



Ultrasound technology restores germination potential and seedling vigor in artificially aged seeds

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ARTICLE INFO

Keywords:

Antioxidant defenses
Enzymatic activity
Germination
Oxidative stress
Seed longevity
Ultrasound treatment

ABSTRACT

Seed longevity and quality are crucial for agricultural sustainability, as aging negatively affects germination and seedling vigor. Prolonged storage and unfavorable environmental conditions promote oxidative stress, leading to lipid peroxidation, enzyme inactivation, and reduced antioxidant capacity. This study explored ultrasound technology as a novel, eco-friendly approach to counteract these aging-induced effects in pea (*Pisum sativum*) and turnip (*Brassica rapa* L.) seeds. Seeds underwent artificial aging followed by ultrasound treatment (19.8 kHz, 200 W, 7.5 min), a process proposed to enhance seed coat permeability, water uptake and gas exchange through cavitation-induced physical effects, thereby facilitating metabolic reactivation, potentially involving modulation of hormone-regulated germination pathways. Principal Component Analysis (PCA) revealed distinct clustering of aged and ultrasound-treated seeds, indicating enhanced germination performance, antioxidant activity, and enzymatic defenses. Post-ultrasound treatment, germination rates increased significantly, by 340% in pea and 38% in turnip, compared to aged controls. Additionally, both species exhibited a 69% improvement in germination value, while turnip seeds showed faster germination. At the biochemical level, ultrasound increased chlorophyll content (57% in pea and full restoration in turnip) and reduced the malondialdehyde (MDA) levels, reflecting reduced oxidative damage. Moreover, antioxidant enzyme activities, including superoxide dismutase (SOD) and catalase (CAT), alongside total antioxidant capacity (DPPH, ABTS, and FRAP assays), were generally enhanced after ultrasound treatment and, in some cases, restored to levels comparable to non-aged seeds. These findings suggested that ultrasound treatment alleviates the seed aging effects by improving membrane integrity and redox homeostasis, thereby mitigating age-related physiological and biochemical deterioration. Consequently, ultrasound treatment emerges as a promising priming strategy for enhancing seed vigor, germination uniformity, and early seedling establishment under aging stress conditions.

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<https://doi.org/10.1016/j.bcab.2026.104010>

Received 20 May 2025; Received in revised form 8 February 2026; Accepted 7 March 2026

Available online 9 March 2026

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1. Introduction

Seed longevity, referring to how long seeds stay viable in storage, is an essential trait for ensuring the continuity of flowering plants across generations. The long-term preservation of seeds in soils, gene banks, or commercial storage holds significant ecological and socio-economic importance (Pritchard and Li, 2009; Solberg et al., 2020). Equally important is seed quality, which plays a pivotal role in successful seedling establishment by directly influencing germination capacity and early plant vigor. High-quality seeds consistently produce robust, uniform seedlings, which are crucial for optimizing agricultural productivity and sustainability. In contrast, poor seed quality – manifesting as reduced viability and diminished vigor – leads to weaker seedlings, irregular crop stands, and lower yields, with far-reaching economic and social consequences (Lu et al., 2022; Sharma et al., 2022; Pirredda et al., 2024). Prolonged storage and uncontrolled abiotic (relative humidity, temperature and oxygen) and biotic factors (insects, fungi and rodents) are some of the main factors that contribute to the decline of seed quality. To mitigate this decline, agricultural practices emphasize the drying and storage of seeds under controlled conditions, as moisture is a primary driver of seed aging and deterioration (Rajjou and Debeaujon, 2008; Prasad et al., 2022).

Seeds are complex biological systems that undergo diverse metabolic and physiological activities throughout their life cycle. These processes are highly sensitive to aging, which disrupts cellular homeostasis, leading to the accumulation of reactive oxygen species (ROS) and oxidative stress that disrupts the antioxidative system, loss of membrane integrity (lipid peroxidation), enzyme inactivation, and DNA and protein degradation. Consequently, this impairs germination and weakens plant vigor (Bewley, 1997; Nonogaki, 2019; Li et al., 2022; Pirredda et al., 2024). For instance, a study on sunflower seeds found that higher moisture content during aging reduced viability, linked to hydrogen peroxide accumulation, DNA fragmentation, mitochondrial dysfunction, and potential programmed cell death (El-Maarouf-Bouteau et al., 2011). Similarly, research on oats and another on sunflower seeds at varying moisture levels during different aging days revealed viability loss linked to an increase in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels and reduced activity of key detoxifying enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Kibinza et al., 2006; Xia et al., 2015).

Crucial biochemical activities in seeds, such as enzymatic functions, antioxidant defenses, and gene expression regulation, are vital for preserving redox balance, protecting cellular structures, and optimizing metabolic functions during germination and stress adaptation (Dumanović et al., 2021). Several studies employing priming treatments showed enhanced germination and mitigated aging effects by boosting antioxidant defenses and enzymatic activity. Commonly used methods include hydropriming, osmopriming, nanopriming, and chemical or hormonal priming (Adetunji et al., 2021; Canizares et al., 2025). For instance, a study employing various seed priming methods, including 24-h treatment with gibberellic acid and ammonium molybdate, reported enhanced seed vigor, increased enzymatic activity, and restoration of protein, esterase, and peroxidase isozyme profiles in aged cowpea seeds (*Vigna unguiculata* (L.) Walp.) (Arun et al., 2021). Also, treatments with ascorbic acid and glutathione have demonstrated benefits in artificially aged oats and safflower, improving germination and antioxidant activity while reducing oxidative damage markers, such as MDA and H_2O_2 (Xia et al., 2020; Tonguç et al., 2023). Despite their proven effectiveness, chemical and hormonal priming methods often require careful optimization of treatment conditions, which can vary among crop species, seed physiological status, and aging levels, potentially influencing their consistency across different experimental contexts (Ashraf and Foolad, 2005; Srivastava et al., 2021; Tu et al., 2022). As a result, exploring innovative alternatives, such as ultrasound-assisted priming, may provide more efficient and reliable solutions for restoring seed quality (Nogueira et al., 2023).

