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Molecular mobility in *Medicago truncatula* seed during early stage of germination: Neutron scattering and NMR investigations ☆

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ABSTRACT

First hours of *Medicago truncatula* (*MT*) seeds germination were investigated using elastic incoherent neutron scattering (EINS) and nuclear magnetic resonance (NMR), to follow respectively how macromolecular motions and water mobility evolve when water permeates into the seed. From EINS results, it was shown that there is an increase in macromolecular mobility with the water uptake. Changes in NMR relaxation parameters reflected microstructural changes associated with the recovery of the metabolic processes. The EINS investigation of the effect of temperature on macromolecular motions showed that there is a relationship between the amount of water in the seeds and the effect of freezing–thawing cycle. The NMR relaxometry results obtained at 253 K allowed establishing possible link between the freezing of water molecules tightly bound to macromolecules and their drastic motion restriction around 250 K, as observed with EINS at the highest water content.

1. Introduction

Seed germination represents an important mean for plants perpetuation. Yet the full understanding and control of the mechanisms giving rise to a new seedling from a desiccated seed (orthodox seed) remain to be established [1,2]. The process involves three phases: water imbibition (phase I), which re-initiates metabolic processes (phase II) and leads to the emergence of the radicle (phase III). The understanding of the full process represents a major challenge for the selection of seeds presenting rapid and homogeneous germinations under wide environmental conditions [1,2]. During phase I, the water imbibition reorganizes membrane structures, reactivates stored proteins and the translation of residual mRNA produced during seed maturation prior to desiccation. Water uptake is heterogeneous and follows different trajectories according to species.

Medicago truncatula (*MT*) seed germination is a model to study radical emergence and development in leguminous plants. Germination lasts about 20 h. Of particular interest is the phase I covering about 2.5 h, which is associated with a water uptake of about 85% of the total water absorption before germination.

NMR relaxometry has been widely used to investigate the physical and physiological properties of water in various plant seeds [3–5]. NMR relaxation times T_1 (longitudinal, or spin–lattice relaxation time) and T_2 (transverse, or spin–spin relaxation time) determination provide insight into the dynamics of water molecules and their local environment [6]. The method is sensitive to the rotation of water molecule in the bulk and when it is interacting with solutes and macromolecules, and reveals water dynamics on a wide range of time scales, from an overlap with neutron scattering in the pico- to nanosecond range to the much slower microsecond range.

In biological tissue, compared with pure water, T_1 and T_2 relaxation times are particularly shortened as water mobility is restricted, and their associated decay curves are usually best fitted with multiexponential function. This multicomponent behavior originates from different water proton environments associated with different constituents/structures that are presented in the sample. It should be also noted that T_1 relaxation curves are usually characterized by less components than T_2 ones. This event is supported by chemical exchange and diffusion phenomena between neighboring environments/compartments that, at some extent, lead to average and merge the T_1 values and so to partly lose the specificity of information attached to the different physical/chemical environments.

Elastic incoherent neutron scattering (EINS) provides insights into molecular dynamics on time scales typically from a few picosecond to nanosecond. It has been shown experimentally [7]

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and by simulations [8,9] that for biological systems a hierarchy of motions exists. Thus, motions at different time scales can correlate, spanning over many orders of magnitude from bond vibrations of the femtosecond scale up to large domain motions on the millisecond scale. For instance, it was possible to relate the dynamics extracted from EINS experiments to the activity of an enzyme which happens on a much longer timescale [10]. Whereas coherent scattering carries information on the structure of a material and on the collective dynamics of the atoms, incoherent scattering contains information on the average over individual proton dynamics [11]. It can be interpreted as a superposition of neutron waves that were scattered from the same nucleus at different times. The collected neutron intensities are then a measure for the macromolecular flexibility of the sample. Due to hydrogen's extremely large incoherent scattering cross section, it tends to dominate neutron measurements in hydrogenous materials. Indeed, *MT* dry matter seeds are composed by 40% proteins, 10% lipids and 50% carbohydrates [12], containing about 52%, 60% and 50% of hydrogen atoms, respectively. As a result, the hydrogen proportion of the seeds can be estimated at about 50%. As hydrogen atoms are distributed almost homogeneously over the sample, they give in first approximation information about the global molecular dynamics in the sample [11]. Moreover, profiting of the drastic difference in neutron scattering incoherent cross section of H (80 barn) with respect to its isotope D (2 barn), the contribution from H belonging to the cellular composition of the seeds is highlighted using heavy water for the seeds imbibition.

