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Changes in seed water status as characterized by NMR in developing soybean seed grown under moisture stress conditions

P. Krishnan *, Ravender Singh, A.P.S. Verma, D.K. Joshi, Sheoraj Singh

Laboratory of Biophysics, Division of Agricultural Physics, Indian Agricultural Research Institute, New Delhi 110012, India

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ABSTRACT

Changes in water status of developing seeds of Soybean (*Glycine max* L. Merrill.) grown under different moisture stress conditions were characterized by proton nuclear magnetic resonance (NMR)- spin–spin relaxation time (T_2). A comparison of the seed development characteristics, composition and physical properties indicated that, characteristics like seed weight, seed number/ear, rate of seed filling increased with development stages but decreased with moisture stress conditions. The NMR- spin–spin relaxation (T_2) component like bound water increased with seed maturation (40–50%) but decreased with moisture stress conditions (30–40%). The changes in seed water status to increasing levels of moisture stress and seed maturity indicates that moisture stress resulted in more proportion of water to bound state and intermediate state and less proportion of water in free-state. These changes are further corroborated by significant changes in protein and starch contents in seeds under high moisture stress treatments. Thus seed water status during its development is not only affected by development processes but also by moisture stress conditions. This study strongly indicated a clear moisture stress and development stage dependence of seed tissue water status in developing soybean seeds.

1. Introduction

Lack of adequate soil moisture is a principal constraint to crop yield. More importantly, timing of moisture stress affects both the magnitude and the quality of seeds [1]. Moisture stress during reproductive stages leads to poorer yield than the stress under vegetative stages [2]. The reproductive growth and development in soybean (*Glycine max* L. Merrill.) a wonder crop of significance to human diet, is divided into 8 stages: flowering by R1 and R2; pod development by R3 and R4; seed development by R5 and R6; plant maturation by R7 and R8 stages [3]. Within the reproductive stages, lack of adequate soil moisture during the seed set period (R1–R5) reduces seed number; stress during the seed development period (R5–R7) reduces the seed size [4]. The reduction in seed size is largely due to the shortening of seed development duration, rather than the alterations in the seed growth rate [5–8].

Water relations play a fundamental role in the development as well as the germination processes of seeds. Even the gene expression and seed water status are intricately interwoven at both the stages [9,10]. The sequence of changes in seed water content is an efficient parameter to characterize the seed development and to determine the physiological maturity [1,8]. Nonetheless, the availability of water or its energy status is critical to characterize the physiological water status during seed development [6].

Low-field proton nuclear magnetic resonance (NMR) relaxometry is an exceptional tool to investigate plant water relations in terms of water compartment, diffusion, and movement. It can detect protons predominantly contributed by ^1H_2O contained in plant tissues; proton spin density, the longitudinal relaxation time (T_1), and the transverse relaxation time (T_2) can be measured [11]. Proton spin density (i.e. the amount of ^1H_2O per unit of volume) could be a marker of bulk water content in tissues [12,13]. Both the longitudinal and transverse relaxation times of ^1H NMR are probably related to water compartmentalized in plant organelles and associated with macromolecules [14–16]. The bulk water gives rise to a mono-exponential decay of the magnetization characterized by a single time constant (T_1 or T_2) while water compartmentalized in heterogeneous systems (e.g., plant tissues) leads to multi-exponential decays with multiple time constants.

In general protons of a short relaxation time are associated with bound/structural water; protons of medium relaxation time are associated, in part, with intracellular/cytoplasmic water and protons of long relaxation time are associated with extracellular water. The differences in seed water status of developing seeds and associated changes with moisture stress need to be discerned

* Corresponding author. Fax: +91 011 25842321.
E-mail addresses: pkrishnan@iari.res.in, prameelakrishnan@yahoo.com (P. Krishnan).

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to gain insight into seed development process. The objective of the present study was to gain more knowledge of the seed development process and the effect of moisture stress on seed development (i.e., from R4 to R8 stage) in soybean by monitoring the changes in water status, as measured by NMR.

