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Full Length Research Paper

Characterization of water uptake and distribution in chickpea (*Cicer arietinum* L.) seeds during germination by NMR spectroscopy

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Experiments were conducted to characterize the changes in water status during imbibition by nuclear magnetic resonance (NMR) spectroscopy in chickpea seeds exposed to static magnetic fields of 100 mT for 1 h. Water uptake during seed germination showed three phases with rapid initial hydration phase I, followed by lag phase II and steady hydration phase III. Comparative analysis of the hydration pattern showed that water uptake was more in phase II and III in magnetically exposed than unexposed seeds. The longitudinal relaxation time (T_1) of seed water showed significantly higher values and hence higher molecular mobility of cellular water in magnetically exposed seeds as compared to unexposed seeds. Analysis of transverse relaxation time (T_2) revealed a three component of water in germinating chickpea seeds. Interesting observation found in this study was the early appearance of hydration water with least mobility and higher values of relaxation times of cytoplasmic bulk water and hydration water in magnetically treated over untreated seeds. Early hydration of macromolecules, membranes, greater molecular mobility of bulk and hydration water fractions in magnetically exposed seeds may be responsible for quicker germination and appearance of early seedling vigour in chickpea. Activities of enzymes related to germination process such as α-amylase, dehydrogenase and protease were higher in magnetically exposed seeds as compared to unexposed seeds. Moreover, a significant correlation between the relaxation time of cytoplasmic bulk water and the activities of germination related enzymes supported our conclusion that this fraction of water plays a major role in the metabolism of germination process.

Key words: *Cicer arietinum* L., imbibition, nuclear magnetic resonance, longitudinal relaxation time (T_1), transverse relaxation time (T_2), germination enzymes.

INTRODUCTION

Exposure of seeds to magnetic field is one of the potential, safe and affordable physical pre-sowing treatments to enhance post germination plant development and crop stand. Harichand et al. (2002) observed that a magnetic field (10 mT, 40 h) exposure as seed treatment increased the plant height, seed weight per spike and yield of wheat crop subsequently. Exposure of maize seeds to a 150 mT magnetic field stimulated shoot development and led to increase in germination, fresh weight and shoot length of maize plants (Aladjadjiyan, 2002). In broad bean and pea cultivars, the magnetic stimulation of seeds improved the sprouting and emergence of seed which resulted in higher pod number and seed yield (Podlesny et al., 2004, 2005).

The process of germination can be divided into three phases. During phase I, dry seeds imbibe water, take up oxygen and increase seed mass. In phase II, there is a metabolic plateau with very little water absorption. In phase III, the germination process is completed, radicle protrusion takes place through the seed coat and absorption of water and oxygen rapidly increases. Analysis of water uptake and its distribution during initial stages of germination is necessary to understand the process of seed germination (Bewley and Black, 1994).

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The water status and the changes during imbibition of seeds influence subsequent development and growth (McDonald, 1999). Nuclear magnetic resonance (NMR) spectroscopy offers the most appropriate non-invasive and non-destructive method for studying seed imbibition and for characterization of seed water. These changes in biological system can be elegantly studied non-invasively using low resolution NMR (Ratcliffe and Shachar-Hill, 2001). In general, these NMR studies on hydrated seeds suggest that protons with a short relaxation time are, in part associated with the bound/structural water; protons of medium relaxation time are associated with inter-cellular/cytoplasmic water and protons of long relaxation are associated with the extra-cellular water (Isobe et al., 1999).

The mobilization of seed storage proteins represents one of the most important post germinative events in the growth and development of seedling. Proteolytic enzymes play central role in the biochemical mechanism of germination (Shewry et al., 1995; Muntz, 1996). These proteases increased in the early stages of germination and decreased later (Ramakrishna and Ramakrishna Rao, 2005). In our laboratory, chickpea seeds were subjected to different magnetic fields (50, 100, 150, 200 and 250 mT) and duration (1, 2, 3 and 4 h) and their germination and vigor characteristics of 8 day old seedlings were evaluated. Among various combinations of magnetic field strength and duration, best results were noted in 100 mT, 1 h fields compared to other magnetic fields (Vashisth and Nagarajan, 2008). Therefore, the present study was undertaken to explain the mechanism of improvement in magnetically treated seeds by characterizing changes in water status of magnetically exposed and unexposed chickpea seeds during hydration and germination by NMR spectroscopy along with measurement of activities of some enzymes related to germination process.

MATERIALS AND METHODS

Magnetic treatment of Seed

Chickpea seeds (Var. Pusa-1053) were obtained from National Seed Cooperation, New Delhi. Seeds were exposed to the magnetic fields of 100 mT for 1 h by electromagnetic field generators. The required strength of the magnetic field was obtained by regulating the current in the coils of the electromagnet. Gauss meter was used to measure the strength of the magnetic field between the poles.

Seed imbibitions during germination

The difference in imbibitions kinetics of magnetically exposed along with unexposed chickpea seeds were studied. Seeds were allowed to imbibe water in covered Petri dishes of 4-inch diameter layered with moist filter paper pads at 20°C. After blotting off excess moisture, the wet weight of the seeds and NMR relaxation times that is, longitudinal relaxation time (T_1) and transverse relaxation time (T_2) were measured periodically until all seeds have germinated. They were quickly put into the pre-weighed NMR tubes of 10 mm diameter, corked to avoid dehydration and placed in the probe of NMR spectrometer after taking the fresh weight. The height of the sample was kept approximately at 2 cm. There were three replications. Seeds were later dried in an oven at 95°C to constant weight and seed moisture was calculated as aforementioned.

Longitudinal relaxation time (T_1)

Seed water T_1 was measured using Bruker NMS 120 minispec NMR analyser operating at 20 MHz. Seed water T_1 was measured by saturation recovery method. The following settings were used for the measurement: data points-18, duration-5 ms, and number of scans-4. The in-built Expspel program of the instrument was used to calculate the T_1 of the seed water.

Transverse relaxation time (T_2)

Transverse relaxation or spin-spin relaxation time was measured by the Carr-Purcell-Meiboom-Gill (CPMG) method (Snarr and VanAs, 1992) in the same sample used for the measurement of T_1 in the same instrument. Each measurement had the following setting: data points 150, pulse separation 0.5 ms, dummy echo 3 and scans 10. Gain was adjusted to maximize the signal to noise ratio. The T_2 value was calculated using the built-in Expspel program with the single exponential decay observed in the CPMG sequence.

