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Ultrafine Bubble Priming Promotes *Adzuki Bean* (*Vigna angularis*) Germination Under Drought Stress via Modulation of Seed Traits and Reactive Oxygen Species Production

Thuy Linh Ha, Masatoshi Yoshimura * and Itaru Sotome

Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-0023, Japan; hathuylinh23397@g.ecc.u-tokyo.ac.jp (T.L.H.); asotome@mail.ecc.u-tokyo.ac.jp (I.S.)
* Correspondence: ayoshimura@mail.ecc.u-tokyo.ac.jp

Abstract

Ultrafine bubble (UFB) priming has recently emerged as a promising technique to enhance seed germination, yet its physiological mechanisms remain unclear. This study investigated the effects of UFB priming on adzuki bean (*Vigna angularis*) seeds under stress-free and drought conditions. Four treatments were tested: Control (non-primed), DW (primed with distilled water), UFB1 (4.56×10^7 particles/mL), and UFB2 (1.13×10^8 particles/mL). For priming, seeds were immersed in each solution at 25 ± 1 °C for 24 h. Seed traits, including water uptake, coat thickness, and total reactive oxygen species (ROS) levels in the priming solutions and seeds, were analyzed. UFB priming reduced seed coat thickness and slowed early water uptake without affecting final moisture content. Total ROS levels displayed a concentration-dependent pattern, with higher UFB increasing ROS in water but reducing them on the seed surface. Under stress-free conditions, all priming treatments accelerated germination, although higher UFB slightly prolonged T50. Under drought simulated by polyethylene glycol (PEG 10 and 15%), non-primed seeds were strongly inhibited, whereas primed seeds maintained high germination rates (>97%). Our results demonstrated that UFB priming improved water uptake, seed coat structure, and ROS signaling, enhancing seed performance of adzuki bean under favorable and drought conditions.

Keywords: ultrafine bubble priming; *Adzuki bean*; water uptake; coat thickness; seed physiology; reactive oxygen species (ROS); drought tolerance; sustainable agriculture

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1. Introduction

Adzuki bean (*Vigna angularis*), commonly known as red bean, is an annual crop of the Vigna genus in the Fabaceae family [1]. It is economically and nutritionally valuable and widely cultivated in East Asia, particularly in Japan, China, and Korea [2]. In Japan, adzuki bean is the second most important legume after soybeans, with the main production in Hokkaido [2]. It is rich in protein, complex carbohydrates, dietary fiber, minerals, and bioactive compounds, such as polyphenols, flavonoids, saponins, and peptides [3]. This legume is widely grown in poor-soil regions due to its broad adaptation and tolerance to abiotic stress; however, it remains sensitive to drought during seed germination and seedling stages [4].

Seed germination plays a crucial role in establishing a new plant and directly affects seedling vigor, crop productivity, and overall agricultural outcomes [5,6]. It begins with Phase I (imbibition), where rapid water uptake reactivates basal metabolism, including

transcription, protein synthesis, and mitochondrial activity [7]. This is followed by Phase II (lag phase), when water uptake stabilizes and metabolic activity intensifies, leading to enzyme synthesis for reserve mobilization and embryo development, such as amylases, endoxylanase, and phytase [7]. Finally, Phase III marks a resurgence in rapid water uptake, culminating in embryo growth and radicle protrusion [7]. In addition, seed germination is tightly regulated by signaling molecules, particularly reactive oxygen species (ROS) and phytohormones, which interact to control cell wall loosening, water uptake, and the balance between germination and dormancy phytohormones [5]. ROS also modulate gene expression and phytohormone homeostasis, then coordinate processes such as cell elongation, seed germination, and early seedling development [8].

Drought stress is a complex factor that affects plant physiology, morphology, biochemistry, and molecular responses. It reduces the osmotic potential of dehydrated seeds, thereby limiting metabolism and impairing regeneration, growth, and survival [9]. Under drought, ROS production dramatically increased in different cellular compartments, disrupting the balance between ROS production and ROS scavenging and leading to oxidative stress [8]. This stress induces lipid peroxidation, damages nucleic acids and proteins, and alters carbohydrate metabolism, ultimately causing cell dysfunction and death [10]. In laboratory studies, drought is often simulated using polyethylene glycol (PEG), a macromolecule that does not penetrate the root apoplast [11,12]. PEG 6000 effectively removes water from cells without toxicity under well-aerated conditions, making it a reliable agent for mimicking drought or water stress [13,14].

Seed priming is an effective method to improve germination performance under optimal and drought conditions [15]. Conventional priming methods, such as hydropriming, can enhance germination speed and uniformity [16]; however, their effectiveness under water-limited conditions is often restricted. To address this issue, alternative priming approaches, such as osmopriming (which exposes seeds to relatively low external water potentials) [17,18], biopriming (which utilizes beneficial microorganisms) [19,20], and hormonal priming [21], have been developed to improve seed germination and drought tolerance. Nevertheless, these techniques rely on chemical inputs or require complex processing, restricting their practical and environmental applicability. Therefore, there is a growing demand for simple and sustainable priming methods that minimize chemical use while maintaining high efficacy under stress conditions.