Ultrasound technology is a cost-effective and scalable method that offers a promising eco-friendly solution to improve seed vigor and germination for sustainable agriculture applications. This novel technology utilizes high-frequency sound waves that exert mechanical effects to enhance seed treatment by promoting permeability, inducing biochemical changes, and improving germination performance and seedling vigor (Nogueira et al., 2023). Previous studies have shown the potential of ultrasound to improve the seed hydration, germination performance and seedling growth and vigor of multiple species, including maize, tomato (Nogueira et al., 2024), rice (Huang et al., 2024) and bean (Miano et al., 2016). Other reports showed ultrasound-induced biochemical responses in seeds of maize, soybean and rice, including increased expression of genes encoding water transport proteins (aquaporins), hormone regulation (gibberellin, salicylic acid), activities of key enzymes that promote nutrient mobilization and starch hydrolysis (α -amylase and β -amylase), or enzymatic activity (SOD, CAT), alter lipid metabolism (reducing MDA levels), making its application potentially beneficial for invigorating aged seeds (Alfalahi et al., 2022; Gong et al., 2024; Huang et al., 2024; Nogueira et al., 2025). However, despite these promising findings, crucial knowledge gaps remain regarding the extent to which ultrasound-induced invigoration persists beyond germination to support seedling establishment in aged seeds is not well documented. Additionally, species-specific physiological and biochemical responses to ultrasound treatment in aged seeds remain poorly understood.

The present study aimed to evaluate the potential of ultrasound treatment to mitigate the effects of seed aging by investigating the physiological and biochemical processes triggered by ultrasound that contribute to reversing deterioration and improving germinability. Furthermore, the study intended to determine whether these restorative effects at seed level persist throughout seedling development and to identify the associated processes triggered. Therefore, to achieve these objectives and to validate the robustness of the ultrasound treatment across species with contrasting seed size and physiological characteristics, seeds of two economically important crop species, pea (*Pisum sativum*) and turnip (*Brassica rapa* L.), were subjected to artificial aging.

2. Material and methods

2.1. Seed materials

Commercially available seeds of pea (*Pisum sativum* - cv. Lincoln) and turnip (*Brassica rapa* L.- cv. Purple Top White Globe) were purchased from a local market. The seeds were stored in a dry place at 21 °C until use.

2.2. Artificial aging

Prior to the artificial aging procedure, pea and turnip seeds were surface sterilized in 70% (v/v) ethanol for 2 min, followed by a 5-min treatment with 50% (v/v) commercial bleach (containing approximately 5% sodium hypochlorite as the active ingredient), and thoroughly rinsed several times with distilled water. The artificial aging of the seeds was undertaken in a sealed plastic container with a high surface-to-volume ratio to ensure maximum seed exposure and humidity control. The seeds were distributed in a single layer of plastic mesh tray attached to the container. The bottom of the container was filled with 300 mL of distilled water. The equipment was autoclaved prior to use, and the containers were tightly sealed with parafilm to maintain near 100% relative humidity. The aging conditions were as follows: pea seeds were aged for 1 day at 46 °C, while turnip seeds were aged for 4 days at 45 °C, which were adapted from previous studies and optimized for each species to achieve a balance between reduced vigor and seed viability (López-Ribera and Vicient, 2017; Huang et al., 2022).

2.3. Ultrasound processing

2.3.1. Ultrasonic Instrumentation

The seed processing experiments were conducted on a laboratory scale using multi-frequency, multimode and modulated (MMM) technology according to Nogueira et al. (2024). The system included a piezoelectric transducer, a booster, an acoustic waveguide, and a probe specifically designed and optimized for the system's functionality with oscillation capabilities to exhibit various vibration modes and harmonic ultrasonic frequencies. MMM technology activates multiple vibratory modes within the acoustic load, promoting a uniform distribution of high-intensity acoustic energy. This design overcomes traditional efficiency limitations by preventing stationary standing wave patterns.

2.3.2. Ultrasonic seed treatments

For the ultrasonic treatment, continuous pulse trains were applied using the ultrasonic unit by immersing the probe 2 cm into the medium (water and seed mixture). The treatment was conducted at a frequency of 19.8 kHz with a ± 100 Hz sweep and a power output of 200 W, parameters optimized based on previous work (Nogueira et al., 2024). This operating window falls within the low-frequency ultrasound range, in which acoustic cavitation is the principal mechanism underlying biological effects, providing a controlled and biologically safe treatment optimized for reproducibility and mechanistic analysis.