Components of transverse relaxation time (T_2)

In plant and seed systems, at least three water components can be identified with the transverse relaxation times T_{2c}, T_{2b} and T_{2a}. T_{2c} accounts for the hydration water of macromolecules and is tightly bound, T_{2b} for the cytoplasmic bulk water with lower mobility and T_{2a} for the extracellular free water (Di Nola et al., 1991; Brosio et al., 1992).

Enzymes activities during germination

Enzymes related to germination in magnetically treated and untreated germinating seeds of chickpea were assayed at different hours of imbibition in distilled water at 20°C. One gram (1 g) germinating seeds were taken for enzyme extraction at different time. α-Amylase activity was estimated following the method described by (Berbfeld, 1995). Protease activity was estimated following the method (Kunitz, 1947). Dehydrogenase activity was estimated following the method described by Kittock and Law (1968). There were three replications for each measurement and for estimating dehydrogenase activity; five embryonic axes were taken in quadruplicate.

RESULTS AND DISCUSSION

Water uptake during germination

Water uptake during seed germination showed three phases namely; rapid hydration (imbibition, phase I), lag phase (phase II) and steady hydration phase (germination growth, phase III). The rapid hydration phase I and lag phase II were observed until the 10 h and between

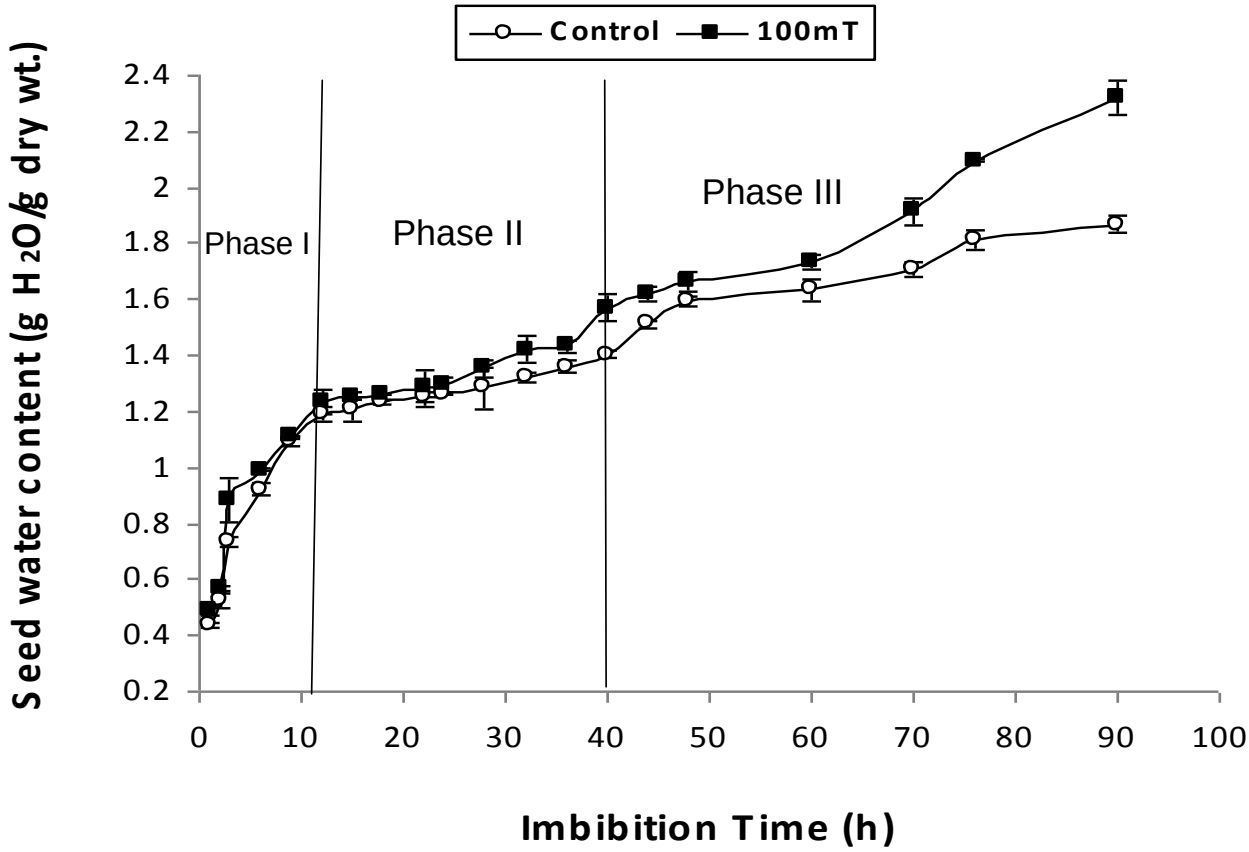

Figure 1. Changes in seed water content with hours of imbibition in water at 20°C for magnetically exposed and unexposed chickpea seeds.

10 and 40 h, respectively (Figure 1). The third phase, which coincided with the radicle protrusion showed a steady hydration phase III from 40 h. During the rapid hydration phase I, water uptake was similar for magnetically exposed and unexposed seeds. In magnetically exposed seeds water uptake was marginally greater during the second half of phase II and significantly greater during phase III of imbibition. The moisture content increased from the initial level of 0.44 to 1.868 g H_20 g^{-1} in control seeds, from 0.49 to 2.321 g H_20 g^{-1} in seeds exposed to 100 mT (1 h).

In the present study, germinating seed showed these three distinct phases of hydration. Compared to un-exposed seeds, marginal increase during lag phase and significantly higher uptake of water in phase III of hydration was observed in magnetically exposed seeds. A further rapid increase in water uptake occurred after radical emergence (phase III) until the storage tissue and growing seedling have water contents of 70 to 90% (Steiner, 1998). Kavi (1977) observed that soybean seeds exposed to magnetic fields have increased capacity of moisture absorption. Garcia-Reina et al., (2001) observed a significant increase in the rate of absorption of water in lettuce seeds exposed to a magnetic field. He correlated the observed increase of germination rate of the seeds with the theoretically calculated variations induced by magnetic fields in the ionic currents across the cellular membrane. The fields originate in changes in the ionic concentration and thus in the osmotic pressure which regulates the entrance of water to the seeds. Hence, there is strong evidence that the magnetic field alters the water relations in seeds, and thereby alters the germination rate of seeds.