Given these challenges, recent attention has turned to ultrafine bubbles (UFBs), a novel water-based technology with unique physicochemical properties. UFB are fine bubbles with diameters smaller than 1 μm, characterized by low buoyancy and high stability, allowing them to persist for several months without external stimuli [22,23]. Reactive oxygen species can be detected in the UFB production process and after some months of storage [24–26]. During UFB soaking, these ROS are proposed to act as signaling molecules that regulate seed germination and modulate the oxidative window, thus contributing to promotive or suppressive outcomes [27–29].

Previous studies have shown that UFB water can influence seed germination across various species through two distinct approaches. One approach involves continuously soaking seeds in UFB water throughout the germination period (UFB soaking), with promotive effects dependent on seed species and UFB concentration. For example, optimal germination responses have been reported for spinach at 1×10^8 particles/mL and carrot at 2×10^7 particles/mL [27]. The other approach involves briefly dipping seeds in UFB water before germination (UFB priming), offering a straightforward and more energy-efficient alternative. Under stress-free conditions, UFB priming at an optimal concentration positively affects germination rate, capacity, and uniformity across different species and storage periods [30,31]. Under drought stress, UFB treatment has been

reported to promote seedling growth in soybean and coffee, while its impact on seed germination remains unclear [32,33].

Furthermore, several key factors influencing UFB-seed interactions beyond species and concentration remain unexplored, particularly the correlations between seed physiological traits and the physicochemical properties of UFB. Although adzuki bean is a common and economically important crop, the mechanisms underlying UFB effects on its seeds remain poorly understood. Therefore, this study aims to elucidate the mechanisms of UFB-induced germination enhancement by integrating three key aspects: (1) seed physiological traits (seed coat thickness, water uptake kinetics, and total ROS levels); (2) physicochemical properties of UFBs (concentration and total ROS content); and (3) adzuki bean responses under drought conditions. By integrating these factors, this research seeks to elucidate the mechanism underlying UFB-induced germination enhancement and lay the groundwork for developing an efficient and eco-friendly UFB-priming protocol applicable to diverse crop species under normal and stress conditions.

2. Materials and Methods

2.1. Seed Materials

Adzuki bean (*Vigna angularis*) seeds were bought from Takii Shop (Takii Seed Co., Ltd., Kyoto, Japan). According to the product label, the seeds were harvested in Kyoto Prefecture, Japan, and had a minimum germination percentage of 75% before their expiration date in January 2026. Seeds were stored in a refrigerator at 10 °C before using.

2.2. Seed Priming Procedure

2.2.1. Preparation of Priming Solutions

Two types of priming solutions were prepared: distilled water (DW; Autostill WA-53, Yamato Scientific Co., Ltd., Tokyo, Japan) and ultrafine bubble (UFB) water. UFB water was produced using an Ultrafine GALF generator (FZ1N-10, IDEC Co., Osaka, Japan) for 60 or 120 min. After preparation, UFB water was stored overnight at 28 ± 1 °C to stabilize the bubbles.

The number concentration and size distribution of UFB were measured at room temperature using a commercial particle tracking analyzer (NanoSight LM10, Quantum Design Inc., Tokyo, Japan) equipped with a black-and-white CCD camera and NTA 3.4 Build 3.4.4 software. The measured UFB concentrations were 4.56×10^7 particles/mL for UFB generated for 60 min (UFB1) and 1.13×10^8 particles/mL for those generated for 120 min (UFB2). These concentration ranges were similar to those reported in a previous study [27].

2.2.2. Priming Treatments

For priming, seeds were immersed in each solution at a ratio of 10 mL/seed and incubated in a water bath at 25 ± 1 °C under dark conditions for 24 h. Four treatments were conducted: Control (non-priming), DW (priming with distilled water), UFB1 (priming with UFB1 water), and UFB2 (priming with UFB2 water).

2.3. Water Uptake Kinetics and Mathematical Modeling

The initial moisture content of 25 seeds per replicate (3.01 ± 0.05 g) was determined using a halogen moisture analyzer (HB43-S, Mettler Toledo, Greifensee, Switzerland) at 105 °C, with an average measurement time of 18.18 ± 0.37 min. The obtained values were expressed as initial moisture content on a wet basis (% w.b.) and subsequently converted to dry basis (M_0, % d.b.).

Water uptake kinetics were evaluated by recording changes in seed mass during water absorption. For each replicate, 25 seeds were randomly selected, weighed, and placed in

glass beakers containing 250 mL of priming solution. At predetermined intervals (4, 19, and 24 h), seeds were removed, drained, gently blotted with filter paper to eliminate surface moisture, weighed, and immediately returned to the solution. A digital chronometer and an analytical balance (MS4002S, Mettler Toledo, Greifensee, Switzerland; precision 0.01 g) were used for timing and weight measurements.

The initial dry mass ($w_{0,dry}$) was calculated from the initial mass (w_0) and the initial dry basis moisture content (M_0) as:

$$w_{0,dry} = w_0 \times \frac{M_0}{100} \quad (1)$$

The experimental moisture content at time *t* ($M_{t,exp}$, % d.b.) was then determined from the mass at time t (w_t) and initial dry mass ($w_{0,dry}$) as:

$$M_{t,exp} = \frac{w_t - w_{0,dry}}{w_{0,dry}} \times 100 \ (\%\ \text{d.b.}) \quad (2)$$

Moisture absorption kinetics were described using a Weibull distribution model [34], which predicts the model moisture content ($M_{t,model}$, % d.b.):

$$M_{t,model} = M_0 + (M_\infty - M_0) \times \left[1 - \exp\left[-\left(\frac{t}{\beta}\right)^{\alpha}\right]\right] \quad (3)$$

where $M_{t,model}$ is the moisture content at time t, M_0 is the initial moisture content, M_∞ is the equilibrium moisture content, β is the scale parameter, and α is the shape parameter. The scale parameter β represents the time required to achieve ~63% of total water uptake, while the shape parameter α indicates the uptake mechanism: α = 1 reduces the model to a first-order growth, α > 1 predicts a lag phase, and higher α values correspond to the slower process at short times [34].