The tests were conducted in a glass flask with specific dimensions (0.2 cm wall thickness, 17.0 cm height, 6.0 cm inner diameter). Seed samples were suspended in 500 mL of sterilized distilled water, with a sonication time of 7.5 min. To maintain equivalent temperatures across both the treatment and control sets, frequent checks were conducted. Chilled water was periodically added to both groups to prevent excessive heat buildup. For the treatment group, a magnetic stirrer operating at 250 rpm continuously mixed the seeds and liquid, ensuring even distribution of ultrasonic waves and reducing the risk of overexposure. Meanwhile, the control seeds were submerged in an identical amount of water for the same duration as their treated counterparts, though they did not undergo sonication.

2.4. Germination experiments

Two types of germination tests were conducted immediately following ultrasonic treatment: i) pea seeds were sown in plastic trays with Nutrofert, a natural soil substrate characterized by high organic matter content (>80%), pH ranging from 5.5 to 6.5, total nitrogen of 150–200 mg/L, total phosphorus (P_2O_5) of 150–190 mg/L, and a total potassium (K_2O) of 200–300 mg/L. The trays featured 50 individual cells each filled with 4 pea seeds; ii) turnip seeds were sown in transparent re-sealable plastic boxes (17 × 13.5 × 9.5 cm) containing 1% (w/v) agar and half-strength basal Murashige and Skoog (MS) medium. The experiments for each species were conducted in triplicate, with each replicate consisting of 50 seeds of turnip, and 40 seeds of pea. Seeds were incubated at 24 °C under an 8-h dark/16-h light photoperiod (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Germination was monitored daily for 14 days, with seeds considered germinated: i) once the cotyledons emerged above the soil surface for pea; ii) once the radicles were visibly protruding and reaching a length of 2 mm in agar for turnip.

At the end of the germination period, various germination parameters were calculated. The final germination percentage (GP) represents the total number of seedlings at the end of the test, and was calculated as follows (Eq. (1)):

$$GP (\%) = \frac{\text{Germinated seeds}}{\text{Total number of seeds}} \times 100 \quad (1)$$

The germination value (GV) was calculated as a composite index that takes into account both the speed and total germination

percentage of the seeds (Czabator, 1962), with higher values indicating more vigorous seed performance. The GV was estimated as follows (Eq. (2)):

$$GV = \frac{\sum DGS}{N} \times GP \times 10 \quad (2)$$

where, DGS represents the number of newly germinated seeds per day and N the total number of seeds.

The time to 50% germination (T_{50}) was determined as an indicator of germination speed and calculated using Eq. (3) (Coolbear et al., 1984):

$$T_{50}(\text{days}) = ti + \frac{\left[\left(\frac{N}{2} \right) - ni \right] \times (tj - ti)}{(nj - ni)} \quad (3)$$

where, ti is the time when the cumulative germination reached less than $N/2$; tj is the time in which the cumulative germination exceeded $N/2$; ni represents the cumulative number of seeds germinated at time ti and nj is the cumulative number of seeds germinated at time tj .

2.5. Crude extractions

After the germination period, seedlings were immediately frozen in liquid nitrogen and ground to a fine powder using a pre-chilled mortar and pestle. The frozen samples were then lyophilized during 3 days. Following the freeze-drying process, the completely dehydrated samples were carefully stored in a desiccator for subsequent analyses. To obtain pea seed material, experiment was repeated as above, but seeds with and without aging treatment were incubated for 2 days and, prior to radical protrusion, were frozen in liquid nitrogen. Additionally, ungerminated seeds (T_0), both with and without aging treatment, were frozen prior to germination to allow assessment of the immediate biochemical effects of aging on the seeds before any germination-related changes occurred.

2.5.1. Sample extraction for antioxidant assays

The antioxidant activity assays of seeds (50 mg) and seedlings (5 mg) powder were homogenized in 1 mL of 80% methanol. The homogenate was centrifuged at 5000 g for 30 min at 4 °C and the supernatant was collected for the antioxidant activity assays.

2.5.2. Sample extraction for enzymatic assays

In order to extract antioxidant enzymes, seeds (100 mg) and seedlings (5 mg) powder were homogenized in 5 and 2.5 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing 1% (w/v) polyvinylpyrrolidone and 0.5 mM ethylenediaminetetraacetic acid (EDTA), respectively. The homogenate was centrifuged at 5000 g for 30 min at 4 °C. The supernatant was collected for the enzymatic assays of CAT and SOD activity.

2.6. Chlorophyll and carotenoids content

Chlorophyll content was measured following the method of (Curtis et al., 1996). In brief, 2 mg of dry seedling powder samples were homogenized in 1 mL of methanol (90%). After filtration, the solutions were incubated in the dark to prevent photo-oxidation. Chlorophyll *a* (Chl_a) and Chlorophyll *b* (Chl_b) contents were quantified by measuring the absorbance at 665.2 nm ($A_{665.2}$) and 652.4 nm ($A_{652.4}$), respectively. Carotenoid content was determined at 470 nm (A_{470}). Calibration was performed using 90% methanol as a blank. The Chl_a , Chl_b , total chlorophyll ($Chl_a + b$) and total carotenoids concentration (Car_c) ($\mu\text{g}/\text{mg DW}$) were calculated using the following equations (Lichtenthaler, 1987):