Longitudinal and transverse relaxation time

Except for an initial dip, seed water T_1 nearly followed the same trend as seed water during imbibition for both magnetically exposed and un-exposed seeds (Figure 2). However, the values for seeds exposed to magnetic field were significantly higher from phase II imbibition onwards. The transverse relaxation (T_2) of magnetically exposed and unexposed seeds of chickpea showed an initial decrease when dry seeds were subjected to hydration during germination over a 3 h period of imbibition (Figure 3). During subsequent period of hydration, marginal increase in T_2 value until 48 h was observed and magnetically treated seeds in general showed slightly higher values than untreated controls.

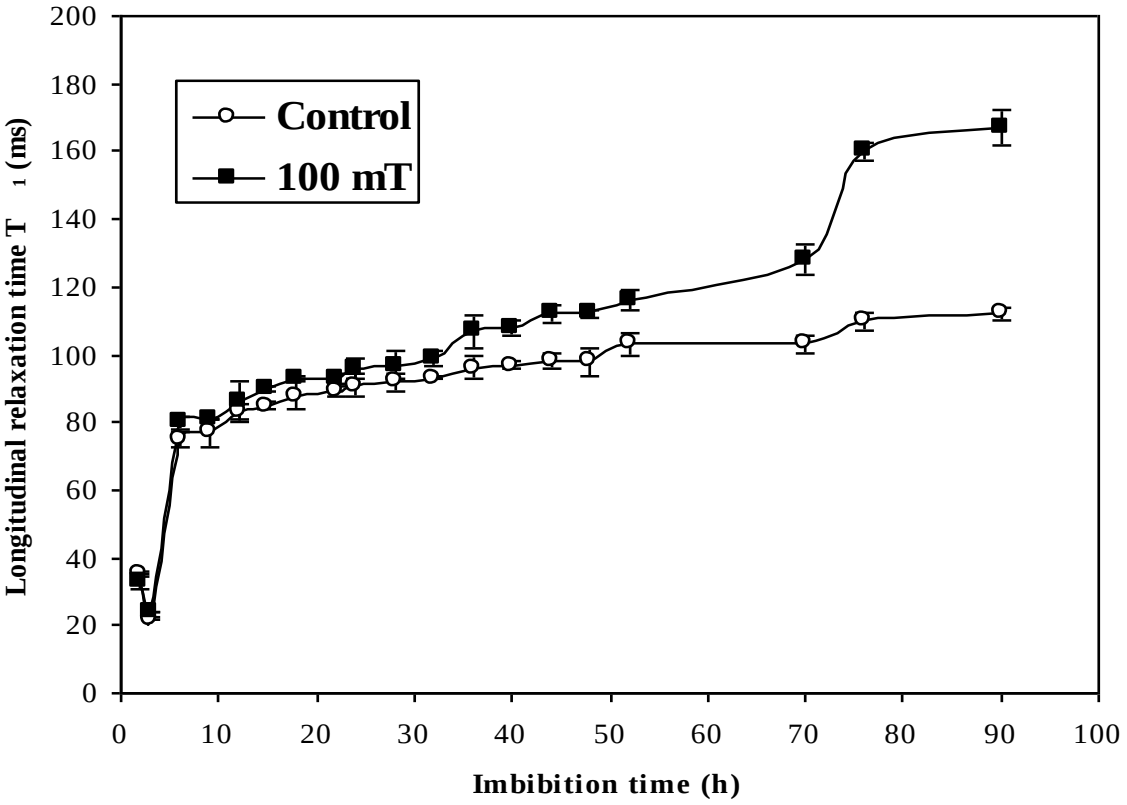

Figure 2. Changes in weighted average longitudinal relaxation time, T_1 of seed water with hours of imbibition in water at 20°C for magnetically exposed and unexposed chickpea seeds.

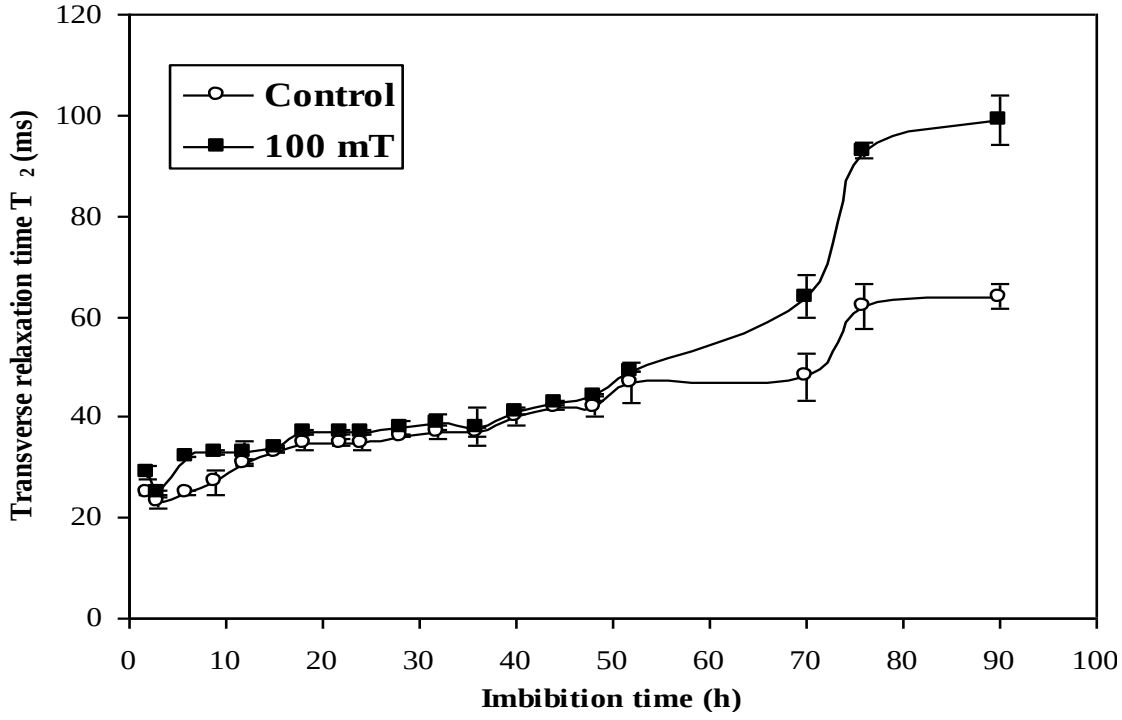

Figure 3. Changes in weighted average transverse relaxation time, T_2 of seed water with hours of imbibition in water at 20°C for magnetically exposed and unexposed chickpea seeds.