2. Materials and methods

2.1. Plant material

Seeds of soybean (*Glycine max* L. Merrill., cultivar JS35) were sown in the experimental fields of the Indian Agricultural Research Institute, New Delhi. Fertilization and crop protection were designed to ensure optimal growth. Three levels of soil moisture namely W1: −0.05 to −0.1 MPa; W2: −0.5 to −1.0 MPa and W3: −1.5 to −2.0 MPa were maintained. The quantity of irrigation water to be applied was calculated on the basis of soil moisture deficiency by sampling soil periodically. Soybean pods at different stages of growth were hand collected at 3 d intervals from 14 to 65 DAF (days after flowering), at the level of the second node to ensure pods of similar age. Seeds were removed from pods after harvest and used immediately in order to determine seed weight per plant, number of seeds per plant, single seed weight and seed moisture content (% FW) from a random sample each of 100 seeds in triplicate [14].

2.2. Biochemical and physical parameters

Frozen sap from twenty seeds was used to determine seed osmotic potential (Ψs) measured with a vapor pressure osmometer (Wescor 5500) [17]. Seed leachate (%) was determined from 2 g of seeds soaked in 50 ml distilled water at 25 °C for 16 h [14]. High density seeds Index (HDSI) (those having a specific gravity greater than 1.20) was determined by the specific gravity method using sodium chloride solution [17]. The concentrations of oil and protein in seeds were determined using a Near Infrared spectrometer (FOSS Infratec-1241) calibrated for soybean seeds. The concentrations of oil and protein were reported as percentage (%) of the seed weight [17]. Sugar and starch concentration were estimated from dried sample (50 mg) extracted in 10 ml of 80% ethanol [14,17].

2.3. NMR relaxation measurements

Seed samples (2 g, ~25 seeds) were immediately placed in the tubes (10 mm diameter), corked to avoid dehydration and placed in the probe of a low resolution (23 MHz) pulsed NMR spectrometer (MQC, Oxford Instruments, UK). The column height of the seed sample in each tube was kept at about 2 cm and sample temperature maintained at 30 °C. Each measurement was done with six replicates. Non-exponentiality of spin–spin relaxation is accounted for by the presence of clearly recognizable multi-components with different relaxation times (T_{2a}, T_{2b} and T_{2c}) [18,19]. Three components of spin–spin relaxation with three different relaxation times were identified.

3. Results

3.1. Changes in growth parameters

Fig. 1A shows the changes in dry weight of soybean seeds grown under three different moisture treatments (W1: −0.05 to −0.1 MPa; W2: −0.5 to −1.0 MPa and W3: −1.5 to −2.0 MPa).