$$Chl_a = 16.82A_{665.2} - 9.28A_{652.4} \quad (4)$$

$$Chl_b = 36.92A_{652.4} - 16.54A_{665.2} \quad (5)$$

$$Chl_{a+b} = 0.28A_{665.2} - 27.64A_{652.4} \quad (6)$$

$$Car_c = \frac{1000A_{470} - 1.91Chl_a - 95.15Chl_b}{225} \quad (7)$$

2.7. Lipid peroxidation – MDA content

Lipid peroxidation was assessed by measuring MDA content following the method described by (Cakmak and Horst, 1991). Lyophilized seed and seedling samples were homogenized in 1.2 mL of 0.1% (w/v) trichloroacetic acid, followed by centrifugation at 12,000 g for 10 min. A 300 μL aliquot of the supernatant was then mixed with an equal volume of 0.5% (w/v) thiobarbituric acid. The mixture was heated at 95 °C for 30 min, and the reaction was stopped by immediately placing the tubes on ice. After cooling for 5 min, the tubes were centrifuged again at 12,000 g for 10 min. The absorbance of the supernatant was measured at 532, 600, and 450 nm to determine the MDA content. Interference from soluble sugars at 532 nm and 450 nm was corrected by subtraction. The MDA content

($\mu\text{mol/g DW}$) was calculated using the formula:

$$[\text{MDA}] = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450} \quad (8)$$

where, A_{532} , A_{600} and A_{450} represent the absorbance of the mixture at 532, 600, and 450 nm, respectively.

2.8. Antioxidant enzyme activity

2.8.1. SOD activity

The SOD activity was determined following the method described by (Vuleta et al., 2016), which is based on the enzyme's ability to inhibit the photoreduction of nitroblue tetrazolium (NBT). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.4), 50 μM NBT, 0.1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 10 μM riboflavin, and 20 μL of crude extract, with a final volume of 300 μL . A reaction mixture without the crude extract was used as the blank control. The reaction was initiated by exposing the mixture to white light for 30 min at room temperature, after which the absorbance was measured at 560 nm. One unit (U) of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT. The SOD activity was expressed as U/mg DW.

2.8.2. CAT activity

CAT activity was measured following the method described by (Aebi, 1984). The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2 , and 40 μL of crude extract, with a total volume of 200 μL per well. The activity was monitored spectrophotometrically at 30 °C by tracking the decrease in absorbance at 240 nm, corresponding to the decomposition of H_2O_2 . The CAT activity was expressed as μmol of H_2O_2 oxidized per minute per gram of dry weight.

2.9. Total antioxidant activity

2.9.1. DPPH radical scavenging assay

Antioxidant activity was assessed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay (Ferreira-Santos et al., 2022). Dry seed and seedling powder samples were mixed with 0.4 mM DPPH in 80% methanol and thoroughly shaken. The mixture was incubated in the dark at room temperature for 30 min. After incubation, absorbance was measured at 517 nm with 80% methanol as the blank. The DPPH radical scavenging activity of the extracts was calculated using the following formula:

$$\text{DPPH scavenging activity (\%)} = \frac{(Abs_{\text{control}} - Abs_{\text{sample}})}{Abs_{\text{control}}} \times 100 \quad (9)$$

where, Abs_{control} indicates the absorbance value of the reaction mixture without sample extracts. Abs_{sample} represents the absorbance of the reaction mixture with sample extracts.

2.9.2. ABTS radical scavenging assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay was carried out following the method of (Youn et al., 2019), with minor modifications. The ABTS + solution was prepared by mixing 2.45 mM potassium persulfate with 7 mM ABTS+ in distilled water at a 1:1 ratio. The mixture was stored at room temperature in the dark for 16 h to allow the formation of the ABTS radical cation. Before use, the solution was diluted with 90% methanol to achieve an absorbance of 0.700 at 734 nm. The sample methanolic extracts were then mixed with the diluted ABTS⁺ solution and incubated in the dark for 5 min before measuring the absorbance at 734 nm. The ABTS radical scavenging activity was analyzed considering the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \frac{(Abs_c - Abs_s)}{Abs_c} \times 100 \quad (10)$$

where, Abs_c refers to the absorbance at 734 nm of the blank and Abs_s refers to the absorbance at 734 nm of the different samples analyzed.

2.9.3. Ferric reducing antioxidant power (FRAP) assay

The antioxidant capacity based on the reduction of the Fe^{3+} tripyridyltriazine (TPTZ) complex (colorless) to Fe^{2+} tripyridyltriazine (blue), was measured spectrophotometrically following the Benzie & Strain method (Benzie and Strain, 1996). The FRAP reagent was prepared by combining 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM hydrochloric acid, and 20 mM FeCl_3 in a 10:1:1 ratio at 37 °C. Sample methanolic extracts were mixed with the FRAP reagent and incubated for 30 min. The absorbance was measured at 593 nm using a blank (without sample extract) as reference. A calibration curve was plotted using a stock solution of Trolox (2 mg/mL in 80% methanol) and diluted to concentrations ranging from 0.1 to 0.5 mM. Results were expressed as mM of Trolox equivalents (TE) per g of dry sample.