There was substantial increase in T_2 during the subsequent stage of germination and magnetically exposed seeds showed significantly higher T_2 values than unexposed seeds.

NMR longitudinal and transverse relaxation times of tissue water are used to study changes in structure and integrity of cellular membranes as the relaxation characteristics indicate the distribution of water and its molecular mobility (Maheswari et al., 1999). The changes in seed water T_1 with imbibition time showed a decline initially and then an increase subsequently. Initial dip in T_1 values during hydration and then a gradual increase has been observed in maize (Ratkovic, 1987) and in wheat seeds (Gambhir et al., 1997). Magnetically exposed seeds had higher T_1 values compared to unexposed seeds. Though moisture content has a direct relationship with relaxation time, in this study even where seed moisture levels were similar, the corresponding T_1 values were higher for magnetically exposed seeds. Longitudinal relaxation time of water in leaves has been directly related to water activity (a_w) of the cell water (Gambhir et al., 1997), which in turn is related to availability for metabolic activities. This may explain the greater speed of germination and increased seedling vigour of magnetically exposed seeds as reported by Vashisth and Nagarajan (2008).

The results of T_2 measurements showed distinct changes during various stages of hydration and germination of seeds. This shows that NMR relaxation time T_2 reflects the *in vivo* changes in dynamic and physical states of water in seed. Seed water transverse relaxation time (T_2) decreased initially during the rapid hydration phase and increased slowly during phase II of hydration followed by a rapid increase during subsequent hydration in seeds (Figure 3). Such trend has been reported in cowpea (Brosio et al., 1992) when the seeds were soaked in water. The reduction in seed water T_2 values, in spite of an increase in seed water content in the initial phase can be explained on the basis of the reorganization of the different water fractions within the seed tissues. The relaxation times are influenced by a delicate balance between total water content, macroscopic and microscopic distribution of water at different sites, macromolecular–water interactions and exchange (slow or fast) between different phases with increase in seed moisture content (Mathur-De Vre, 1984). In magnetically treated seeds, T_2 values were higher than untreated controls at later stages of imbibition. This may be explained on the basis of higher water uptake by magnetically treated seeds and higher values of the components of relaxation times.

Components of transverse relaxation time

The actual relaxation curve showed a marked non-exponentiality that could be accounted by the presence of three clearly recognizable components with different relaxation times. According to the individual values, these three components have been identified with the transverse relaxation times T_{2a}, T_{2b} and T_{2c}, respectively. $T_{2a,}$ corresponding to extra-cellular free water decreased with increase in hydration time in magnetically exposed and unexposed seeds until 40 h and increased thereafter. Increase in T_{2a} coincided with sprouting in seeds (Figure 4a). $T_{2b,}$ which corresponds to cytoplasmic bulk water increased until 44 h in magnetically exposed seeds and until 48 h in unexposed seeds of chickpea. However, during subsequent period of germination there was considerable decrease in T_{2b} in both cases (Figure 4b). The values of T_{2b} were higher for magnetically treated seeds compared to control during most of the seed hydration period. Component T_{2c} was not detectable in a dry seed but resolved at 1 and 2 h after imbibition in magnetically exposed and unexposed seeds, respectively (Figure 4c). T_{2c}, which corresponds to the hydration water of macromolecules, initially increased and then decreased to 6 ms in 100 mT (1 h) and 3 ms in unexposed seeds, respectively. Thereafter, there was continuous increase in T_{2c} in all seeds and was significantly greater in magnetically exposed seeds than unexposed. However, this third component disappeared after 48 h of hydration when radicle protrusion took place.

As seen, Figure 5a-b gives the fractional populations of different water protons of varying mobilities during germination in chickpea. After the appearance of the least mobile fraction, both magnetically exposed and unexposed seeds showed an increase in spin population of T_{2b} and T_{2c} together and a decrease in T_{2a} until the radicle emergence took place. However, during the subsequent period of imbibition, only two populations of water were observed with T_{2a} fraction being larger than T_{2b} fraction. Figure 6a-c gives the amount of seed water corresponding to different T_2 components in magnetically treated and untreated seeds. Amount of water in more mobile form (extracellular free water) remained stable and increased only after radicle emergence. The values for untreated seeds were marginally higher than value of magnetically treated seeds till radicle protrusion and then they were greater for treated seeds. Amount of water in less mobile form (cytoplasmic bulk water) increased continuously in both treated and untreated seeds at different rates. Amount of water with least mobility remained between 0.17 to 0.35 g g-1 dry wt in magnetically treated seeds and between 0.14 to 0.30 g g-1 dry wt. in untreated seeds and then disappeared when radicle protrusion took place. These two fractions of water were larger for magnetically treated seeds compared to untreated seeds. This may be explained as the initial hydration of water tightly held by macromolecules and membranes with least mobility and subsequent layers of water had relatively greater mobility due to protein hydrolysis (Bewley and Black, 1994) and due to exchange with bulk or free water.

