellular compartmentalization is disrupted, an exchange of water protons occurs between the more mobile intracellular water and the less mobile

extracellular water as well as the hydration water of tissue, causing decrease in the T_2 relaxation time (Hills and Remigereau 1997; Maheswari et al. 1999). Some of the stresses causing the loss of cellular compartmentalization are osmotic stress (OS), ultrasonication (US) and high hydrostatic pressure (HHP).

Osmotic stress (OS) is known as a process leading to the partial water removal from plant tissue by dipping in a hypertonic aqueous solution consisting of salt, sugar, glycerol or other humectants. During OS treatment, plasmolysis occurs that influences the rheological properties, water mobility and water distribution of the cell (Dellarosa et al. 2016; Nieto et al. 2013; Sereno et al. 2001). Ultrasonication (US) is a process that is based on the exposition of a liquid sample with ultrasonic waves ranging from 20 to 10 MHz resulting in agitation. Sound waves propagate into the liquid media and creates cavitation. When cavitation bubbles burst, very high temperature and pressure change occurs resulting in deterioration of the cell wall (Mason et al. 1996). High hydrostatic pressure (HHP) processing, which is mostly used as an alternative to pasteurization of food products, is an advanced technology. High-pressure values range from 100 up to 900 MPa and in commercial system pressure values commonly range between 400 and 700 MPa. In plants tissues, HHP treatment changes cell structure in a destructive manner by affecting cell compartmentalization and tissue integrity (Butz et al. 1994; Préstamo and Arroyo 1998; Basak and Ramaswamy 1998).

In light of the above mentioned information, the objectives of the present study were set to investigate the effect of osmotic stress (OS), ultrasonication (US) and high hydrostatic pressure (HHP) on the cell membrane integrity of tomato seeds using variations in T_2 relaxation times and to analyze the use of H-NMR relaxometry as a tool for the identification of physiological changes in tomato seeds.

Materials and methods

Materials

Tomato seeds used in this study were provided by the Department of Plant Protection Faculty of Agriculture Science and Technology of Isparta University in Isparta, Turkey. The seeds with an average weight and diameter of 0.004 g and 1.25 mm, respectively were stored in a refrigerator at 4 °C prior to experiments. The initial moisture content of these tomato seed samples was found to be $9 \pm 0.5\%$. The number of tomato seeds required for one NMR measurement is approximately around 25–30. Thus, for all of the experiment including OS treatment, US treatment, HHP treatment and control, approximately 250–300 tomato seeds are required.

Osmotic stress (OS) treatment

The osmotic stress was performed with three different aqueous solutions: 10 (w/w), 20 (w/w) and 30% (w/w) NaCl solution, which was prepared with NaCl (Sigma-Aldrich, 746398) at 25 °C. Tomato seed samples were weighed and immersed into solutions containing NaCl with a ratio of 1:4 (w/w) between the seed and the solution. Soaking of tomato seeds with NaCl solution was performed for a 1-day period with three replicates.

Ultrasound (US) treatment

Tomato seed samples were soaked with distilled water at a ratio of 1:4 between the seed and the solution to increase the moisture content of the seeds. After 1-day period, samples were subjected to probe type ultrasonicator using MS73 probe (Bandelin Sonoplus HD 3100, Bandelin electronic GmbH & Co. KG, Berlin Germany) at 75% amplitude for 5, 10 and 20 min. Each system was prepared with triplicates for the three-ultrasonication time.