2.10. Statistical analysis

Statistical data for pea and turnip seeds/seedlings were analyzed separately using one-way ANOVA to compare the effects of the three treatment conditions (non-aged control, artificially aged control, and artificially aged + ultrasound treatment) on the measured variables. Given the small sample size per treatment, residual normality was assessed by visual inspection of Q-Q plots, an approach recommended for small-sample designs. All data were analyzed for statistical significance using Prism 10 (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as means $\pm$ standard error ($n = 3$). Different lowercase letters indicate significant differences between treatments ($p \leq 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc tests. Principal Component Analysis was performed using the factominer package for R.

3. Results

3.1. PCA trait clustering by aging and ultrasound treatment

PCA of seed and seedling traits showed distinct clustering patterns when seeds were subjected to aging and ultrasound treatments prior to germination (Fig. 1). Regarding pea cultivar (Fig. 1A), the first two principal components explained a cumulative variance of

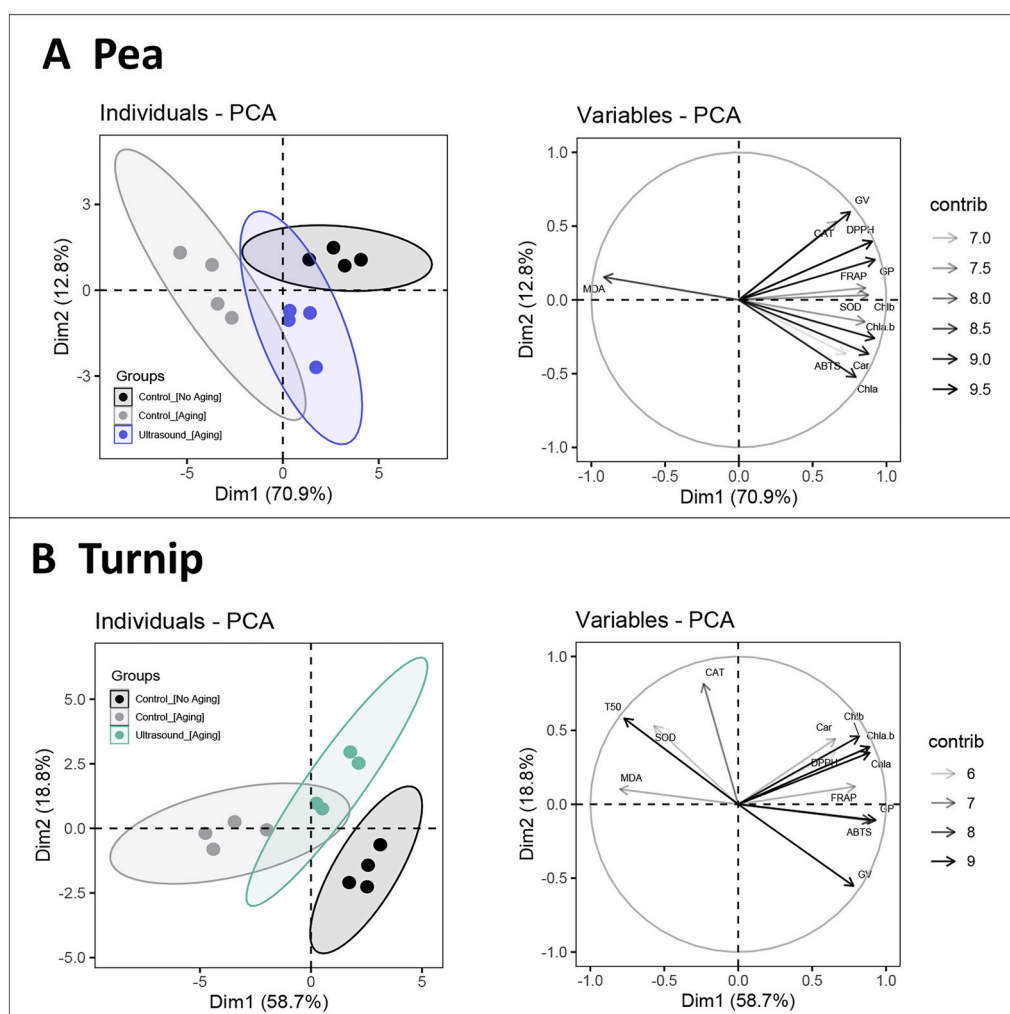


Fig. 1. Principal Component Analysis (PCA) of physiological and biochemical parameters in pea (A) and turnip (B) seeds/seedlings under different treatments. Left: Individual factor map showing the distribution of samples from control non-aged (Control_[No Aging]), control aged (Control_[Aging]), and ultrasound-treated aged seeds (Ultrasound_[Aging]). Ellipses represent 95% confidence intervals. Right: Variable factor map displaying the correlation between measured parameters and their contribution to principal components. Arrow length indicates the contribution strength of each variable to the principal components. Variables include germination vigor (GV), germination percentage (GP), time to 50% germination (T50), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), ABTS and DPPH radical scavenging activities, ferric reducing antioxidant power (FRAP), chlorophyll a (Chla), chlorophyll b (Chlb), chlorophyll a+b (Chla.b) and carotenoids (Car).

