Determining the effects of LFHV-PEF treatment on the water distribution and vigor of aged rice seeds using LF-NMR

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Abstract: This study aimed to investigate the effect of low-frequency high-voltage pulsed electric field (LFHV-PEF) treatment on the germination of aged rice seeds. Aged rice seeds were subjected to LFHV-PEF treatment with different electric field strengths, and low-field nuclear magnetic resonance (LF-NMR) was performed to acquire the LF-NMR data of rice seeds at different germination periods during a standard seed germination test to analyze their internal patterns of water state and distribution. Optimal treatment conditions were determined based on the physicochemical data collected during germination, and the improvements in seed vigor were verified. The findings indicated that during germination, the contents of bound and semi-bound water within the aged rice seeds initially increased and then decreased, whereas free water and total water contents increased continuously and rapidly. Side peaks were also observed within the seeds. Under the LFHV-PEF treatment, the semi-bound water within the seeds was more easily converted to free water, and the water absorption rate, germination potential, germination rate, germination index, and vigor index of these seeds improved. Further, the optimal electrical field strength was 12 kV. By analyzing the internal patterns of water state and distribution in seeds, the mechanism by which electric field treatment improved seed vigor was elucidated, thus, providing theoretical support and data evidence for research on water absorption during the germination of rice seeds, and methods for improving seed vigor.
Keywords: aged rice seeds, germination, water phase, seed vigor, LF-NMR, LFHV-PEF
DOI: 10.25165/j.ijabe.20221504.7590

Citation: Cheng E S, Song P, Hou T G, Wu L Y, Zhang B H. Determining the effects of LFHV-PEF treatment on the water and vigor of aged rice seeds using LF-NMR. Int J Agric & Biol Eng, 2022; 15(4): 190–196.

1 Introduction

High-vigor crop seeds can ensure proper germination and robust plants[1]. Thus, seed vigor considerably influences crop quality[2]. High-voltage pulsed electrical field (HV-PEF) treatment can enhance seed vigor and regulate seed germination and seedling growth[3]. Several studies have examined the mechanisms underlying the improvements in seed vigor using the HV-PEF treatment[4-7]. These studies demonstrated that the application of reasonable electric field strength and treatment time can positively affect seed metabolism[8], optimize nutrition[9,10], and stimulate the activity indices of various enzymes, such as antioxidant enzymes, superoxide dismutase, peroxidases, and catalase. During seed germination[11,12], water plays an important role in maintaining the functioning and activity of seeds[13]. The state and distribution of water can affect the catalytic functions of various enzymes[14,15], which in turn can alter the metabolic rates of biological macromolecules, such as carbohydrates, starch, lipids, and proteins, consequently[16], affecting seed germination and emergence rates[17-21]. Research on the patterns of changes in the internal enzymes of seeds treated with HV-PEF has been conducted extensively and has produced remarkable results. However, the effects of HV-PEF on seed vigor considering the characteristics of the internal water state in seeds have rarely been studied.

Low-field nuclear magnetic resonance (LF-NMR) technology can test samples rapidly, accurately, and non-destructively[22-24], and can analyze the internal water phase, distribution characteristics, and flow characteristics of samples at a submicroscopic level[25-27]. Thus, it can reveal the internal water state and patterns of change during the germination of crop seeds[28,29].

This study aimed to elucidate the mechanisms underlying the improvement of seed vigor through electric field treatment by analyzing the internal water state and distribution patterns of aged rice seeds. The seeds were treated with low-frequency high-voltage pulsed electric field (LFHV-PEF) through different electric field strengths. Subsequently, LF-NMR was employed to analyze the internal states of sideband water, bound water, semi-bound water, and free water, and the status of water mobility in treated rice seeds at 0, 24 h, 48 h, 72 h, 96 h, and 120 h during germination to obtain the optimal electrical field strength that improves seed vigor. Later, a standard seed germination test was performed to determine the fresh weight, root length, shoot length, and the number of seeds germinated at the corresponding periods during rice seed germination. The corresponding results were then used to calculate the water absorption (WA) rate, germination potential (GP), germination rate (GR), germination index (GI), and

Received date: 2022-04-12 **Accepted date:** 2022-07-30
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vigor index (VI) of the rice seeds to verify the improvements in seed vigor[30].