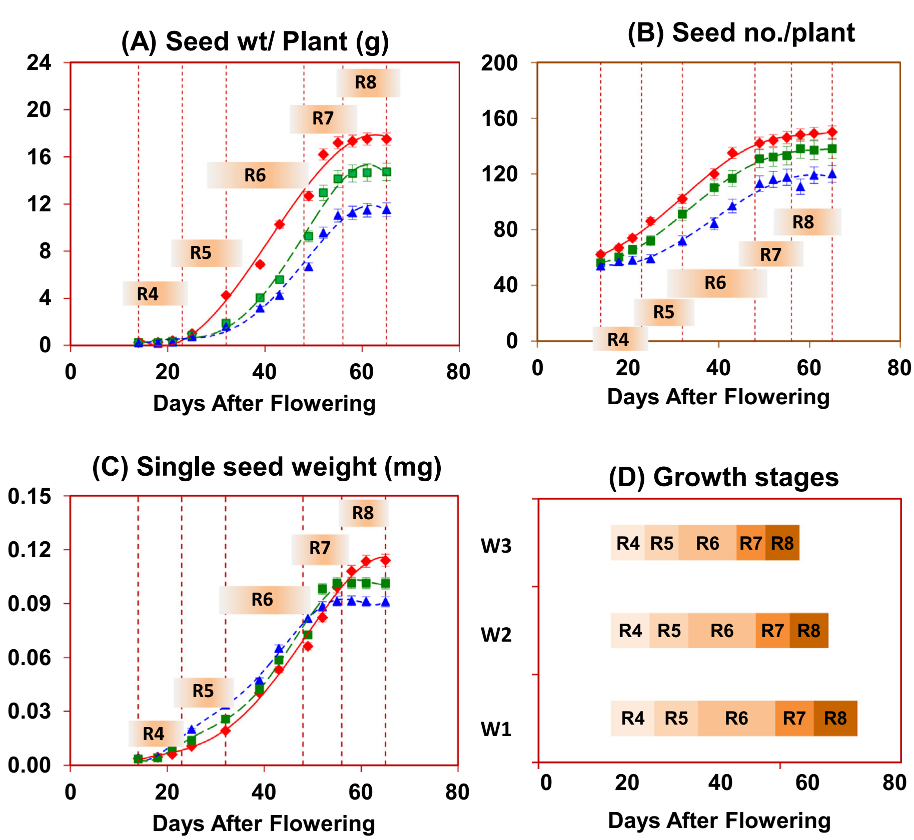

Fig. 1. Changes in growth parameters during seed development and maturation ◆ W1: −0.05 to −0.1 MPa ■ W2: −0.5 to −1 MPa ▲ W3: −1.5 to −2 MPa; R4- full pod, R5- beginning of seed, R6- full seed, R7- beginning of maturity; R8- full maturity. Error bars on data points represent the standard error of the mean.

The dry seed weight of soybean grown at the W1 treatment increased significantly until R8 stage, and thereafter it declined slightly (Fig. 1A). However, the dry weight of seeds in the treatments W2 and W3 linearly increased until R7 and did not change significantly thereafter.

Moisture stress significantly reduced the number of seeds per plant, and plants subjected to severe moisture stress (W3) established less number of seeds (4 and 12 seeds per plant per day) than those subjected to less moisture stress (W2) (Fig. 1B). No significant changes were observed after the R7 stage in all the treatments.

Due to the advancement in the rate of seed growth, the single seed weight was higher under W2 and W3 in all the sampling dates between R4 and R7 stages. However, the single seed weight reached a constant weight by the R7 stage in W3 and W2 and only by R8 in W1 (Fig. 1C). At harvest the single seed weight was maximum in W1 (0.114 mg/seed) than W2 (0.1011 mg/seed) and W3 (0.0910 mg/seed). Seeds under moisture stress (W2 and W3) attained their development stages sooner (podfill stage R6 at 10 d earlier) than those under the control (W1) condition (Fig. 1D).

3.2. Seed composition

The protein concentration in the mature soybean seeds varied from 36% to 46% (dry basis), with seeds under W1 treatment having more than 45% protein (Fig. 2A). The amounts of protein decreased by 3% to 10% during R4 and R5 stages but rapidly increased during R6 stage, followed by a steady increase during the R7 stage and then a constant at maturity (R8). This was attributed to the rapid synthesis of oil and starch in the early seed developmental stages. The increase in protein concentration due to moisture stress probably resulted from stability (or slight increases) of protein content. The stability of protein concentration was determined by an increase in the rate of protein accumulation that compensated for seed protein accumulation.

Unlike protein concentration, oil accumulation decreased with increase in moisture stress (Fig. 2B). Oil was accumulated rapidly from R4 to R5 stage and remained at a similar level during R6–R8. The control (W1) treatment had the highest oil content at all the stages of seed development whereas the moisture stress treatments (W2 and W3) had significantly lower oil content at the beginning of seed development-R5 stage.

Moisture stress resulted in more starch concentration at all the stages of seed development (Fig. 2C). Amounts of starch in seeds decreased at R4 stage, remained relatively unchanged at R5 and increased towards the end of seed maturation R6 and R7. Under higher moisture stress (W3) condition, the starch concentration reached a maximum of 9.52% at R7 itself, whereas those in the treatments W2 and W1, it was 8.10% and 6.25%, respectively, at R8 stage only.

Amounts of sugar in the seeds from those grown under different moisture stress conditions were 5–9% at R4, reached its maximum (10–13%) at R5, drastically decreased during the R6 and R7 stages, and reached the level of about 1% at maturity (R8) (Fig. 2D). Among the different treatments, the sugar content of seeds in W3 significantly increased from R4 to R5 and then decreased rapidly, however it was only at R6 in W2 (40 DAF). Under control (W1) condition, the R6 stage was reached around 47 DAF followed by a concurrent decrease in sugar concentration. These results suggest that the initiation of sugar degradation commenced during maturation, around R6 stage coincided with the increase in soluble sugars.