The fitting goodness of the Weibull model was assessed using three statistical parameters: (4) coefficient of determination (R^2) [35], (5) root mean square deviation (RMSD), and (6) normalized root mean square deviation (NRMSD) [36]. The model is considered to have a good fit when the value of R^2 is close to one, which indicates a close relation between experimental data and the mathematical model.

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(M_{i,exp} - M_{i,model})}{\sum(M_{i,exp} - \overline{M_{exp}})^2} \quad (4)$$

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n}\left(M_{i,exp} - M_{i,model}\right)^2}{n}} \quad (5)$$

$$NRMSD = 100 \times \frac{RMSD}{M_{max,exp} - M_{min,exp}} \quad (6)$$

where $M_{i,exp}$, $M_{i,model}$ are the experimental and model moisture content at the observation ith, $\overline{M_{exp}}$ represents the mean of the experimental moisture content value, and n is the number of experimental data points.

All experiments were conducted with three replicates, and results are presented as mean ± standard error. Parameter estimation was performed using nonlinear least-squares regression in MATLAB R2023a (MathWorks, Natick, MA, USA).

2.4. Seed Coat Thickness Measurement

Measurements were taken before and after priming treatments to evaluate the effect of hydration. Seed coat thickness before (Control) and after priming (DW, UFB1, UFB2) was determined using a digital micrometer (Model 293-821, Mitutoyo Corporation, Kanagawa, Japan) with an accuracy of ±0.001 mm. Six seeds were randomly selected from each treatment group for measurement. A single measurement was taken at the equatorial region of each seed to ensure consistency across all samples. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test to identify significant differences between treatment groups ($p < 0.05$).

2.5. Total ROS Levels Quantification

Total ROS levels were quantified using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Cayman Chemical, Ann Arbor, MI, USA) as a fluorescent probe. DCFH-DA was hydrolyzed with sodium hydroxide to form the non-fluorescent compound DCFH, which was subsequently oxidized by ROS to the fluorescent product dichlorofluorescein (DCF). Fluorescence was detected using a fluorescence microspectrophotometer (F-7000, Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 502 nm and 523 nm, respectively [33,34]. The workflow of DCFH preparation and ROS quantification is illustrated in Figure 1. In this workflow, Figure 1a represents the preparation and hydrolysis steps of the DCFH working solution described in Section 2.5.1, while Figure 1b depicts the ROS measurement procedures for water and seed samples detailed in Section 2.5.2.

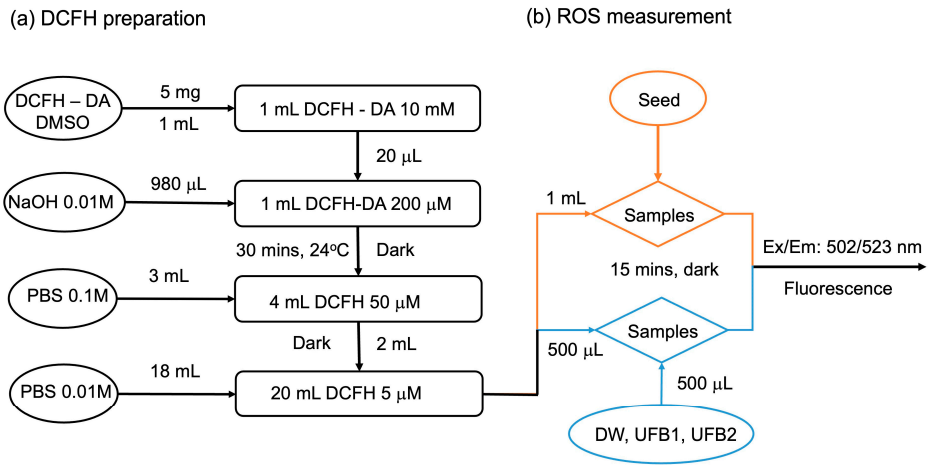


Figure 1. Workflow of DCFH preparation and ROS measurement in water and seed surfaces. Blue lines indicate water samples (DW, UFB1, UFB2), and orange lines indicate seed samples. (**a**) DCFH preparation; (**b**) ROS measurement.

2.5.1. Preparation of DCFH Working Solution

According to Figure 1a, a 10 mM DCFH-DA stock solution was prepared by dissolving 5 mg of DCFH-DA in 1 mL dimethyl sulfoxide (DMSO, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Sodium hydroxide (0.1 M, Kanto Chemical Inc., Tokyo, Japan) and phosphate-buffered saline (PBS, 0.1 M, pH 7.2, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) were diluted with distilled water (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) to 0.01 M solutions. Hydrolysis was performed by incubating the stock solution in 0.01 M NaOH for 30 min at room temperature (24 °C) in the dark, followed by neutralization with 0.1 M PBS. The resulting 50 µM DCFH solution was further diluted with 0.01 M PBS to a 5 µM working solution, which was stored on ice until use.