High hydrostatic pressure (HHP) treatment

HHP treatment was carried out with 760.0118 type high hydrostatic pressure equipment (supplied by SITEC, Zurich, Switzerland). The equipment comprises a cylindrical design pressurization chamber with two end closures, a tool for limiting the end closures, a hydraulic unit, a device for temperature control and a pressure pump. The pressurization chamber had a capacity of 100 mL with length of 153 mm and an inner diameter of 24 mm. A built-in heating-cooling system (Huber Circulation Thermostat, Offenburg, Germany) was used in order to maintain the required temperature. A type of K thermocouple was used to monitor the temperature in the chamber. The pressure-transmitting medium which is composed of a mixture of water and glycol was filled in the chamber. By built-in system, before applying pressure, the medium was heated up to the required temperature. Tomato seed samples were soaked with distilled water with a ratio of 1:4 between the seed and the solution and pressurized in 20 mL sterile cryotubes at pressure levels of 300, 400 and 500 MPa for 20 °C for 15 min.

Nuclear magnetic resonance (NMR) relaxometry measurements

T_2 relaxation times were measured for each sample exposed to OS, US and HHP treatments and the for the untreated control sample. Samples were placed into a 10 mm NMR tube and analyzed. H-NMR Relaxometry experiments were carried out by using 0.5 T (22.40 MHz)

low-field bench top 1H Nuclear Magnetic Resonance (LF-NMR) instrument (SpinCore Technologies, Inc., Gainesville, USA) with 10 mm r.f. coil. The spin-spin relaxation times (T_2) of tomato seed samples were measured using Carr–Purcell–Meiboom–Gill (CPMG) sequence with an echo time (TE) of 1000 μ s, 512 points, spectral width of 300 kHz, 1000–5000 number of echoes, repetition delay of 3 s, and 24 scans. In order to obtain T_2 relaxation curves, obtained T_2 signals were analyzed with MATLAB, displaying mono and bi-exponential fitting. A mathematical transformation PROSPA software (Magritek Inc., Wellington, New Zealand), which uses Non-Negative Least Square (NNLS), was conducted to analyze the multiexponential decay of T_2 relaxation curves. 1D-NNLS analysis by PROSPA software revealed T_2 relaxation spectrum. All measurements were performed in triplicate.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) images were obtained at the Scanning Electron Microscopy Laboratory, Metallurgical and Materials Engineering Department, Middle East Technical University (METU), Turkey. Since the SEM works under a vacuum, it is essential that the specimen is completely dry to obtain an image (Karcz 2009). Tomato seeds had a moisture content of between 4 and 8% naturally. For that reason, untreated tomato seeds and seeds exposed to ultrasonication for 5, 10 and 20 min were freeze dried in the freeze dryer (Christ Alpha 2-4 LO Plus Freeze Dryer, Ankara, Turkey) before getting the SEM images. After freeze drying, samples were coated at room temperature with a thin layer of Au–Pd (6–11 nm; 10 mA; 40 s) and the analysis was carried out with a scanning electron microscope (SEM, Quanta SC7620, England).

Statistical analysis

Analysis of variance (ANOVA) with Tukey's multiple range test was used to compare the means of the relaxation times. Differences were considered significant for $p < 0.05$. T_2 relaxation times of peaks were not analyzed statistically since some peaks disappeared after applied treatments.

Results and discussion

NMR relaxometry

1D T_2 relaxation spectra

One dimensional T_2 relation measurements provide information on water content, interaction of water with the

surrounding macromolecules and physical properties of water (Zhang and McCarthy 2012). The changes in relaxation spectrum give information about changes within food systems related to proton such as change in water content, proton exchange between compartments and physiological events that result in occurrence of new proton pools (Belton and Capozzi 2011). The measured signal in a CPMG (Carr–Purcell–Meiboom–Gill) experiment is the weighted sum of the T_2 relaxation decay of water protons in each compartment. The relative water content in each compartment specifies the contribution of T_2 relaxation behavior of water to the observed signal. Therefore, multiexponential inversion of the T_2 relaxation data was obtained as shown in Fig. 1a. It shows T_2 relaxation spectra of an untreated tomato seed sample revealing several components having different relaxation times. In untreated tomato seeds, four peaks were observed. When the relaxation spectra obtained from previous studies, which analyzed the fruit and vegetable tissues by using T_2 relaxation spectra, were compared with those obtained from this study, consistency was observed in the peak numbers (Hernández-Sánchez et al. 2007). Each peak relates to a relaxation component that is associated with a water-proton compartment within the sample. The multicompartment nature of the plant cellular tissue leads to the

multiexponential relaxation behavior in the T_2 relaxation spectra of the plant cell (Hills and Clark 2003).