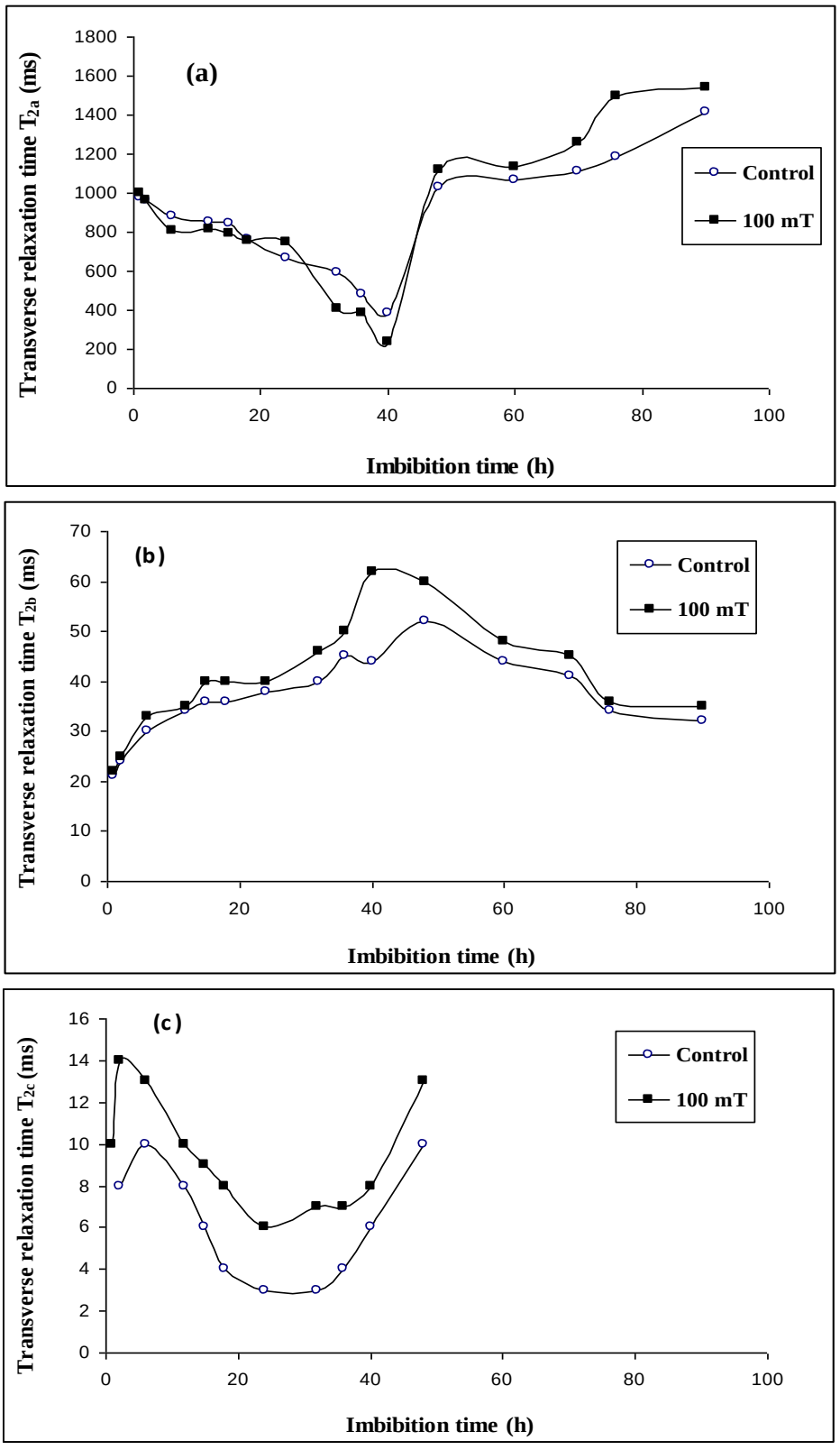

Figure 4a-c. Changes in components of transverse relaxation time, T_2 of seed water with hours of imbibition in water at 20°C for magnetically exposed and unexposed chickpea seeds.

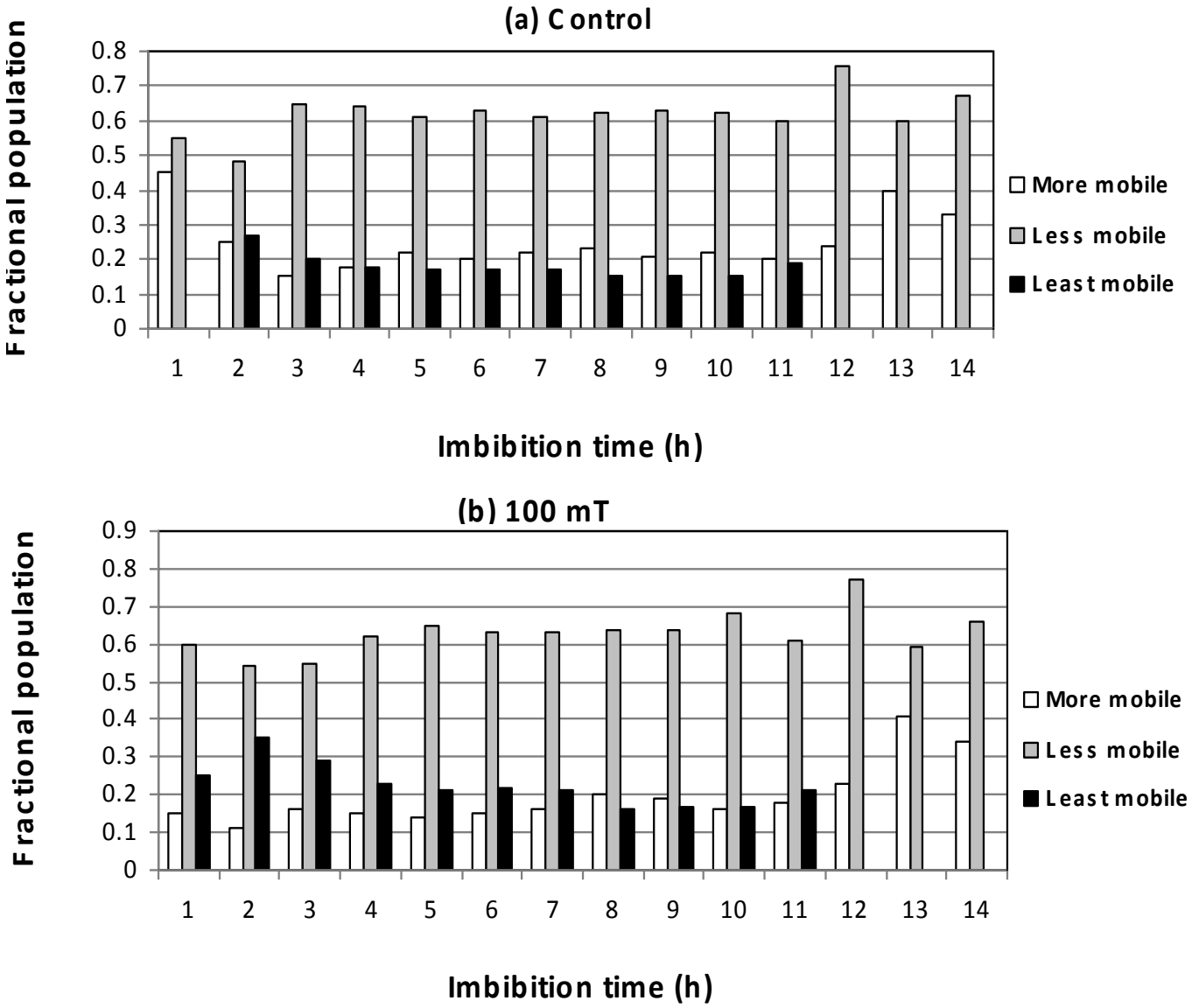

Figure 5a-b. Fractional population of different water protons of varying mobilities with hours of imbibition in water at 20°C for magnetically exposed and unexposed chickpea seeds.