2.5.2. ROS Measurement Protocol

As shown in Figure 1b, 500 µL of each water sample (DW, UFB1, UFB2) was mixed with 500 µL of 5 µM DCFH working solution and incubated for 15 min at room temperature. For seed samples, seeds from each treatment group were incubated in 1 mL of 5 µM DCFH working solution for 15 min at room temperature. During incubation, DCFH was oxidized to DCF in the presence of extracellular ROS in water or surface-associated ROS on seeds. After incubation, 1 mL of each measurement sample was transferred to a transparent tube for fluorescence measurement, with DCFH solution used as a control. The achieved data is expressed as Relative Fluorescence Units (RFU). All procedures were performed in a container to minimize photo degradation of the fluorescent probe.

2.5.3. Data Analysis and Normalization

All experiments were conducted with three replicates, and results are presented as mean ± standard error. Statistical analysis was performed using MATLAB R2023a (MathWorks, Massachusetts, USA). One-way ANOVA followed by Tukey's post hoc test was used to identify significant differences between treatment groups ($p < 0.05$).

2.6. Germination Test

2.6.1. Experimental Design and Treatment

After priming treatments, seeds were subjected to germination tests under drought stress conditions. As shown in Figure 2, an experimental design was implemented with twelve treatment combinations: three priming treatments (DW, UFB1, UFB2) and a no priming as Control (CT), each tested under three levels of drought stress (0%, 10%, and 15% PEG 6000).

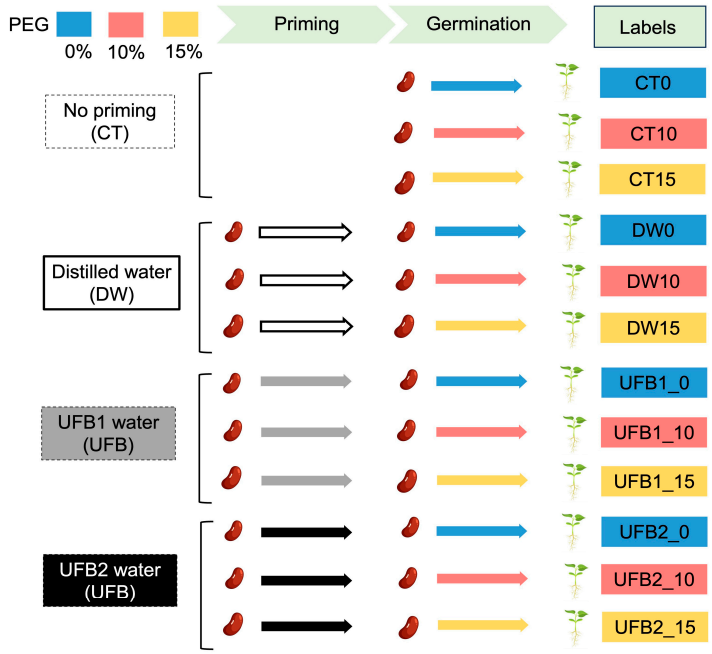


Figure 2. Experimental design for seed germination under drought stress. Twelve treatment combinations were tested, including three priming treatments (DW, UFB1, UFB2) and a no-priming control (CT), each applied under three levels of osmotic stress (0%, 10%, 15% PEG 6000).

2.6.2. Germination Setup

In each replicate, 25 seeds were arranged between layers of filter paper (No. 2 Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan) pre-saturated with 30 mL of polyethylene glycol solution (PEG 6000; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) at concentrations

of 0%, 10%, or 15% (*w*/*v*). The seeds were then transferred to seed boxes (164 × 150 × 33 mm), with three replicates per treatment. To maintain constant moisture and prevent evaporation, the seed boxes were sealed in clear plastic zip bags (Model IG-4, Japack's, Tokyo, Japan) throughout the germination period.

2.6.3. Germination Conditions

Germination was carried out in a controlled growth chamber (Eyelatron FLI-301NH, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) set to maintain a temperature of 26 ± 2 °C, relative humidity of 75–85%, with an 8 h photoperiod for 6 days (3 July 2025~9 July 2025).

2.6.4. Data Collection Analysis

Germination was monitored daily, and a seed was considered germinated when the radicle tip emerged from the seed coat. Germination percentage was calculated as the mean of three independent replicates.

The germination process was analyzed using a dose–response log-logistic model [37] by MATLAB R2023a (MathWorks, Massachusetts, USA):

$$G(t_i) = \frac{G_{max}}{1 + \exp[B(\log(t_i) - \log(T50))]} \quad (7)$$

where G_{max} is the maximum germination ratio, t_i is the time for each inspection, T50 is the time at which the inferred germination ratio is 50% of G_{max}, $G(t_i)$ is the observed germination percentage for each inspection, and B is the slope at the time T50.

Final germination percentages were presented as mean ± standard error. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to identify significant differences between treatment groups ($p < 0.05$).

3. Results

3.1. Water Uptake Kinetics During Priming Treatments

Moisture absorption of adzuki bean seeds during priming followed a sigmoidal pattern (Figure 3) and was well fitted by the Weibull model ($R^2 > 0.99$) (Table 1).