3.3. Physical properties of seeds

Seeds from all treatments showed similar patterns of Osmotic potential (Ψs) during their development. Seeds at the early-stages (R4 and R5) of development had lesser potential of 415 to 550 mmol/kg (Fig. 3A). In W3 Ψs reached a minimum value at R5 stage and those of W2 at R6. Irrespective of treatments seeds

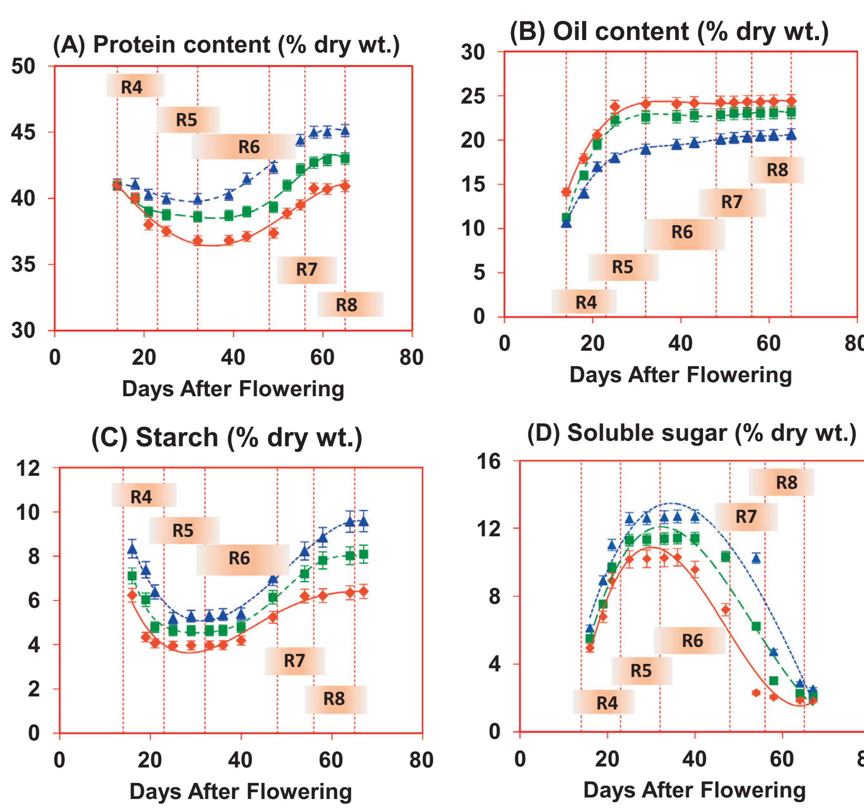

Fig. 2. Changes in biochemical parameters during soybean seed development and maturation ◆ W1: −0.05 to −0.1 MPa ■ W2: −0.5 to −1 MPa ▲ W3: −1.5 to −2 MPa. Error bars on data points represent the standard error of the mean.