NMR studies on hydrated seeds suggest that protons with short relaxation time are associated with bound water, protons of medium relaxation time are associated with cytoplasmic water and protons with long relaxation time are associated with extracellular or free water (Isobe et al., 1999). Water in biological systems is characterized by great structural complexity. This water forms a part of network of biological interfaces and exhibit restricted motion (Sun, 2000). Subsequent layers of water have little interaction with polymer surface and are assumed to have bulk properties. Free water has properties associated with pure liquid-state water. Results of transverse relaxation time analysis indicated the complex water exchange process taking place between components inside magnetically treated and untreated seeds of chickpea. During imbibition the third component with least mobility appeared immediately after 1 h in magnetically exposed seeds and after 2 h in unexposed seeds. Magnetic resonance imaging of lupine seed also showed protons corresponding to long relaxation time in dry seeds in vascular bundles adjacent to embryonic axis (Garnczarska et al., 2007).

The 1H NMR 2D micro imaging of low-hydrated tobacco seeds showed that there were localized tissue areas with higher proton mobility (Leubner-Metzger, 2005). This shows that the state and quality of different water components during early stages of imbibition could provide a medium suitable for metabolic activity to

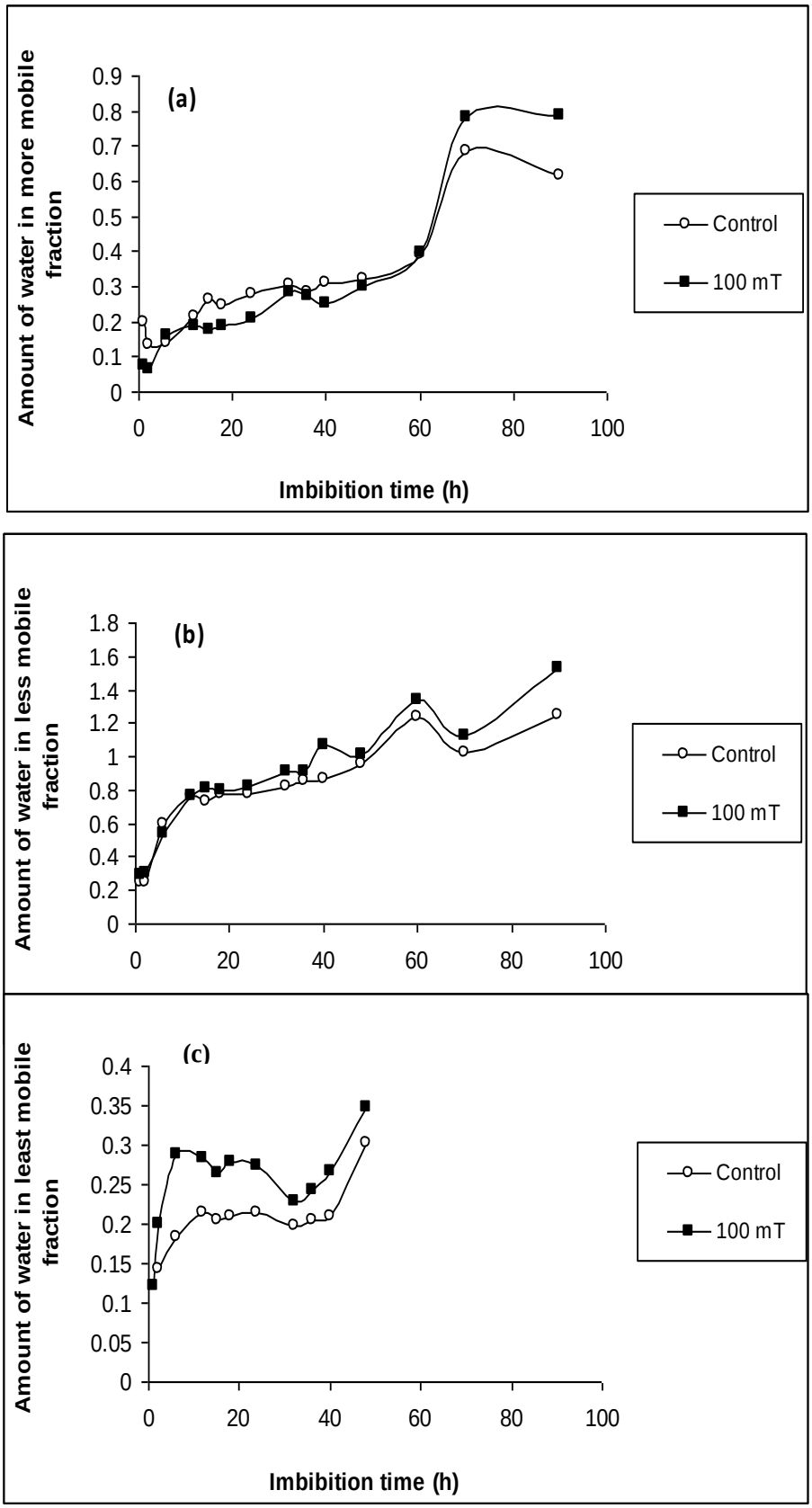

Figure 6a-c. Amount of water corresponding to different T_2 components with hours of imbibition in water at 20°C for magnetically exposed and unexposed chickpea seeds.

proceed, although the total water content is still low. Component T_{2a}, which represents the relaxation time of extracellular free water decreased with imbibition time till radicle protrusion then increased sharply (Figure 6a). Once the radicle protrusion takes place, tissue growth begins and the bound fraction disappears, the relaxation time of this free component increased. T_{2b}, which represent the relaxation time of bulk water fractions in seeds increased in all seeds until radicle protrusion then decreased (Figure 6b). T_{2c}, representing the relaxation time of macromolecular hydration water fraction decreased initially until 20 h of imbibition then increased (Figure 6c).