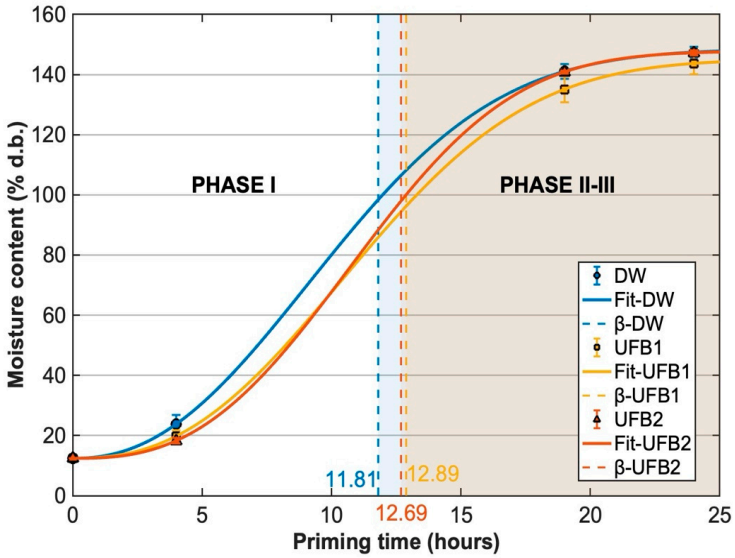


Figure 3. Water uptake kinetics of seeds primed with distilled water (DW) and ultrafine bubble water (UFB1, UFB2) over 24 h. Phase I: initial lag phase with slow water uptake that then accelerates. Phase II/III: slower uptake approaching a plateau. Values represent the mean ± SE of three replicates.

Table 1. Weibull model parameters and goodness-of-fit statistics for different priming treatments.

Parameter	DW	UFB1	UFB2
M_∞	148.4320	145.0555	147.7628
β	11.8064	12.8856	12.6875
α	2.2523	2.4476	2.7004
R^2	0.9977	0.9954	0.9998
RMSD	3.0551	4.2088	0.9276
NRMSD (%)	2.22	3.10	0.68

The initial seed moisture was consistent across treatments, starting at 11.05% (w.b.), equivalent to 12.43 g water per 100 g dry matter (12.43% d.b.). Differences between the treatments emerged in the first four hours. Seeds primed in distilled water (DW) absorbed water faster initially, reaching 23.8 ± 3.0% moisture. In contrast, seeds treated with ultrafine bubble water (UFB1 and UFB2) absorbed less water in this early stage, reaching only 19.8 ± 2.0% and 18.3 ± 0.9%, respectively. After this initial lag, water uptake accelerated sharply in all treatments. By 19 h, the moisture levels had converged, with no significant differences between DW (141.1 ± 2.5%), UFB1 (135.1 ± 4.4%), and UFB2 (140.9 ± 0.5%). All treatments plateaued near the same equilibrium moisture content (≈145–148%) by 24 h.

Fitting the water uptake data to the Weibull model provided insights into the hydration kinetics (Table 1). The scale parameter β, representing the time to reach ~63% of total water uptake, was slightly higher for UFB1 and UFB2 than for DW (11.8 h), indicating a modest delay in the overall hydration rate under UFB treatments. The shape parameter α, which reflects the curvature of the uptake profile, was greater than 1 for all treatments, confirming the presence of a lag phase. Notably, the shape parameter α increased from DW (2.25) to UFB1 (2.45) and UFB2 (2.70), indicating that all treatments exhibited a lag phase ($\alpha > 1$). The higher values α for UFB treatments suggest a slightly slower initiation of water uptake, but once absorption began, it proceeded more rapidly and synchronously across the seed population. The model fit was excellent for all treatments, particularly UFB2 ($R^2 = 0.9998$, NRMSD = 0.68%), suggesting highly consistent and predictable hydration behavior among replicates. These results indicate that UFB priming modulates early-stage water uptake kinetics while maintaining final moisture content to DW.

3.2. Seed Coat Thickness Before and After Priming Treatments

Seed coat analysis revealed minor structural modifications in response to priming. In the Control group, the average seed coat thickness was 0.076 mm. After priming, UFB treatments reduced thickness by 6.6% and 9.9% relative to the Control, with UFB1 and UFB2 measuring 0.071 mm and 0.069 mm, respectively. In contrast, DW priming caused a slightly nonsignificant increase to 0.077 mm. ANOVA indicated no significant differences among treatments ($p > 0.05$), with standard errors remaining consistent across groups (<0.003 mm).

3.3. Total ROS Levels on Seed Surface and in Priming Solutions

Fluorescence measurements revealed clear differences in ROS levels in water and seeds during priming (Figure 4). Before priming (Figure 4a), all water samples showed significantly lower ROS levels than Control ($p < 0.0001$), with a 28–32% decrease. Among the priming treatments, a clear trend was observed: UFB2 had the highest ROS (7117.3 RFU), followed by UFB1 (6893.3 RFU) and DW (6695.3 RFU), although no significant differences were detected among them.