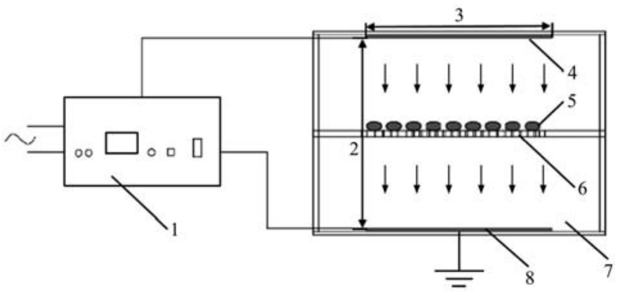
2 Materials and methods

2.1 Materials

Wuyou No. 4 (WY4) rice seeds from a conventional japonica rice variety cultivated in northern China were selected for this study. Grains having a thousand weight of (27.7±0.5) g and a moisture content of (12.5±0.3)% were harvested in 2019 and stored for 20 months under conditions in a cool and dry environment at (18.0±0.5)°C temperature. In total, 4000 rice seeds with plump kernels and intact husks were selected, of which 1000 and 3000 seeds were used for the LF-NMR and standard seed germination tests, respectively. All tests were performed at the Liaoning Engineering Research Center for Information Technology in Agriculture (Shenyang city, Liaoning province, China), and the LF-NMR test was conducted in an LF-NMR laboratory.

2.2 LFHV-PEF treatment

The LFHV-PEF treatment apparatus, consisting of the LFHV-PEF generator and sample treatment chamber, was developed by the Shenyang Agricultural University. The output voltage was 4-20 kV and the frequency was 0.1-15.0 Hz, both of which were continuously adjusted. The sample treatment chamber comprised two copper electrode plates (50 cm×50 cm) separated by a distance of 5 cm. A perforated plexiglass plate was fixed between the two electrode plates to place the rice seed samples (Figure 1).



1. LFHV-PEF generator 2. Copper plate spacing 3. Copper plate size 4. Copper anode 5. Seed 6. Plexiglass with holes 7. Sample disposal room 8. Copper cathode

Note: LFHV-PEF: Low-frequency high-voltage pulsed electric field. The same as below. The one-way arrow indicates the direction of electric field intensity.

Figure 1 Schematic diagram of LFHV-PEF device

The LFHV-PEF treatment was conducted in an air-conditioned laboratory at (25.0±0.5)°C and (30±1)% relative humidity (RH). In total, 10 test gradients, including nine different voltage treatment groups and one control check (CK) were included in the test, with 400 rice seeds for each test gradient. Prior to each treatment, rice seeds in each test gradient were spread evenly in a single layer on the perforated plexiglass plate. The anode and cathode of the LFHV-PEF generator were connected to the upper and lower copper plates of the sample treatment chamber, respectively, with the cathode grounded. After switching on the power, the rice seeds were treated with LFHV-PEF, with the direction of the electric field strength being vertically downwards. Based on previous studies and pilot testing, the treatment time was set at 30 min; voltages were set at 4 kV, 6 kV, 8 kV, 10 kV, 12 kV, 14 kV, 16 kV, 18 kV, and 20 kV; and the frequency was set at 0.2 Hz. Subsequently, the treated rice seeds were stored in a sealed bag until further use.

2.3 LF-NMR test

The basic principle of LF-NMR is to use the frequency signal (spectral signal) converted from the nuclear magnetic resonance signal through the Fourier formula, and then use it as a method for quantitative analysis and detection. LF-NMR is frequently employed for non-destructive testing of the internal water phases, distribution characteristics, and flow characteristics of samples. The characteristics of the LF-NMR spectrometer (NMI20-015V-I, Niumag Electronic Technology Co., Ltd., China) were as follows: permanent magnet type, (0.50±0.08) T magnetic field strength, 21 MHz radiofrequency (RF) pulse frequency, 32°C magnet temperature, and 15 mm probe coil diameter (Shanghai Niumag Electronic Technology Co., Ltd., China). In total, 100 rice seeds were collected from each of the 10 test gradients and divided into twenty groups containing 5 seeds each. A glass test tube containing the rice seeds was placed vertically at the center of the LF-NMR coil, and the Carr-Purcell-Meiboom-Gill (CPMG) pulse train was used to acquire the transverse relaxation time (T_2) decay curve of the seeds. The parameters for the CPMG pulse train were as follows: spectrometer frequency SF_1=21 MHz, frequency offset O_1=650488.66 Hz (slight offset for each test), RF 90° pulse width P_1=18 μs, number of sampling points TD=90016, the interval of scan repetitions TW=1000 ms, pre-amplification gain PRG=3, RF 180° pulse width P2=36 μs, number of echoes NECH=3000, TE=0.150 ms, receiver signal frequency SW=200 kHz, sampling starting point RFD=0.080 ms, analog gain RG1=20 db, digital gain DRG1=3, and cumulative number of scans NS=64. The acquired data were transferred into the NMR inversion software to conduct inversion with 100 000 iterations to eliminate variations in the initial moisture content of the rice seeds due to differences in their initial masses. Further, mass normalization was performed on the signal amplitude data for all spectra.