Enzymes related to germination

Activities of α-amylase, dehydrogenase and protease measured in magnetically exposed seeds maintained significantly higher values as compared to unexposed control in most stages of germination. An increase in enzyme activity occurred with germination process, reached a maximum value then the activities decreased. α-amylase activity was significantly increased until 48 h of imbibition and reached maximum that was 18% higher in 100 mT for 1 h exposure as compared to corresponding values of unexposed controls (Figure 7a). Dehydrogenase activity was significantly increased until 36 h of imbibition and reached maximum value that was 19% higher in 100 mT, 1 h as compared to corresponding value of unexposed control (Figure 7b). Protease activity increased significantly until 40 h of imbibition reached maximum value that was 13% higher in magnetic treatments as compared to corresponding values of unexposed control (Figure 7c).

α-Amylase is responsible for the degradation of food reserves of the seedling during germination. In the present study α-amylase activity increased up to 48 h in chickpea (Figure 7a) beyond which it started declining. Faster germination in magnetically treated seeds might be partially explained with the increased activity of α-amylase. Rajendra et al. (2005) reported that α-amylase activity in broad bean significantly decreased at 5, 50 and 100 μT on day 2 and 4 of growth. In their experiment the seed exposure was at very low magnetic fields. The investigation of Lebedev et al. (1975) indicated profound influence of magnetic field on the basic aspects of metabolism of winter wheat, soybean and sunflower plants, including photosynthesis, respiration and enzymatic activity.

Bhatnagar and Deb (1978) observed that the seeds treated at 150 and 100 mT had significantly higher ($P<0.01$) α- amylase activity than the controls. Lebedev et al. (1975) had also reported a similar result in wheat. Pitman and Ormrod (1970) found more reducing sugars in germinating magnetically treated wheat seeds. In the present study, dehydrogenase activity in both magnetically exposed and unexposed control seeds increased with imbibition, reached maximum of 36 h beyond which it started declining. Increase in dehydrogenase activity has been reported in primed soybean (Saha et al., 1990) and tomato (Pandita et al., 2003) compared to unprimed seeds. Also greater glucose-6-phosphate dehydrogenase activity has been reported in primed sweet corn seeds (Smith and Cobb, 1991). Exposure to magnetic field seems to act like priming with similar enhancement effects. Protease activity in seeds showed an increase up to 40 h of imbibition followed by a decrease with onset of germination. The activity of this enzyme was significantly higher in magnetically treated seeds compared to controls. Protease are involved in the degradation of proteins in the germinating seeds and the reduction being initiated by endoproteases which convert the water insoluble storage protein into soluble peptides that can hydrolyzed to amino acids by exopeptidases (Callis, 1995; Shutov and Vaintraub, 1987). Vidyavathi et al. (1983) observed maximal proteolytic activity on the third day of germination in germinating finger millet seeds. The storage protein degradation and the increase in proteolytic activity observed in germinating chickpea seeds are consistent with various reports (Ahmed et al., 1995; Alvarez and Guerra 1985; Nielsen and Liener, 1984).

Also, significantly increased activity of germination related enzymes (α-amylase, protease and dehydrogenase) over control during germination in magnetically treated seeds was observed and followed similar trend as the cytoplasmic bulk water component T_{2b} albeit reaching their peak values at different imbibition times. Also, T_{2b} showed highly significant positive correlation with the activities of all the three germination enzymes (Figure 8a-c). This corroborated the hypothesis that T_{2b} represents the molecular mobility and availability cytoplasmic bulk water for various metabolic functions including germination related enzyme activities.

Conclusion

The study shows evidence for re-arrangement of cellular water in chickpea seeds exposed to static magnetic field and in un-exposed controls. NMR relaxation time of seed water and its analysis indicated early appearance of structural/hydration water and greater amount of cytoplasmic bulk water and hydration water in magnetically exposed seeds compared to unexposed controls. Also, in these treated seeds, molecular mobility of cytoplasmic bulk water and hydration water of macromolecules were higher as indicated by their respective relaxation times. This may be responsible for early germination and higher seedling vigour of these seeds over untreated controls. A highly significant correlation between the relaxation time of cytoplasmic bulk water and the activities of germination related enzymes in general emphasised the fact that this fraction of water is vital for metabolic activities taking