83.7% (Dim1: 70.9%, Dim2: 12.8%) with each condition occupying different quadrants. The variable factor map revealed that germination parameters, antioxidant capacity markers (DPPH, FRAP, ABTS) and enzymatic activities (SOD and CAT) were positively correlated with Dim1, while MDA showed strong negative correlation, indicating its association with seed deterioration. Regarding turnip cultivar (Fig. 1B), the first two components accounted for 77.2% of the total variance (Dim1: 58.7%, Dim2: 18.8%). The clustering plot showed that aged seeds treated with ultrasound exhibited a pattern similar to non-aged control seeds, occupying the positive quadrants of Dim1, while aged control seeds were observed in the negative quadrants. The variable factor map demonstrated similar trends to pea seeds, although enzymatic activities (SOD and CAT) along with T50 were strongly correlated to Dim2, suggesting species-specific responses to the ultrasound treatment (Fig. 1B).

3.2. Ultrasound treatment effects on the germination of artificially aged seeds

Artificial aging significantly reduced seed germination parameters, which were partially mitigated by ultrasound treatment in both artificially aged pea and turnip seeds (Fig. 2). Pea seeds of non-aged control showed the highest germination percentage (53%) and germination value (24). The application of artificial aging plus ultrasound treatment significantly improved (340%) the germination percentage of seeds when compared to aged control seeds. Similar results were observed in the germination value parameter (increase of 69%). Turnip seeds with artificial aging plus ultrasound treatment showed a 38% increase in germination percentage when compared to aged control seeds, almost reaching the levels observed in the control seeds without aging (Fig. 2B). The germination value followed a similar trend, with ultrasonic treatment showing significant improvement (69%) above aged seeds. Interestingly, it was observed that the artificial aging increased T50 from 1.5 days (non-aged seeds) to 3.6 days (aged seeds), which decreased significantly to 3 days in aged seeds subjected to ultrasound treatment.

3.3. Effects of ultrasound treatment on the photosynthetic pigments of seedlings

Seedlings from seeds subjected to artificial aging showed a significant reduction in photosynthetic pigments but a significant increase was observed in seedlings from artificial aging plus ultrasound treatment (Table 1). Aging reduced the chlorophyll content of

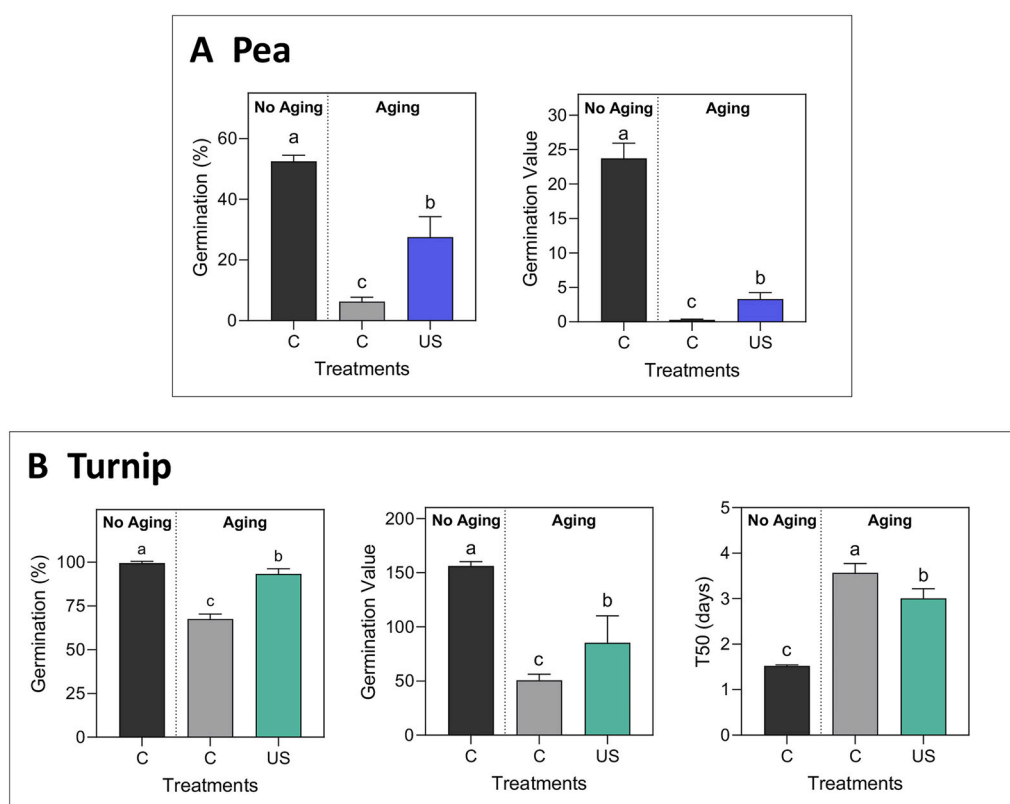


Fig. 2. Germination percentage under ultrasound treatment. For pea (A): Germination percentages (%) and germination value; for turnip (B): germination percentage and value and time to 50% germination (T50; days) under ultrasound (US) treatments and respective controls (C), without aging (left side) and with aging (right side) conditions. Pea and turnip seeds underwent artificial aging (45 °C, 1 day). Data are presented as means $\pm$ SE (n = 3). Lowercase letters denote statistically significant differences between treatments ($p < 0.05$). Statistical significance was assessed using one-way ANOVA followed by Tukey's post-hoc tests.

Table 1

Chlorophyll and carotenoid content in pea (A) and turnip (B) seedlings. Seedlings were analyzed under control (C) and ultrasound (US) treatments, both without aging (No Aging) and with aging (Aging). Data are presented as means $\pm$ SE ($n = 3$). Different lowercase letters indicate significant differences between treatments as determined by one-way ANOVA followed by Tukey's post-hoc tests.