According to Fig. 1a and Table 1, the peak having the smallest relative area, peak 2, may be associated with the signals coming from the water in the cell wall. The water in cell wall tightly held by strong water-binding sides and surrounding matrix and exists in very small pores (Taiz and Zeiger 2006). This feature of cell wall water restricts the water mobility and thus increases proton exchange between water and surrounding macromolecules. Thus, the smallest T_2 relaxation time was supposed to belong to the water in the cell wall. Peak 3 is associated with the cytoplasm. A part of the water in cytoplasm forms hydrogen bonds with the side chains of the proteins, setting the gel-like framework of cytosol (Kramer 1983; Raffo et al. 2005) and an intermediate T_2 relaxation time is expected to be displayed. Peak 4, having the longest T_2 relaxation time and relative area could be assigned to the vacuole. In plant cells, the majority of water is stored in the vacuoles by containing 50–80% or more of cellular water (Kramer 1983). The vacuole water is in a remarkably mobile liquid state since it does not include intensive amount of water binding molecules (Zhang and McCarthy 2012). Thus, water in vacuole should reveal the longest T_2 relaxation times. Peak 1 with the smallest T_2 relaxation time has a short average T_2 relaxation time that is appropriate for T_2 values of solids rather than water (Hashemi et al. 2010). Thus, Peak 1 may be associated with the protons of sugar and protein molecules.

Figure 1b shows the relaxation spectrum of tomato seeds one day after soaking with distilled water. It was observed that the relative areas of the four peaks became equal with each other. This may be due to water diffusion through the cells. Water influx continues until the osmotic pressure reaches equilibrium with the turgor pressure. The cell is still intact at this stage since the cell wall and semi permeable membrane prevents the cell lysis in the plant cell (Dellarosa et al. 2016).

The identification of several peaks in the untreated tomato seed sample demonstrated that, on the time scale of NMR acquisition, the exchange of water between compartments is slow, since the cell membranes, plasma membrane and tonoplast acted as barriers by separating plant cell into sub-cellular compartments. The compartments, consisting of different content and structure, influence the molecular movement of water protons and resulting in different T_2 relaxation times (Snaar and Van As 1992; Vandusschoten et al. 1995). In case of deterioration of the subcellular membranes, serving as permeability barriers to proton exchange, fast diffusive exchange occurs resulting in loss of cell integrity. This causes merging in the distinct peaks of slow diffusive exchange regime and decrease in the number of peaks in the

Fig. 1 T_2 relaxation spectrum of tomato seeds 1 h (a) and 1 day (b) after soaking with distilled water

Table 1 T_2 relaxation times and % relative areas (RA) of tomato seed samples exposed to different treatments

Treatment	T_2 (ms)		T_2 (ms)	RA (%)
Untreated	952.333 ^a	Peak 1	4.4	13.85
		Peak 2	35	23.13
		Peak 3	140	29.68
		Peak 4	1200	33.34
OS 10%	619.203 ^b	Peak 1	3.8	14.67
		Peak 2	33.7	19.19
		Peak 3	126.7	31.57
		Peak 4	900	34.15
OS 20%	741.333 ^b	Peak 1	24.7	18.79
		Peak 2		
		Peak 3	113.3	38.81
		Peak 4	1046.7	42.40
OS 30%	764.667 ^b	Peak 1	35.3	21.26
		Peak 2		
		Peak 3	136.7	33.49
		Peak 4	963.3	39.97
US 5 min	710.423 ^b	Peak 1	29	12.50
		Peak 2		
		Peak 3	260	25.33
		Peak 4	880	62.10
US 10 min	698,767 ^b	Peak 1	28	12.03
		Peak 2		
		Peak 3	270	37.52
		Peak 4	730	50.45
US 20 min	712.455 ^b	Peak 1		
		Peak 2		
		Peak 3	290	60.53
		Peak 4	360	42.67
HHP 300 MPa	650.351 ^b	Peak 1	290	15.19
		Peak 2		
		Peak 3	69	29.87
		Peak 4	53	23.45
HHP 400 MPa	590.623 ^{b,c}	Peak 1	420	16.50
		Peak 2		
		Peak 3	90	29.15
		Peak 4	29	17.09
HHP 500 MPa	585.307 ^c	Peak 1		
		Peak 2		
		Peak 3	290	16.68
		Peak 4	28	40.90