In the present work, three different investigations have been carried out at different temperatures and imbibition times. Firstly, the time evolution at room temperature of *MT* seeds soaked with heavy water for 0, 1 and 2.5 h was followed by EINS measurements every 10 min over a total monitored time of 1000 min. In parallel, *MT* seeds soaked with light water were studied by NMR at the same imbibition times (0, 1 and 2.5 h), and the corresponding T_1 and T_2 relaxation time values were determined at 293 K. Secondly, freezing/thawing cycles were performed on *MT* seeds soaked with heavy water, for 0, 0.5, 1 and 2.5 h, between 200 K (220 K for 1 h) and 295 K and followed by EINS to study hysteresis effects. Finally, at the subzero temperature of 253 K, T_1 and T_2 were determined by NMR after soaking with nutritive medium during 0, 1 and 2.5 h. At that temperature, only tightly associated water in the seed remained observable. The existence of unfrozen water at freezing temperatures can be attributed to mainly three effects: (i) the freezing point depression due to small solutes (ii) the freezing point depression due to macromolecules, membranes and other hydrophilic ultrastructure and (iii) the effects of viscosity [13]. Consequently, the freezing temperature of water in biological environments is less than that of pure water. Ice, which has short relaxation times of a few micro-seconds, is not detected on the millisecond timescales of the classical NMR pulse sequences. Lowering the temperature reduces the water self-diffusion coefficient and slows the exchange of water molecules between the various compartments. This leads to shift the water relaxation time distribution into the slow exchange regime so that compartments are potentially more easily resolved.

In conclusion, this double analytical approach by NMR and neutron scattering techniques allows studying the imbibition effect by two complementary ways through water mobility and macromolecular motions.

2. Materials and methods

2.1. Plant material

Seeds of *Medicago truncatula* cv. Jemalong A17, harvested in 2009, were obtained from INRA UMR 1334 AGAP (Amélioration Génétique et adaptation des plantes méditerranéennes et tropicales).

For all conditions, about 40 seeds were soaked at 298 K in darkness in a 9 cm diameter Petri dish on Whatman paper soaked with 4 ml of water, heavy water or nutritive solution. The nutritive solution consisted in (1 mM $CaCl_2$ ($2H_2O$), 1 mM $MgSO_4$ (7 H_2O), 2 mM KH_2PO_4, 1 mM NH_4NO_3, 4 mM KNO_3, 100 μM $MnSO_4$ (H_2O), 30 μM $ZnSO_4$ ($7H_2O$), 0.1 μM $CuSO_4$ ($5H_2O$), 1 μM $NaMo_4$ ($2H_2O$), 100 μM H_3BO_3, 5 μM KI, 0.1 μM $CoCl_2$ (6 H_2O), 25 μM Na_2Fe-EDTA). To prepare samples in heavy water, all handling was realized in a glove box in a cold room (controlled temperature 277 K), to minimize isotope exchange between deuterium and hydrogen. The degree of hydration (water content) of the seeds was estimated by the measurement of the mass change upon imbibition with heavy water. Dry seeds having residual water content of approximately 9%, the seed hydration was 13%, 15% and 31% after 0.5, 1 and 2.5 h of imbibition, respectively.

2.2. Neutron scattering measurements

EINS temperature scans were performed on the high resolution backscattering spectrometer IN13 [14,15] at the Institut Laue-Langevin (ILL, Grenoble–France). With an energy resolution of $\Delta E = 8$ μeV (FWHM) and an accessible momentum transfer range of $0.2 \leqslant Q \leqslant 4.9$ Å^{-1}, IN13 allows the investigation of molecular motions in the time-scale up to 100 ps and amplitude from 1.3 to 31.4 Å. For background corrections, the signal of the empty cell was subtracted from raw data, which were all normalized to the neutron flux. The scattering signal of the totally incoherent scatterer Vanadium was used to provide the normalization factor for the detector efficiency.

Data were collected every 10 min covering a maximum total period of 1200 min. Short (10 min) EINS scans, acquired at 300 K, shown an important standard deviation. Thus, to optimize signal to noise ratio, the elastic intensity was integrated over the total Q range accessible ($\sum I_{inc}(Q,t)$).

2.3. NMR measurements

Seeds were packed in 10 mm diameter NMR tubes to reach 1 cm height. For measurements at 293 K on fresh seeds, the NMR tubes were immediately placed in the spectrometer and left 10 min for temperature stabilization. For measurements at 253 K, the NMR tubes were immersed in liquid nitrogen for 10 min, transferred to the spectrometer at 253 K and left 10 min for equilibration. For each condition, NMR measurements were made on five biological replicates. NMR analyses were done using a Minispec mq20 (Bruker), with a 0.47 Tesla (20 MHz proton resonance frequency) operating at controlled temperature.

The transverse relaxation time (T_2) was determined using a Carr Purcell Meiboom Gill (CPMG) sequence. Recycle delay varied from 1 to 5 s depending on temperature of measurement and imbibition time. 600 to 16,000 data points (even echoes) were acquired per echo train, with a 180°–180° pulse spacing time (2τ) of 86.3 μs for an overall sampling time of T_2 relaxation curve of 138 ms. Signal was averaged over 128–512 scans for a total acquisition time of about 15 min. The longitudinal relaxation (T_1) time was determined using an inversion-recovery (IR) sequence. Sixteen points were acquired by incrementing (geometric series) the 180°–90° pulse spacing from 0.12 ms to a value of 500 ms at 253 K and from 0.5 ms to a value of 5000 ms at 293 K. Signal was averaged over from 8 to 64 scans for a total acquisition time of about 15 min.