		Chlorophyll Content (µg/mg)			Carotenoids Car c (µg/mg)
Treatments		Chl a	Chl b	Total Chl (a+b)	
Pea					
C	[No Aging]	7.19 ± 0.77 ^a	4.01 ± 0.74 ^a	11.60 ± 1.23 ^a	1.87 ± 0.06 ^a
C	[Aging]	5.12 ± 0.77 ^b	1.65 ± 0.27 ^b	6.77 ± 1.04 ^b	1.27 ± 0.17 ^b
US	[Aging]	7.94 ± 0.72 ^a	2.79 ± 0.38 ^b	10.73 ± 1.09 ^a	1.90 ± 0.13 ^a
Turnip					
C	[No Aging]	5.02 ± 0.12 ^a	1.68 ± 0.10 ^a	6.69 ± 0.14 ^a	1.22 ± 0.14 ^a
C	[Aging]	3.70 ± 0.32 ^b	1.24 ± 0.04 ^b	4.93 ± 0.34 ^b	0.96 ± 0.19 ^a
US	[Aging]	5.29 ± 0.37 ^a	1.82 ± 0.26 ^a	7.11 ± 0.57 ^a	1.34 ± 0.10 ^a

pea seedlings up to 60% while a 57% improvement was observed in seedlings from artificial aging plus ultrasound treatment compared to aged control seeds. Similar results were observed in total carotenoid content, declining from 1.9 (non-aged) to 1.3 $\mu\text{g}/\text{mg}$ (aged) within control groups, while seedlings from aged seeds subjected to ultrasound treatment exhibited a significant carotenoid enhancement (Table 1). Artificial aging reduced the total chlorophyll content of turnip seedlings by 27%, but seedlings from artificial aging plus ultrasound treatment of seeds showed an increase of total chlorophyll values similar to the non-aged control values (7.1 and 6.7 $\mu\text{g}/\text{mg}$, respectively). Total carotenoids showed a similar trend of reduction with aging and improvement with ultrasound treatment but no significant differences were observed (Table 1).

3.4. Ultrasound treatment effects on the aging-induced lipid peroxidation

Artificial aging significantly increased lipid peroxidation levels, as evidenced by increased MDA concentrations in both seeds and seedlings (Fig. 3). However, ultrasound treatment demonstrated significant opposite effects on aging-induced oxidative stress. The MDA levels of non-aged pea seeds significantly increased by up to 142% after two days of incubation (from 0.4 to 1.0 $\mu\text{mol}/\text{g}$), but ultrasound minimized MDA levels to 0.8 $\mu\text{mol}/\text{g}$, although no statistically differences were observed (Fig. 3A). Similarly, aging increased the MDA concentration by 86% after incubation, however, ultrasonic treatment significantly decreased the oxidative stress to the levels of quiescent (T0) and non-aged seeds (Fig. 3A). Regarding seedlings, aging increased MDA values by 84% in pea and 21% in turnip, while aging plus ultrasound treatment reduced MDA levels to the same values of the non-aged control plants (Fig. 3B and C).

3.5. Ultrasound treatment impact on the enzymatic activity of aged seeds and seedlings

SOD activity of pea seeds showed no variation between quiescent, artificial aging or ultrasound treatments (Fig. 4A). Similarly, CAT activity showed no variation in aged seeds, however, in non-aged seeds, ultrasound treatment led to an increase up to 165% compared to control group (Fig. 4A). In pea seedlings, artificial aging reduced SOD activity by 47% and CAT activity by 32% compared to the non-aged control, while ultrasound treatment maintained the enzymatic activities of seedlings from aged seeds in values similar to non-aged seedlings (Fig. 4B). In turnip seedlings, aging-induced changes significantly increased the SOD activity, which was equivalent to ultrasound-treated samples. In consonance, the ultrasound treatment significantly increased the CAT activity in turnip

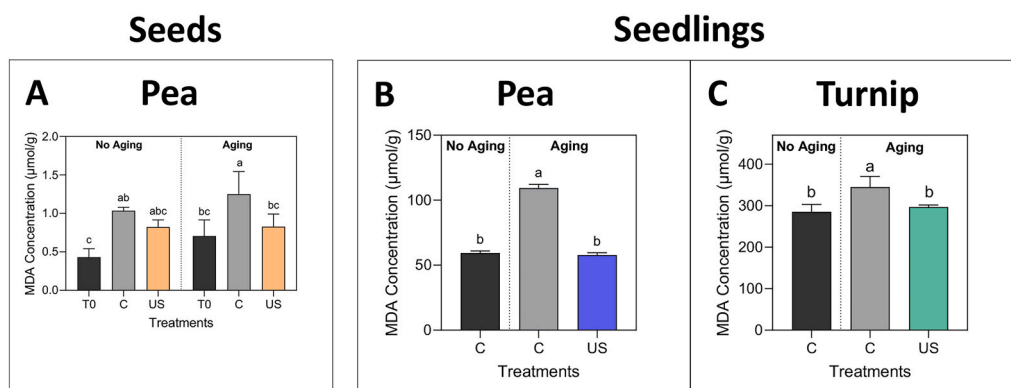


Fig. 3. Lipid peroxidation levels in pea seeds (A) and seedlings of pea (B) and turnip (C). The malondialdehyde (MDA) concentration ($\mu\text{mol}/\text{g}$) was measured under control conditions (C) and after ultrasound treatment (US), both without aging (No Aging) and with aging (Aging). An additional time point (T0) represents initial MDA levels before germination for pea seeds. Data are presented as means $\pm$ SE ($n = 3$). Different lowercase letters indicate significant differences between treatments as determined by one-way ANOVA followed by Tukey's post-hoc tests.