2.4 Standard seed germination test

For this test, 300 rice seeds were obtained from each of the 10 test gradients and disinfected with 3% NaClO solution for 5 min. Subsequently, seeds were rinsed three times with distilled water and placed uniformly in a germination box covered with a germination paper, with each group containing 100 seeds. All sample groups were placed in an intelligent artificial climate chamber (RTOP-268D, Zhejiang Tuopu Instrument Co., Ltd., China, temperature control range was 0°C-50°C, RH control range was 50%-95%, illuminance of 0-5500 lx, illumination method was partition type) and cultured at (28±1)°C, with an alternating 12 h light/dark cycle. The standard seed germination test was conducted according to the procedure described in the International Rules for Seed Testing. During the germination period of the rice seeds, the same amount of distilled water was sprayed for all sample groups at the same time each day to keep the germination paper surface moist. The number of germinated seeds was recorded, and the fresh weight of fixed selected 20 rice seeds was measured until they were germinated.

At the end of germination, 25 seedlings were randomly selected from each group to measure their seedling fresh weight, and root and shoot lengths, and the corresponding mean values were calculated. Finally, the WA(%), GP (%), GR (%), GI, and VI values were calculated for each group according to Equations (1)-(5).

$$\mathrm{WA}=\frac{\text{Seed quality after soaking}-\text{Seed quality before soaking}}{\text{Seed quality before soaking}}\times 100\% \quad (1)$$

$$\mathrm{GP}=\frac{\text{Total number of germinating seeds on Day 3}}{\text{Total number of seed samples}}\times 100\% \quad (2)$$

$$\mathrm{GR} = \frac{\text{Final number of germinating seeds}}{\text{Total number of seed samples}} \times 100\% \qquad (3)$$

$$\mathrm{GI} = \sum G_t/D_t \qquad (4)$$

where, G_t is the number of germinating seeds on Day t; D_t is the corresponding number of days.

$$\mathrm{VI} = \mathrm{GI} \cdot S \qquad (5)$$

where, S is the fresh weight of seed, g.

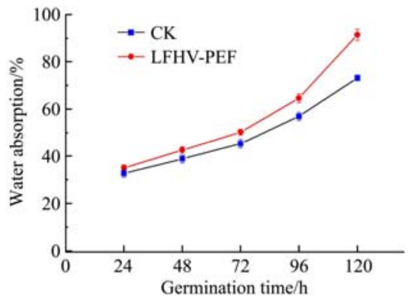
3 Results and analysis

3.1 Effects of the LFHV-PEF treatment on WA changes during the germination of the aged rice seeds

The WA values of the aged rice seeds in the nine LFHV-PEF treatment groups and CK group at 24 h, 48 h, 72 h, 96 h, and 120 h during germination were calculated. LFHV-PEF was found to significantly promote WA during seed germination; additionally, an output voltage of 12 kV was found to be the optimal treatment gradient. Hence, this gradient data were used to plot the relationship between WA and germination time during the entire germination process (Figure 2). All data were expressed as mean±standard deviation (SD).

During germination, WA sharply increased at 0-24 h, followed by a gradual increase at 24-48 h, a slightly steeper increase at 48-72 h, and a rapid increase after 72 h. This could be attributed to the three germination stages of rice seeds, that is, imbibition, radicle emergence, and sprouting stages. During early germination, external water molecules entered the seed through the ruptured seed coat, and the hydrocolloids in the seeds rapidly absorbed water, causing the seed to expand. After 24-48 h of seed imbibition, the seed organelles were activated and repaired and WA decreased. At 48 h, the radicle tip emerged through the seed coat, and the WA capacity of the seed gradually increased. At 72 h, the radicle and plumule emerged, and the growth rate increased, which consequently, increased WA again.