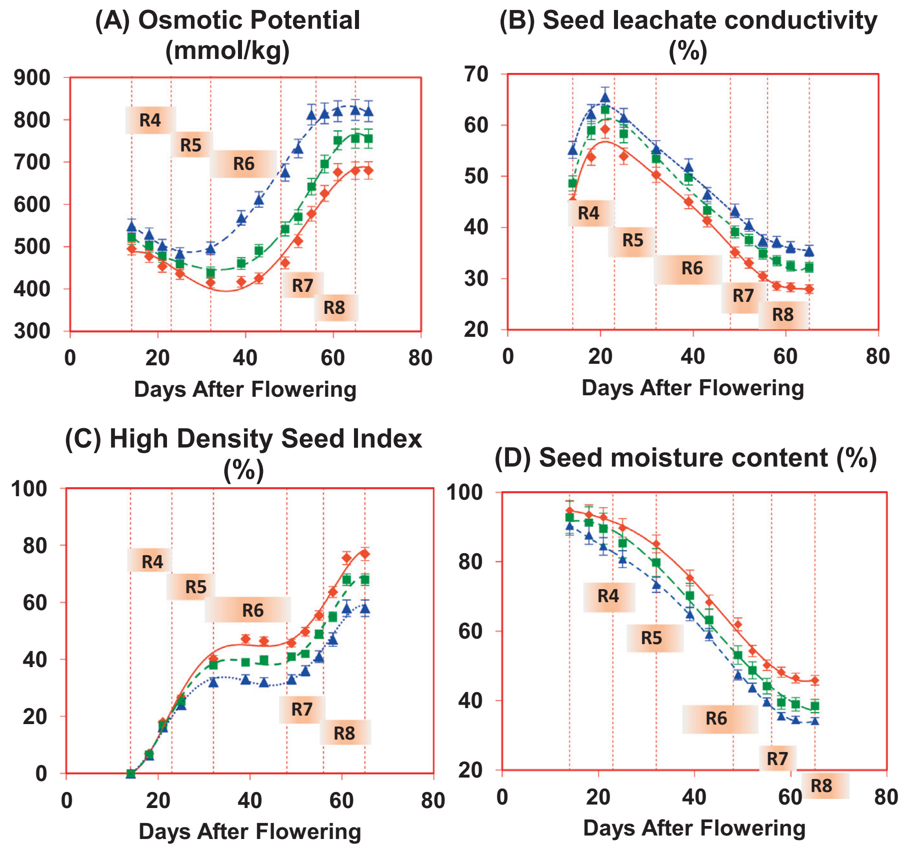

Fig. 3. Changes in physical parameters during seed development and maturation ◆ W1: 0.05 to −0.1 MPa ■ W2: −0.5 to −1 MPa ▲ W3: −1.5 to −2 MPa. Error bars on data points represent the standard error of the mean.

reached their highest Ψs at R8 stage. In general seeds in W2 and W3 showed higher osmotic potential than those from W1 (control); these differences were larger during the R6 and R7 stages.

During the R4 stage of development, seeds from all the three treatments had their maximum value of leachate conductivity (Fig. 3B). Later, during the R5–R7 stages, they showed a decrease with least at the maturity stage (R8). Seeds from moisture stress conditions had higher leachate conductivity (5–10% increase in W2 and 14–20% increase in W3) than those from the control (W1) with highest conductivities observed in W3 during all stages of development.

HDSI of developing seeds of soybean showed rapid increase during R4 and R5 (Fig. 3C). A steady state with no change was observed during R6 stage of seed development. However a second rapid increase was observed during R7 and the seeds reached their maximum HDSI at the R8 stage. Significant changes in HDSI were probably due to the moisture stress. At all the developmental stages, the seeds of W1 showed higher HDSI than those from the W2 (8–11% decrease in HDSI) and W3 treatments (21–25% decrease).

Seeds grown in W2 and W3 conditions showed consistently lower moisture content than those in W1 (Fig. 3D). In addition, the moisture content of seeds grown under W2 and W3 decreased faster than W1 because of greater moisture loss and rapid gain in dry mass. From R6 stage seeds under moisture stress conditions showed more loss of seed water than those under well watered (W1) condition, with differences of 8.2% and 11.5% of seed water observed in W2 and W3 respectively.

3.4. NMR relaxation time measurements

In the course of development, the seeds showed rapid decreases in T_2 during the R4 and R5 stages (Fig. 4A). The seeds at the W2 and W3 treatments showed lower T_2 and greater decrease in the rate of change than those grown under the well watered (W1) condition. During the cell elongation phase (both R5 and R6) the T_2 values of seeds reached the minimum, followed by an increase with a maximum at R7 stage. However, a decrease in T_2 was observed in seeds of all the treatments during maturity-R8.

The component of transverse relaxation time ($T_{2a} \approx 100$ ms) which corresponds to the extra-cellular free water in the seed tissues was detected during all the developmental phases, albeit at different rates at different moisture stress levels (Fig. 4B). It was almost constant during the cell division phase (R4), increased during the R5–R6 stages, and reached a maximum at the end of drying phase (R7). But, it decreased drastically during the maturity phase (R8). The seeds in W2 and W3 (under moisture stress) showed lower T_{2a} values than those in W1. T_{2b} ($\approx$50 ms) that corresponded to the cytoplasmic bulk water decreased significantly in W2 and W3, during R4 and R5 stages. Seeds of W1 showed an increase in cytoplasmic bulk water (T_{2b}) with a maximum reached at the end of elongation phase (R6 and R7). However no such significant changes were observed in seeds grown under the moisture stress (W2 & W3) condition. The third component T_{2c} ($\approx$15 ms) which corresponded to the bound water of macromolecules was not observed in the cell division phase (R4) but was noticed in the early elongation phase (R5) in seeds of W1 treatment and was delayed in those under moisture stressed (W2 & W3) conditions. Significant increase in the T_{2c} component was observed in R7 and R8 stages of seed development, during which seeds lose their moisture rapidly. In general the rate of increase in bound water component was more in W1 than those under the moisture stressed W2 and W3 conditions.