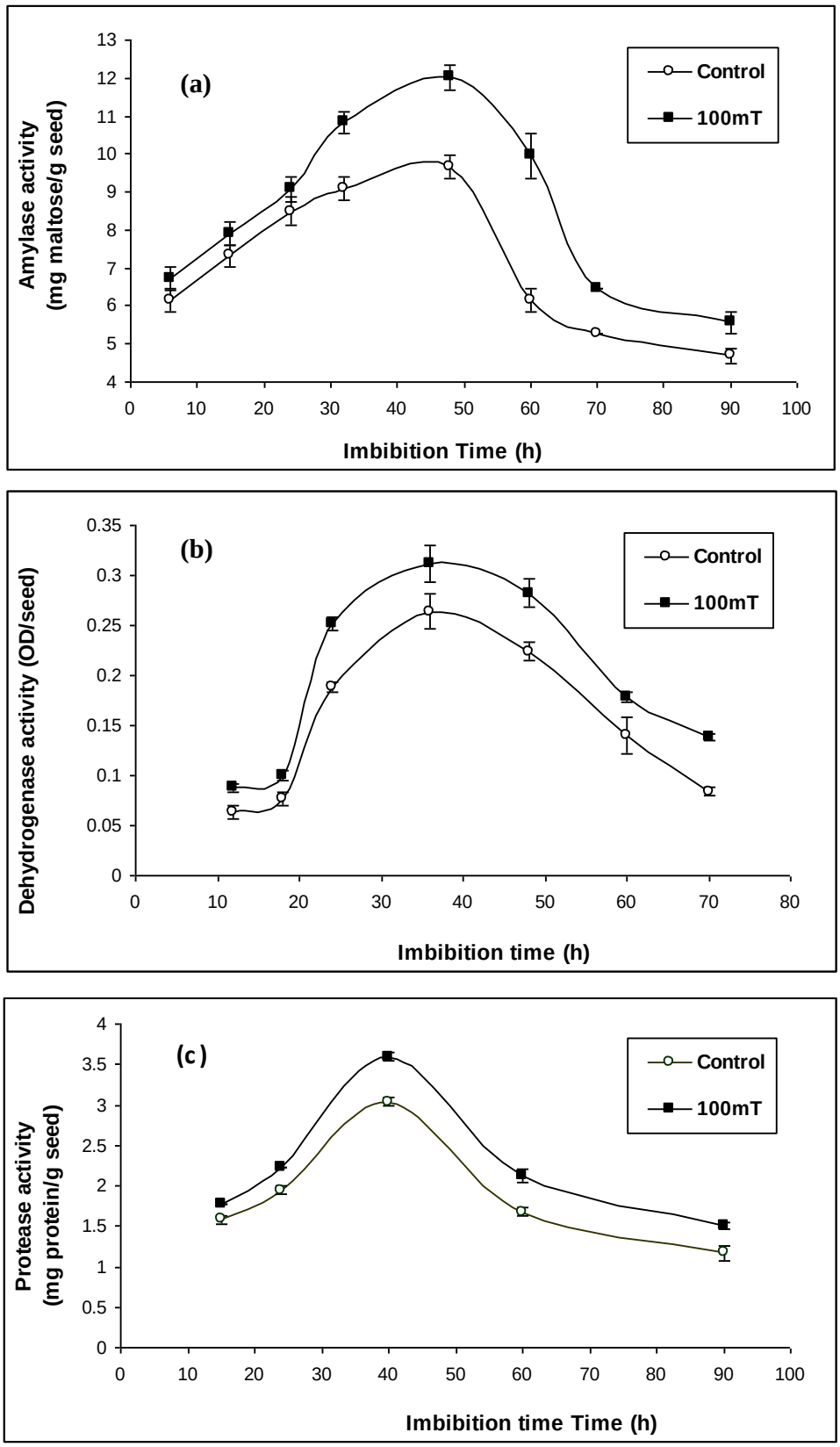

Figure 7a-c. Changes in the germination related enzyme activities, α-amylase, dehydrogenase and protease with hours of imbibition in water at 20°C for magnetically exposed and unexposed chickpea seeds.

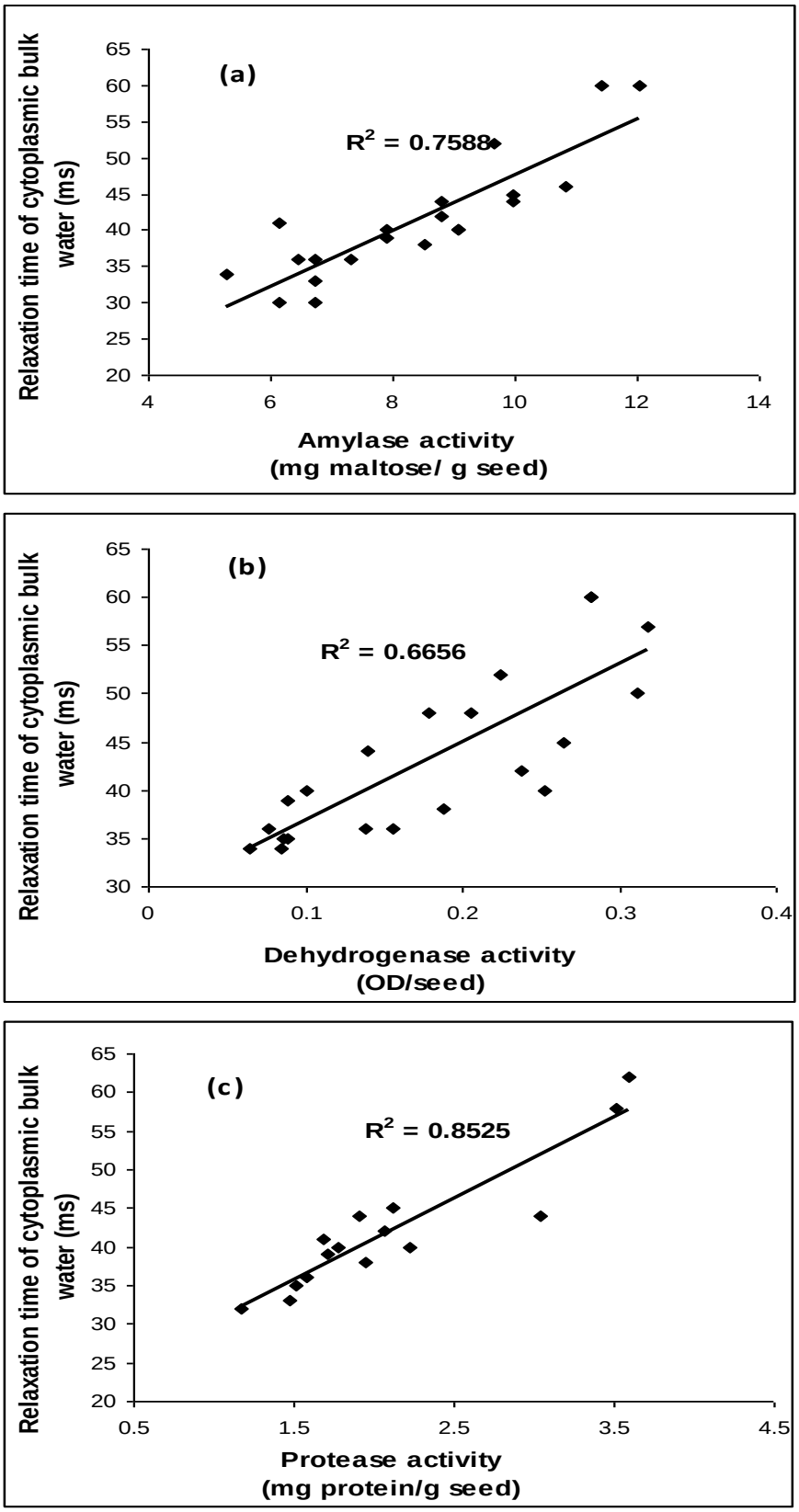

Figure 8a-c. Simple linear correlation of relaxation time of cytoplasmic bulk water of both magnetically exposed and unexposed seeds with α-amylase, dehydrogenase and protease activities.

place during germination process.

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