Based on the curve data shown in Figure 2, the WA of the LFHV-PEF treatment group was higher than that of the CK group by 3.28% (p<0.05), 9.58% (p<0.05), 10.77% (p<0.01), 13.43% (p<0.01), and 24.88% (p<0.01) at 24 h, 48 h, 72 h, 96 h, and 120 h, respectively, the water absorption of LFHV-PEF treatment group increased compared with CK group, and gradually increased with the extension of germination time.

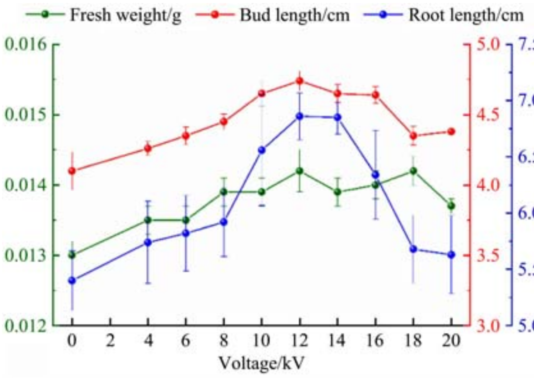


Note: CK: Control check group; WA: Water absorption. The same as below.

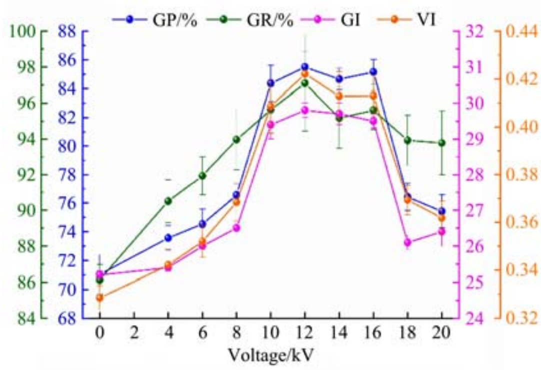
Figure 2 Relationship between WA and germination time during the germination of the aged rice seeds

3.2 Effects of the LFHV-PEF treatment on the physicochemical indicators and vigor indices during the germination of the aged rice seeds

The physicochemical indicators (shoot length, root length, and fresh weight) of the seedlings of the nine LFHV-PEF treatment groups and the CK group were measured, and the corresponding vigor indices (GP, GR, GI, and VI) were calculated. All data were expressed as mean±SD (Figure 3).



a. Physicochemical indicators



b. Vigor indices

Note: GP is the germination potential of the rice seeds, %; GR is the germination rate of the rice seeds, %; GI is the germination index of the rice seeds; VI is the vigor index of the rice seeds. The same as below.

Figure 3 Effects of LFHV-PEF treatment on physicochemical indexes and vigor indices of seeds

The physicochemical indicators and vigor indices of the aged rice seeds treated with LFHV-PEF were significantly higher than those of the CK group (p<0.05, Figure 3). The electric field strength with an output voltage of 12 kV was the optimal treatment gradient, which was consistent with the WA results. Based on the physicochemical indicators and vigor indices, the electric field strengths ranked according to seed vigor (in descending order) were as follows: mid-range electric field strength (output voltage: 10, 12, 14, and 16 kV), high-range electric field strength (output voltage 18 and 20 kV), and low-range electric field strength (output voltage: 4, 6, and 8 kV). Further, pairwise comparisons of the three ranges showed significant differences (p<0.05), implying that voltage can improve the vigor of the aged rice seeds. However, this effect was limited by excessively high or low voltages, with significant differences observed in the effects of different voltage levels.

3.3 Effects of the LFHV-PEF treatment on different water phases during the germination of the aged rice seeds

Germination is accompanied by several complex physical changes and chemical reactions, and water is an important medium for the intracellular metabolism of rice seeds[31,32]. The transverse relaxation time T_2 of the LF-NMR spectrum can reflect the dynamics of water molecules within rice seeds. According to the NMR principles, the T_2 length can reflect the state of water, the size of signal amplitude can reflect the status of water enrichment, and the changes in the signal amplitude can reflect changes in water mobility within the sample.