Untreated (CONTROL); OS (10%), OS (20%), OS (30%), US (5 min), US (10 min), US (20 min), HHP at 300 MPa and 20 °C, HHP at 400 MPa and 20 °C, HHP at 500 MPa and 20 °C

Lower case letters denote significance different at 5%

relaxation spectrum indicating a physiological alteration in the cellular structure of the sample tissue (Hills 1998; Hills and Clark 2003).

Effect of OS treatment on tomato seed samples

In tomato seed samples, exposed to osmotic stress, a decrease in the T_2 relaxation time of tomato seeds as shown in Table 1 was observed. This can be explained by the process known as plasmolysis. When the seed contacts with the osmotic solution, water loss occurs and the semipermeable membrane, plasma membrane and tonoplast start to separate. This water loss causes decrease in the T_2 value of the sample. The T_2 distributions obtained from CPMG experiments of OS treated tomato seeds revealed almost all four peaks in the relaxation spectrum with only minor changes in T_2 relaxation times and relative areas as shown in Fig. 2. The osmotic dehydration caused by OS treatment caused to removal of water partially from inner parts of the cell towards the extracellular space (cytoplasm) thereby, the T_2 value of vacuole was slightly decreased and the T_2 value of cytoplasm was slightly increased. The peak assigned to the cell wall was disappeared in tomato seeds treated with 20% NaCl solution and 30% NaCl solution. This suggests that OS treatment with 20 and 30% NaCl solutions have caused the cell wall disruption resulting in the release of the water that was bounded in rigid cell wall compartments. Peak 1 which is attributed to the protons of starch or non-exchangeable macromolecular protons remained intact implying the rupture of internal cellular membranes.

Effect of US treatment on tomato seed samples

The effect of US was investigated by NMR relaxometry technique and the results were shown in Table 1 and Fig. 3. When T_2 relaxation times of untreated tomato seeds and US treated tomato seeds were compared, a significant decrease ($p < 0.05$) was seen. In Fig. 3, partial merging was observed between the peaks. This can be explained by fast water exchange between compartments which equals the water proton relaxations to an average in response to the disruption of the cell. The merged peaks in a relaxation

Fig. 2 T_2 relaxation spectrum of tomato seeds soaked with 10% NaCl (a), 20% NaCl (b) and 30% NaCl (c) solutions

spectrum is an indication of this water diffusive averaging effect. Cell disruption as result of US treatment causes the increase in permeability or loss of tonoplast integrity. According to Table 1, relative area and T_2 value of the vacuole were decreasing, and relative area and T_2 of extracellular space/cytoplasm were increasing as US treatment duration increased from 5 to 20 min. These results suggested a partial removal of water from inner cellular compartments towards the extracellular space.