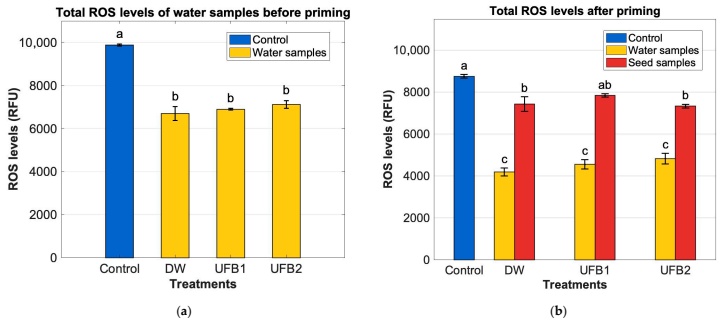


Figure 4. Total ROS levels in water and seeds during priming treatments. (**a**) ROS levels in water before priming. (**b**) ROS levels in water and seed-associated ROS after priming. Different letters above bars indicate significant differences among treatments (one-way ANOVA, Tukey $p < 0.05$). Values represent mean ± SE of three replicates.

After priming (Figure 4b), ROS levels in water decreased substantially compared to Control and their respective pre-dipping values, reaching only 48–55% of Control. A consistent trend was maintained among treatments, with UFB2 showing the highest ROS (4819.3 RFU), followed by UFB1 (4544.0 RFU) and DW (4182.7 RFU). In contrast, ROS levels associated with seeds were considerably higher than in water, ranging from 7328.0 to 7838.3 RFU. UFB1-treated seeds showed a higher value than DW (7419.7 RFU) and UFB2 (7328.0 RFU).

3.4. Germination Performance

Drought stress progressively inhibited seed germination, with inhibition becoming more severe as PEG concentration increased.

In non-primed seeds (Control), germination declined from nearly 100% at 0% PEG to 50.7% at 15% PEG, while T50 increased from 55.3 h to 112.0 h. Slope B also rose under stress, showing reduced germination synchrony (Figure 5).

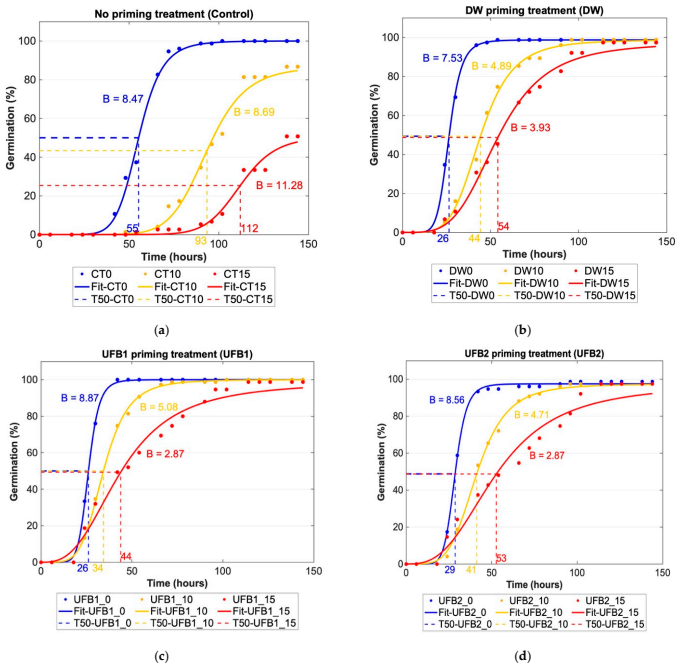
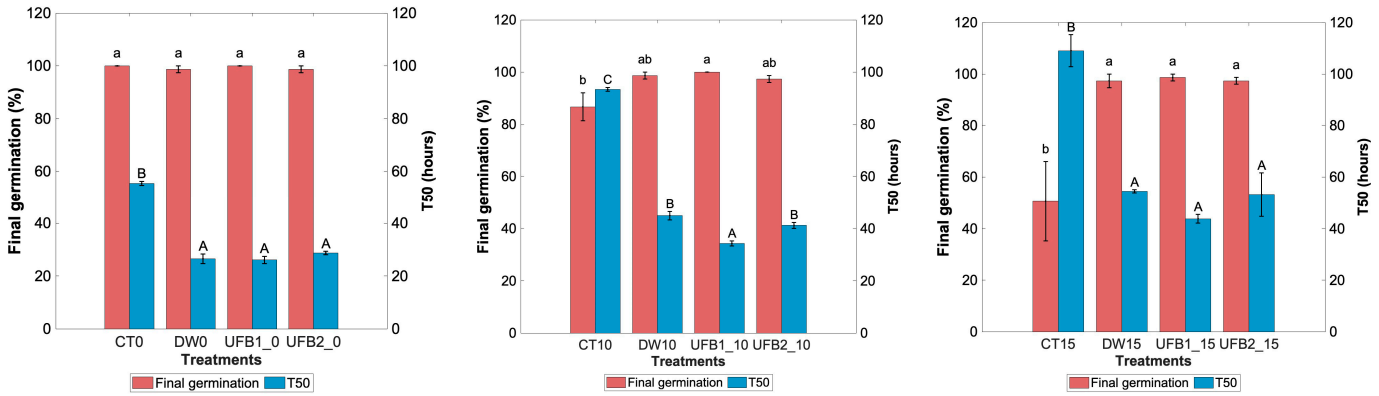


Figure 5. Germination percentages of adzuki bean seeds under stress-free (PEG 0%) and drought conditions (PEG 10% and 15%); (**a**) no priming (Control); (**b**) priming with distilled water (DW); (**c**) priming with UFB1 water (UFB1); (**d**) priming with UFB2 water (UFB2).