A shorter T_2 duration implies that the water molecules have a smaller degree of freedom and are more tightly bound to other substances; conversely, a longer T_2 duration implies that the water molecules have a larger degree of freedom and are more loosely bound to other substances. Thus, water in rice seeds that is tightly bound to hydrocolloids composed of proteins, carbohydrates, and phospholipids has relatively short T_2 durations, whereas water in seed capillaries, intracellular vacuoles, and intercellular spaces has relatively long T_2 durations. In this experiment, the LF-NMR T_2 relaxation spectra revealed four peaks in all curves (except the curve at 0 h of germination), suggesting that water existed in four states during the germination of aged rice seeds. These states were defined, from left to right, as side-peak water (0.01 ms< T_{2a}<0.4 ms), bound water (0.4 ms<T_{2b}<10 ms), semi-bound water (10 ms<T_{2c}<100 ms), and free water (100 ms<T_{2d}<1000 ms). Side-peak water referred to water having a lower degree of freedom than bound water.

The total signal amplitude A in the LF-NMR T_2 relaxation spectrum was directly proportional to the number of hydrogen protons in the rice seeds. Stronger signal amplitude indicated more hydrogen protons and higher relative moisture content. Thus, the corresponding signal amplitude of each peak could also reflect the relative contents of the different water phases. A, A_{2a}, A_{2b}, A_{2c}, and A_{2d} denote the total water, side-peak water, bound water, semi-bound water, and free water contents, respectively. These variables are related as follows: $A=A_{2a}+A_{2b}+A_{2c}+A_{2d}$. Figure 4 shows the T_2 relaxation spectra of the aged rice seeds against different LFHV-PEF treatments.

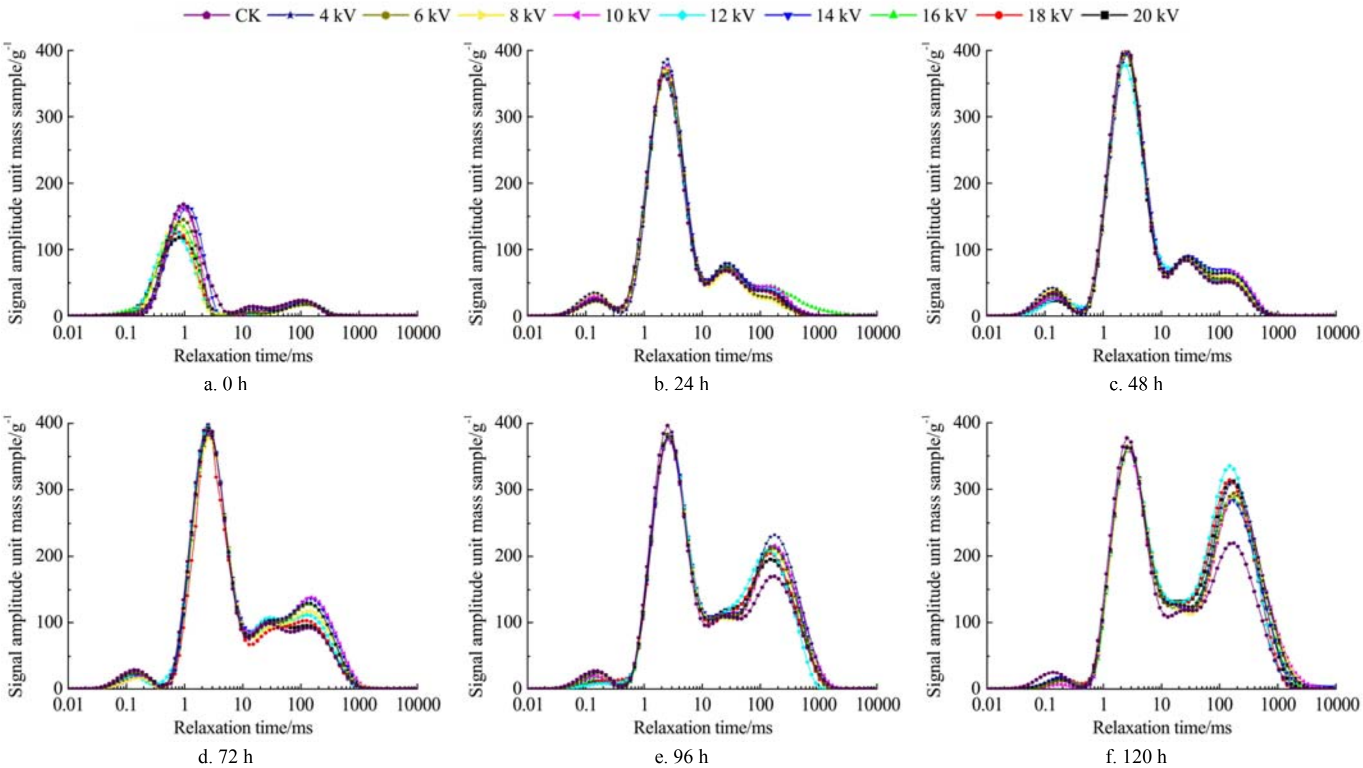


Figure 4 LF-NMR T_2 relaxation spectra during the germination of the aged rice seeds treated with different LFHV-PEF gradients