Effect of HHP treatment on tomato seed samples

NMR measurement results of tomato seeds treated with HHP are shown in Table 1 and Fig. 4. T_2 values of HHP treated tomato seeds were significantly ($p < 0.05$) lower compared to the untreated samples and OS and US treated tomato seeds, which supported water loss. After HHP treatment, T_2 relaxation spectrum gave fewer peaks as compared to the untreated tomato seeds. 300 MPa pressurization resulted in three peaks with lower T_2 relaxation time of vacuole and higher T_2 relaxation time of the cytoplasm. As pressurization level increased to 400 MPa, partially merging between peak 1 and peak 2 was observed. 500 MPa pressurization led to only two peaks in the relaxation spectrum with a lower T_2 relaxation time of vacuole and a higher T_2 relaxation time of cytoplasm. These changes in the relaxation spectrum could be explained by the detrimental effect of high hydrostatic pressure to the cell structure of tomato seed. As previous investigators proved, HHP treatment could cause changes in the functionality of proteins, polysaccharides and lipids that might result in functional and structural changes in the plant tissue (Fister et al. 2002). HHP caused disruption of the cellular compartmentalization that would lead to partial removal of water from inner cellular compartments towards to the extracellular space. When the applied pressurization levels were compared according to the NMR relaxometry measurements (Table 1), it was observed that

Fig. 3 T_2 relaxation spectrum of tomato seeds exposed to ultrasonication for 5 min (a), 10 min (b) and 20 min (c)

Fig. 4 T_2 relaxation spectrum of tomato seeds exposed to HHP at 300 MPa, 400 MPa and 500 MPa at 20 °C, 15 min

500 MPa caused the most detrimental effect in the cell structure since T_2 relaxation spectrum gave two peaks (proton pools) due to the disruption the cell compartmentalization.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) provides an opportunity to view the interior and three-dimensional structure of the cell surrounded by the cell membrane. Untreated, OS, US and HHP treated tomato seed samples were analyzed by SEM (Fig. 5). Figure 5a shows the SEM image of untreated tomato seed and Fig. 5b–d show SEM image of OS treated tomato seed, Fig. 5e–g show SEM image of OS treated tomato seed and Fig. 5h–k show SEM image of HHP treated tomato seed. When the images were analyzed, it could be observed that untreated tomato seed had an organized cellular structure. However, the SEM images of OS, US and HHP treatment revealed the disruption to the parenchyma cell by displaying the damage to the tonoplast and cell membrane and hereby loss of vacuole structure and cell compartmentalization.

Conclusion

It has been proven that H-NMR Relaxometry is an effective method for providing information on water distribution and for quantification of cell membrane damage in a plant cell. Osmotic stress (OS), ultrasonication (US) and high hydrostatic pressure (HHP) resulted in cell permeabilization and changes in the plant parenchyma cell structure. After treatments, important information on water redistribution in tomato seeds were obtained using NMR relaxometry, that is indicating the severity of cell disruption effect of applied treatments. It was observed that OS treatment with 10% NaCl solution had caused the least changes in the T_2 relaxation spectrum and OS treatment with 20 and 30% NaCl resulted in minor changes in the T_2

Fig. 5 SEM images of untreated tomato seeds (a), tomato seeds treated with OS with 10% NaCl solution (b), 20% NaCl solution (c) and 30% NaCl solution (d), tomato seeds treated with US for 5 min (e), 10 min (f) and 20 min (g), and tomato seeds treated with HHP at 20 °C and 15 min at 300 MPa (h), 400 MPa (j) and 500 MPa (k)

relaxation spectrum. However, US and HHP caused significant alterations in the cell structure of tomato seed and HHP at 500 MPa resulted in the most detrimental effect in the cell structure. SEM measurements further supported the results obtained from relaxation measurements observing physiological changes such as alterations in cell membrane integrity. Thus, it was possible that various degrees of membrane damages by different treatments could be distinguished based on changes in the T_2 relaxation spectrum of the sample.

Practical application

NMR Relaxometry has been shown to be a useful tool on understanding cell integrity in various studies. And in this study, it was shown that it could also be used to identify the changes occurring at cellular level as a result of HHP and Ultrasound treatments on tomato seeds. This could give us information about the possible microstructural changes that are observed in the seeds.

Fig. 5 continued

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