In contrast, all priming treatments (DW, UFB1, UFB2) maintained high germination percentages (97–100%) across all PEG levels (Figure 6). Under non-stress conditions, priming accelerated germination, with UFB1 reaching T50 at 26.1 h, followed by DW (26.5 h) and UFB2 (28.7 h), nearly twice as fast as the control. At 10% PEG, primed seeds maintained 97–100% germination, with UFB1 showing the highest final germination (100%) and the shortest T50 (34.4 h), and being significantly different from Control treatment ($p < 0.05$). Even at 15% PEG, primed seeds achieved 97–99% germination with T50 between 44 and 54 h. Although slope B values in primed seeds were generally lower than Control, indicating reduced synchrony, their overall germination performance was consistently superior, with UFB1 showing the most resilient response



(**a**) (**b**) (**c**)

Figure 6. Final germination and T50 of adzuki bean seeds. (**a**) Stress-free (PEG 0%). (**b**) Drought stress (PEG 10%). (**c**) Drought stress (PEG 15%). Different lowercase letters above bars indicate significant differences among treatments for final germination, and uppercase letters indicate significant differences for T50 (one-way ANOVA, Tukey $p < 0.05$). Values represent mean ± SE of three replicates.

4. Discussion

The following discussion provides a detailed interpretation of the experimental results, linking the observed physiological responses of seeds to the potential mechanisms of UFB water during priming, and highlighting the practical implications, limitations, and future perspectives of this priming approach.

4.1. Mechanism of Water Uptake Modulation by UFB

The water uptake of adzuki bean seeds during priming can be divided into three phases. Phase I represents the initial lag stage with limited water uptake, followed by a rapid increase. Phase II begins when ~63% of total hydration is reached (as indicated by the scale parameter β of the Weibull model), showing slower uptake. Phase III corresponds to the relaxation stage, when moisture content approaches equilibrium, marking the termination of soaking or the lag phase of germination [38].

During the first 4 h, seeds exhibited a noticeable lag phase with low water absorption, consistent with their low initial moisture content and soaking temperature. This behavior aligns with previous reports [36], which indicated that seeds with moisture content below 20% d.b. possess an impermeable or hard seed coat restricting water flow, with uptake occurring mainly through the hilum. Interestingly, UFB-treated seeds showed lower water absorption than DW. This difference may be related to the greater number of air bubbles in UFB water, which could temporarily accumulate near the hilum and hinder water penetration. After hydrating from the inside, the seed coat becomes permeable and allows water uptake through the coat [39]. At this stage, the unique physicochemical properties

of UFB water may enhance absorption by increasing the seed coat's water permeability, enhancing cell wall loosening, and facilitating water transport [40].

At 19 h, UFB-primed seeds reached moisture levels comparable to DW, with no significant difference. Ultrafine bubbles are hydrophobic, negatively charged, and have large surface areas, enabling them to attach to the seed surface [40]. Their continuous generation of low ROS could also improve seed coat permeability [40], which may explain the faster uptake after the initial slow phase.

Final moisture at 24 h was 144–148%, consistent with previous observations [41]. These results suggest that UFB primarily influences early-stage absorption without altering equilibrium moisture, potentially reducing imbibitional damage and promoting uniform hydration [42].

These kinetic patterns were well captured by the Weibull model, which provided an excellent fit across treatments. The scale β and shape α parameters corresponded well with the observed hydration patterns. In particular, the higher α values for UFB treatments indicate more synchronized water uptake once imbibition began, supporting more uniform hydration across seeds. Overall, the model parameters align closely with experimental observations, reinforcing the mechanistic interpretation of UFB effects on early-stage seed hydration.

4.2. Interactions of UFB with Seed Coat Structure Relating to Water Uptake

Analysis of seed coat morphology revealed no statistically significant differences in thickness among treatments ($p > 0.05$), consistent with the inherently rigid structure of adzuki bean seed coats. The testa is almost non-porous, composed of a dense layer of macrosclereids and a thin parenchyma layer [36], which likely prevents swelling or deformation after priming. Nevertheless, a clear trend was observed: DW priming slightly increased thickness, whereas UFB treatments reduced it, with UFB2 showing a more pronounced reduction than UFB1. Given that UFB2 contained a higher concentration of nanobubbles, this effect may reflect stronger physical interactions at the seed coat compared with UFB1. The reduced thickness in UFB2 is consistent with its water uptake dynamics, characterized by delayed initial absorption followed by accelerated hydration once uptake occurs through the coat. Although statistically insignificant, the thinning trend observed under UFB treatments corresponds with improved hydration synchronization, suggesting that surface-level interactions with ultrafine bubbles may facilitate more efficient water penetration through the seed coat microstructures.

4.3. ROS Production and Signaling in Response to Different UFB Concentrations

The DCFH-DA assay is a quick and simple method that provides clearly comparable data across treatments. It has been applied to quantify ROS levels in seeds such as soybean, tomato, and wheat [35], and in nanomaterials including ZnO, CuO, Mn_2O_3, and $BaSO_4$ [36]. In this study, the method was used to determine extracellular ROS in water samples before and after priming and surface-associated ROS on seeds after priming, thereby enabling direct comparison of oxidative activity between priming solutions and seeds. To our knowledge, this is the first application of the DCFH-DA assay for evaluating total ROS levels in air ultrafine bubble water.