The LF-NMR T_2 relaxation spectra shown in Figure 4 indicate the variation trends of the LF-NMR T_2 signal amplitudes at 0, 24, 48, 72, 96, and 120 h during the germination of the aged rice seeds were consistent across the 10 gradients. This indicated a consistent variation trend for all water phases across the different treatment gradients. Here, the optimal LFHV-PEF treatment gradient (voltage output 12 kV) was considered to further describe the variation trend of the LF-NMR T_2 signal amplitudes during the germination of the aged rice seeds (Figure 5). All data were expressed as mean±SD.

3.3.1 Effects of the LFHV-PEF treatment on the content of side-peak water in the aged rice seeds

No side peaks were observed in dried rice seeds, whereas side peaks marginally appeared in the LF-NMR T_2 relaxation spectra of the 10 treatment gradients at 24-120 h of germination (Figure 5). These were located to the left of bound water and accounted for less than 5% of the total water content. No significant variation trends were found in the side-peak water content as the germination time increased for each group. The side peaks appeared because of the hydrophilicity of chemical compounds within the rice seeds. The total protein and starch content of aged WY4 seeds (23.9%) contained a large number of hydrophilic groups, such as -OH, -CHO, and -COOH, thus, promoting water states that were extremely tightly bound to macromolecules. The appearance of the side peaks suggested that the germination of rice seeds involved both physical and chemical processes, thus, demonstrating the close association of side-peak water with seed germination.

3.3.2 Effects of the LFHV-PEF treatment on the bound water content in the aged rice seeds

Moisture in the dried aged rice seeds mainly existed as bound water (Figure 5). At this stage, the seeds were dormant, with the seed coat encapsulating the kernel. Moreover, the inner tissues were firm and compact, and the intracellular substances were in a dry gelatinous state. During the germination of the aged rice seeds, water was absorbed rapidly at the imbibition stage at 0-48 h. Thus, the bound water content increased sharply, peaking at 48 h; subsequently, the rate of increase slowed down after 72 h. The LFHV-PEF treatment did not affect the bound water content within the seeds. When the aged rice seeds were directly exposed to external water, their seed coats began to soften, thereby increasing permeability. This allowed hydrocolloids to bind rapidly with external water, thus, promoting the transition of the protoplasm from a gel state to a sol state, along with rapid volume expansion. Rapid WA of the seeds was due to physical effects. No significant changes in the colloid properties were detected even after the LFHV-PEF treatment. High-vigor, low-vigor, and even dead seeds underwent imbibition. Hence, the bound water content increased rapidly during the initial germination stage. On entering the radicle emergence stage after imbibition, vigorous physiological and biochemical activities occurred within the seeds, and a large number of storage substances were hydrolyzed into soluble small-molecule substances. The bound water within the seed was converted to semi-bound water, and the binding ability of water molecules to protoplasmic cells was diminished. Thus, the bound water content decreased gradually.