In general during the cell division phase (R4), nearly 60–70% of water was under cytoplasmic bulk water state, only 30–40% under free state, and no water under bound state (Fig. 4C). During the cell

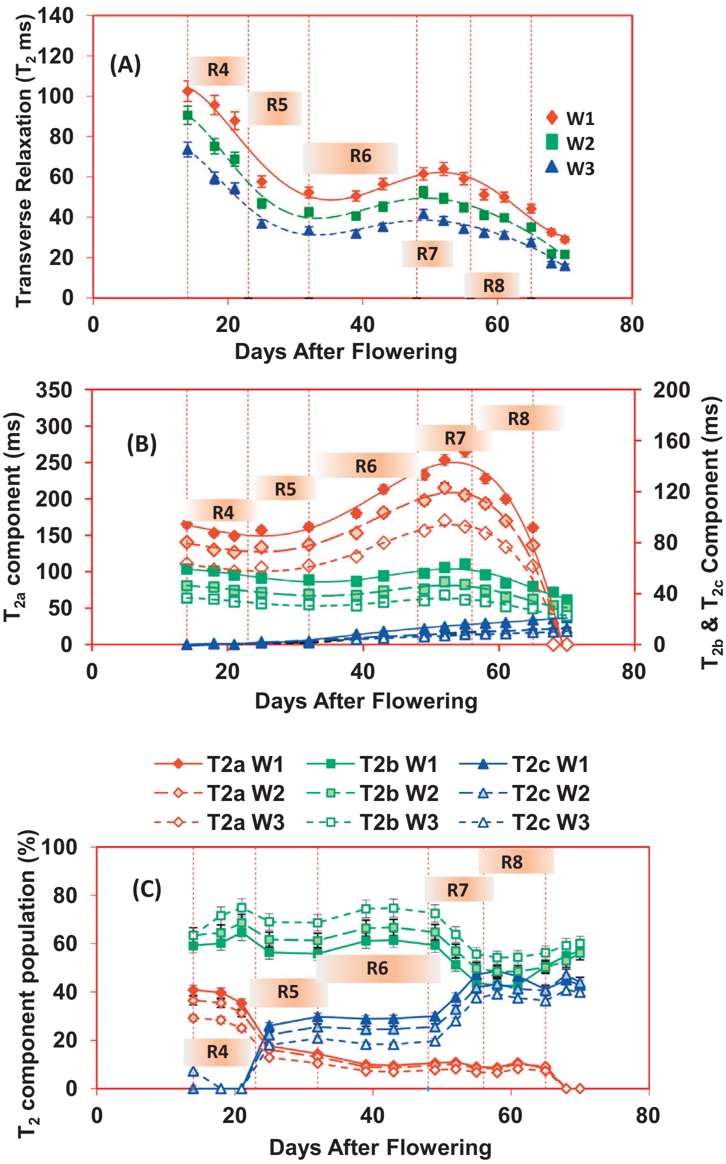

Fig. 4. Changes in seed water status as characterized by nuclear magnetic resonance (NMR) W1: −0.05 to −0.1 MPa W2: −0.5 to −1 MPa W3: −1.5 to −2 MPa. Error bars on data points represent the standard error of the mean.

elongation phase (R5 and R6), 60–70% of water was under cytoplasmic bulk state, 20–30% under bound state and only 10–20% under free state. In the drying phase (R7 and R8), greater amount of water (40–60%) was in the cytoplasmic bulk state, 40–50% under the bound state and only 0–10% under free state. The moisture stress led to less amount of water under the cytoplasmic bulk state and more amount of water under the free- and bound states. The differences between the treatment WI with W2 and W3 were highly significant during the cell elongation phase (R6) but became less significant at the end of drying phase (R8).