Before and after priming, total ROS levels of water samples followed the order UFB2 > UFB1 > DW, although differences were not statistically significant ($p > 0.05$). This aligns with the acquired results of seed coat thickness and water uptake process, in which higher UFB concentration slightly decreases seed coat thickness and promotes water transport. After priming, ROS in water decreased markedly (~48–55% of the Control), suggesting consumption or transfer during priming. Moreover, total ROS levels in seeds were substan-

tially higher than in water samples ($p < 0.05$), possibly due to different DCFH incubation volumes and ROS on the seed surface. Notably, UFB1-treated seeds accumulated more ROS than DW and UFB2 treatments.

These findings suggest that UFB priming involves two concurrent processes: (i) ultrafine bubbles enrich the priming medium with a low-level ROS background, and (ii) seeds actively modulate ROS accumulation at their surface, which does not appear to be directly proportional to the total ROS levels in the priming solution. The elevated seed-associated ROS in UFB1 suggests a response dependent on UFB concentration, in which moderate ROS may stimulate additional ROS production at the seed surface or trigger endogenous ROS signaling. By contrast, higher ROS input from UFB2 could exceed the optimal threshold and activate antioxidant defenses, thereby limiting further accumulation [28]. This interpretation is consistent with the "oxidative window" concept, whereby ROS within an optimal range enhances signaling and promotes physiological activation; excessive ROS is generally thought to induce protective mechanisms [43].

4.4. Integrated Effects of UFB on Seed Germination

Under stress-free conditions, all priming treatments accelerated germination relative to the non-primed control, as reflected by shorter T50 values. Among them, higher UFB concentrations (UFB2) appeared to slightly limit these benefits, possibly due to the early activation of antioxidant defenses within the oxidative window [28]. This observation is consistent with findings in carrot, where high number-density UFB water (1×10^8 particles/mL) exceeded the toxic threshold and reduced germination, and relatively low number-density UFB water (2×10^7 particles/mL) promoted germination [27].

In contrast, drought stress markedly reduced germination in non-primed seeds, with higher PEG concentrations causing both delayed germination and an increase in T50. This pattern agrees with previous studies employing PEG 6000 to simulate drought conditions [44]. All primed seeds maintained high germination percentages even at 15% PEG, demonstrating the promotive effect of the priming method in enhancing stress tolerance and mitigating drought stress. Notably, UFB treatments further promoted faster germination than DW, consistent with reports showing that UFB application alleviated osmotic stress in soybean seedlings at 15% PEG 6000 [33]. The superior performance of UFB1 may be attributed to optimized ROS signaling or improved seed coat permeability induced by UFB treatment [40].

4.5. Practical Evaluation, Limitations, and Future Perspectives

The present study demonstrates that UFB priming is a promising, purely physical technique for enhancing seed germination. Unlike conventional chemical or hormonal priming methods, UFB generation modified water properties such as reduced surface tension, altered charge distribution, and ROS production without chemical usage [24]. Therefore, it can be applied more simply and scaled up for agricultural use with minimal environmental impact. However, the efficacy of the UFB priming method also depends on many factors, such as optimal UFB concentration, seed species, and dipping time. Therefore, further comparative studies are essential to systematically evaluate its efficiency, reproducibility, and practical scalability against established priming techniques.

In addition, although the initial number density of ultrafine bubbles was quantified before seed soaking, no direct microscopic imaging observations were conducted to monitor bubble dynamics on the seed surface during the imbibition process. Such investigations are crucial to verify the proposed physical interactions, including whether UFBs attach, persist, or detach from the seed coat. Therefore, it is necessary to perform microscopic observations in future studies.

Furthermore, the analysis of oxidative activity was limited to the DCFH-DA assay, which quantifies total ROS levels but cannot distinguish specific ROS types, their subcellular localization, or their interplay with key phytohormonal pathways. Given the tightly coordinated roles of ROS and hormonal aspects in seed germination [45], the absence of hormone profiling limits our understanding of the redox–hormone crosstalk underlying UFB priming.

Finally, while this research highlights the potential of UFB technology for improving the germination of hard-coated seeds like adzuki bean, its findings are based on a single species under one type of stress condition. The applicability of these results to other species with different seed coat permeability, physiological traits, or environmental conditions remains uncertain and requires further investigation.

5. Conclusions

In conclusion, this study elucidates the mechanisms by which ultrafine bubble (UFB) priming enhances the performance of adzuki bean seeds, highlighting its influence on physiological traits, physicochemical properties, and germination under stress conditions. Physiologically, UFB priming modestly reduced early water uptake and seed coat thickness, without affecting the final moisture content. From a physicochemical perspective, DCFH-DA assays revealed that high-concentration UFB water elevates total ROS levels, triggering a response dependent on UFB concentration at the seed surface. These combined physiological and physicochemical effects resulted in faster germination and enhanced tolerance to drought stress. While further studies are needed to confirm these mechanisms across species and environmental conditions, the findings indicate that UFB priming modulates early seed–water interactions and oxidative signaling, offering a sustainable and effective approach to improve crop establishment under challenging environmental conditions.

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