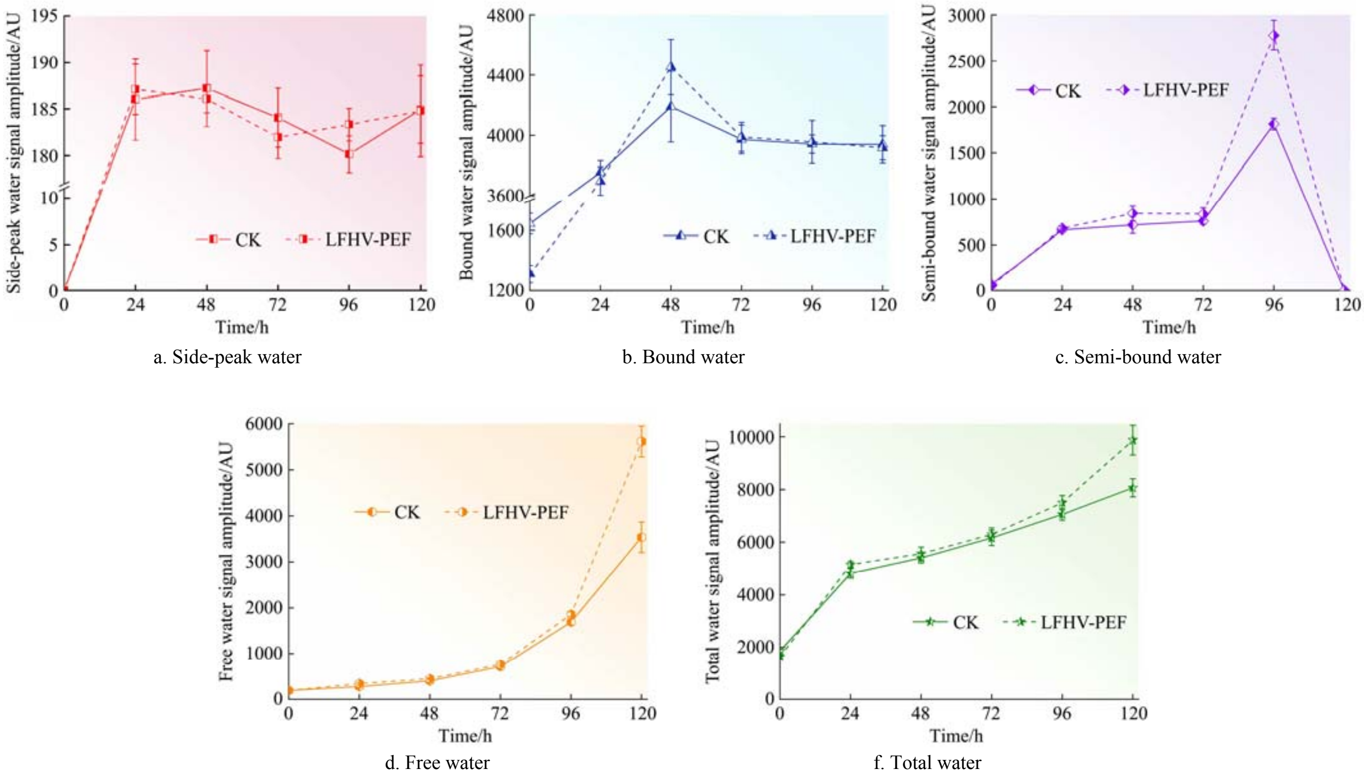


Figure 5 Comparison of the variation trends in LF-NMR T_2 signal amplitudes between the optimal LFHV-PEV treatment group and CK group

3.3.3 Effects of the LFHV-PEF treatment on semi-bound water in the aged rice seeds

The aged rice seeds completed dormancy at 0-96 h of germination (Figure 5), during which the semi-bound water content in the seeds increased sharply. As shown in Figure 4, the peak of semi-bound water shifted to the right, which implied that some semi-bound water had converted to free water. At 120 h of germination, the existing semi-bound water in the 10 groups of aged rice seeds had been completely converted to free water, and the peak area of the semi-bound water dropped to 0. Although seed WA showed an S-shape pattern (fast-slow-fast) during the entire germination process, this trend was not reflected in the changes in the semi-bound water content. During the radicle emergence stage, a temporary lag was observed in the overall WA of the aged rice seeds, but the internal metabolism of the seeds remained active. When external water entered the seeds via the micropyle, the embryo was preferentially exposed to water, which intensified the cellular respiration of the embryo, causing radicle emergence through the seed coat. Subsequently, the bound and free water inside the seed were converted to semi-bound water for transport, thus, allowing water to serve as a medium and solvent for the seed protoplasm and enzymes, which accelerated the degradation and transport efficiency of biological macromolecules, and provided nutrients to the new cells in the radicle and plumule.