4. Discussion

Water status is vital for biological activities such as enzymatic reactions, transport and accumulation of proteins, lipids, starch and other materials in seed tissues. The physical status of water is generally expressed in terms of amount and mobility; both are important determinants for regulation of various biological activities [14,18]. The deficits of water (moisture stress) can severely affect the mobility of water, which is negatively related to the levels of stress, from low to high. In the actively growing tissues during the seed development, there are many membrane reorganization events including water uptake, solute leakage, synthesis of metabolites and lipid turnover; moisture stress can affect all these events significantly. Even at the whole plant level, there are explicit changes depending on the severity and duration of the soil moisture stress. The moisture stress during the anthesis and seed development decreases the number of pods and seeds including the alteration in the dynamics of seed development. The present investigation clearly showed that the seed development processes due to the moisture stress treatments (W2 and W3) were severely affected leading to decreases in seed weight per plant, seed number per plant and single seed weight. In addition, the increases in the osmotic potential with concomitant decreases in moisture content suggested the altered assimilation process leading to poorly filled seeds, which were evident from the indices of high density seeds.

The concentrations of protein increased while those of oil and starch decreased in seeds from the moisture stressed (W2 and W3) soybean plants. Evidently, the accumulation of lipid and carbohydrate in seeds is more sensitive to the cellular water status than the protein accumulation. Protein content of seeds depends more on C and N remobilization from leaves than the oil and residual content, which are reliant on the current photosynthesis [20]. The inorganic N uptake is less sensitive to cellular water changes (drought) than the biological fixation of nitrogen which soybean is capable of [21]. Increased protein synthesis might be due to the continued uptake of inorganic N from the soil or redistribution from other plant organs even under moisture stress condition. The legume plants are known to accumulate sugars like oligosaccharides in seeds during maturation, which serve as energy source for germination [22]. Nevertheless, soybean seeds among different legume seeds are considered to have substantially low levels of starch at maturity [1]. The present study showed that decrease in sugar content was initiated at the commencement of seed filling (R6 stage). Concomitantly, there were increases in the concentration of starch. All the traits measured showed that the interaction effect between the treatments and growth stages were significant, implying that the trends over time of seed metabolite accumulation or degradation were significantly different among treatments.

Loss of free water and the subsequent increase in bound water (R7 and R8) is a characteristic change during drying phase of seed maturation [23]. The decrease in moisture content of seeds, which was also observed in the present study, could be due to the removal of free water (loosely held water) by desiccation caused by the surrounding environment, besides water utilization by various metabolic activities [2,22]. The pattern of changes in electrolyte leakage is often used to estimate the damage to the cellular membranes [14,18,23]. The changes in the electrolyte leakage patterns due to the changes in hydration of the cellular membranes supported the inferences based on the NMR measurements of seed water by analyzing the transverse time components. At the physiological maturity, seed development ceases and seeds reach their maximum dry weight. The physiological maturity of soybean seeds is attained when the moisture content naturally decreases to about 40%. At this stage, significant changes in seed water status are generally observed; free water decreases and bound water increases [14].

In the present study during the development of soybean seeds significant relationship were observed among the different levels of moisture stress and the NMR spin–spin relaxation time (T_2) of seed tissue. The relaxation characteristics of the developing soybean seeds indicate that molecular mobility and biophysical state of tissue water, are related to the changes in synthesis of seed metabolites, cellular membrane structure and integrity [23,24]. Spin–spin relaxation time measurements of seed cellular water are dependent on membrane permeability [18], which was corroborated by the results of the present study. The changes in T_2 components and the hydration dependent transverse relaxation time of seed water during seed development are interrelated. Even though the total water content of seeds was low, the state and quantity (Fig. 4C) of water present in the localized cellular sites could provide a medium, suitable for metabolic activities. Various catabolic processes during seed development help to synthesize the metabolites to control biosynthetic fluxes and improve energy supply to developing seed.

The applicability of NMR spectroscopy to study seed development during maturation in soybean grown under different moisture stress condition has been demonstrated. This paper, characterizes water distribution and water status in maturing soybean seeds, makes a convincing contribution to a better understanding of water loss events in leguminous seeds. Comparative analysis of soybean seeds from individual developmental stages with seeds grow under normal and moisture stress conditions shows that, at a given developmental stage, the same water components are observed in seeds developed under different moisture stress condition. It seems that it is possible to identify related stages of seed development on the basis of common water status, although the mechanisms by which the water content and dry matter increase/decrease in developing seeds are different under different moisture stress conditions.

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