3.3.4 Effects of the LFHV-PEF treatment on the free water content in the aged rice seeds

During the entire germination process of the aged rice seeds, the free water content gradually increased, followed by a sharp increase and a gradual increase (Figure 5). At 0-24 h of germination, the free water content of seeds between the LFHV-PEF treatment and the CK group did not significantly differ. Subsequently, at 24-120 h of germination, the free water content was significantly higher in the seeds treated with LFHV-PEF compared to those in the CK group. Previous studies have shown that the effect of LFHV-PEF on the germination of aged rice seeds significantly promotes the metabolic WA of plant cells in the later stages of seed germination[33,34]. Free water in intracellular vacuoles and intercellular capillaries not only catalyzes enzymes to accelerate internal chemical reactions but also transports nutrients to individual cells. Free water can limit the metabolic capacity of cells. Hence, seeds with high free water content have more vigorous metabolism, and increased seed vigor. LFHV-PEF mainly targets the cell membrane of the aged rice seeds by stimulating the membrane ions, with different intensities, frequencies, pulse widths, and treatment times having varying biological effects on the cell membrane[35]. When the extracellular potential is greater than the intracellular potential, and the transmembrane potential exceeds the perforation threshold (electric field strength ≥0.7 kV/cm), the lipid bilayer of the cell membrane is reassembled to form hydrophilic micropores, resulting in electroporation in the cell membrane. Once the aged rice seeds treated with LFHV-PEF were exposed to external water, the hydraulic conductivity and cell membrane permeability of the seed coat increased, causing water molecules to enter the cell via hydrophilic channels on the cell membrane. This increased the intracellular free water content, which resulted in rapid hydrolysis of nutrients (e.g., starch and proteins) in the seeds for embryo development and fast seedling growth, thereby improving the vigor of the aged rice seeds.

3.3.5 Effects of the LFHV-PEF treatment on the total water content of the aged rice seeds

The total peak area of the T_2 relaxation spectrum of the aged rice seeds gradually increased in the 10 treatment gradients (Figure 5). Further, at 0-24 h, water was rapidly absorbed during the imbibition stage, sharply increasing WA, it was absorbed steadily at 24–72 h during the radicle emergence stage, gradually increasing WA, and a large amount of water was absorbed in 72-120 h during the sprouting stage, which sharply increased WA. Furthermore, the total peak area of the T_2 relaxation spectra for the aged rice seeds treated with LFHV-PEF was significantly higher than that of the CK group. At 120 h of germination, the group with the lowest total water content, which was treated with LFHV-PEF at 6 kV, was 12.1% higher than that of the CK group. Conversely, the group with the highest total water content, which was treated with LFHV-PEF at 12 kV, was 22.5% higher than that of the CK group.

In addition, the LF-NMR signal amplitude data were consistent with the results of the previously conducted analysis on physicochemical indicators and vigor indices. The physicochemical indicators and vigor indices reflected an improvement in the vigor of the aged rice seeds from a macroscopic perspective, while the LF-NMR spectral data provided the possible reasons for the improvements in seed vigor from a submicroscopic perspective with respect to the content and distribution of different water phases.

4 Conclusions

During the germination of the aged rice seeds, both LF-NMR and physicochemical data revealed that WA during seed germination involved three stages, namely, the imbibition stage with rapid WA at 0-24 h, radicle emergence stage with slow WA at 24–72 h, and sprouting stage with a rapid increase in WA at 72–120 h. Moreover, the upward trend of WA in LFHV-PEF treatment rice seeds gradually became steeper with increasing germination time.

Along with a standard seed germination test, the physicochemical indicators and vigor indices of the aged rice seeds treated with LFHV-PEF were significantly higher than those of the CK group and the optimal treatment gradient was the electric field strength with an output voltage of 12 kV. The three ranges of electric field strengths ranked according to improvements in seed vigor (in descending order) were as follows: mid-range electric field strength (output voltage: 10, 12, 14, and 16 kV), high-range electric field strength (output voltage: 18 and 20 kV), and low-range electric field strength (output voltage: 4, 6, and 8 kV). Significant differences in the effect of the three ranges were found in the vigor of aged rice seeds.

Four states of water were found during the germination of the aged rice seeds that were defined according to the LF-NMR spectral signals (from left to right) as side-peak water, bound water, semi-bound water, and free water. As the germination time increased, the bound and semi-bound water contents initially increased, followed by a decrease, whereas the free water and total water contents increased continuously at a gradual steep rate of increase. The appearance of side peaks in the seeds was related to seed germination. Further, during the germination of the aged rice seeds, the LFHV-PEF treatment promoted the conversion of semi-bound water to free water within the seeds, and accelerated the increase in the free water content, thereby improving seed vigor.

The findings of this study may provide a theoretical reference for analyzing seed vigor, and highlight the potential methods for improving seed vigor.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant No. 31701318; 31811540396) and the basic research project of Liaoning Province (Grant No. LSNJC201916; LSNJC201908). The authors would also like to acknowledge the Rice Research Group, the Liaoning Engineering Research Centre for Information Technology in Agriculture, and Shenyang Agricultural University for their experimental support.

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