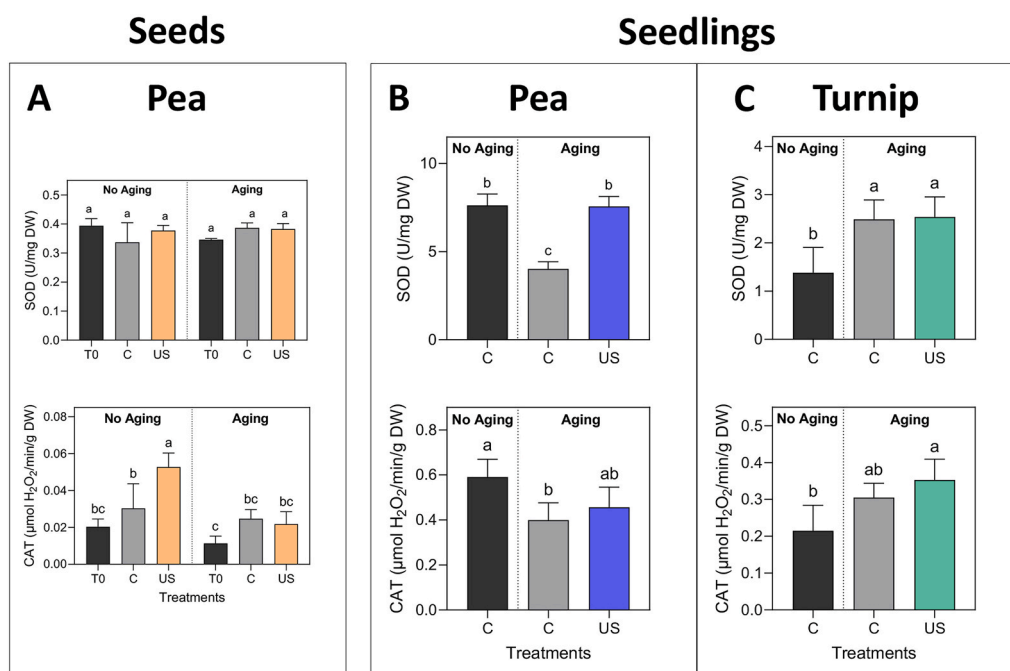


Fig. 4. Superoxide dismutase (SOD) and catalase (CAT) activities in seeds and seedlings under control and ultrasound treatments, with and without aging conditions. (A) Enzymatic activities in pea seeds and seedlings of pea (B) and turnip (C) showing SOD (upper panel, U/mg DW) and CAT (lower panel, $\mu\text{mol H}_2\text{O}_2/\text{min/g DW}$). (B and C). An additional time point (T0) represents initial enzyme activities before germination for pea seeds. Data are presented as means $\pm$ SE ($n = 3$). Different lowercase letters indicate significant differences between treatments as determined by one-way ANOVA followed by Tukey's post-hoc tests.

seedlings by up to 67% compared to non-aged and aged control groups (Fig. 4C). The Pearson correlation reveal physiologically coherent relationships among the evaluated parameters in both species. A consistent negative correlation between MDA levels and the activity of antioxidant enzymes (SOD and CAT) was observed, indicating that enhanced antioxidant capacity is associated with reduced oxidative damage (Fig. S1).

3.6. Ultrasound treatment impact on the antioxidant activity of aged seeds and seedlings

Antioxidant activity of pea seeds and seedlings of pea and turnip was evaluated using three different assays (DPPH, ABTS, and FRAP). The antioxidant activity of pea seeds varied distinctly across treatments in the three assays. The DPPH assay performed on non-aged seeds treated with ultrasound showed an increase in activity of 37% when compared to the non-aged control (Fig. 5A). The ABTS assay results showed that ultrasound enhanced antioxidant activity in aged seeds compared to quiescent seeds (T0), an effect that was absent in the aged control. Apart from that, and similar to FRAP assay results, the control and ultrasound treatments in both non-aged and aged seeds increased the antioxidant activity relatively to T0, however, no significant differences between controls and ultrasound were observed (Fig. 5A).

Antioxidant activity on pea seedlings showed that artificial aging caused a substantial decrease of DPPH activity by 44% and FRAP by 29% (Fig. 5B). However, ultrasound treatment significantly returned the antioxidant activity to values closer to the non-aged seeds (an increase of 34%). Contrarily, ABTS activity remained relatively stable, with no significant difference found across treatments (Fig. 5B).

It was observed that the ABTS activity in seedlings from artificially aged turnip seeds was reduced by 32%, while ultrasound treatment increased the antioxidant activity by 30% in seedlings from aged seeds (Fig. 5C). Similarly, FRAP activity values were significantly enhanced (from 299 to 377 mM TE/g) in seedlings from artificially aged seeds plus ultrasound treatment compared to aged control, approaching the values observed in non-aged control (385 mM TE/g). Although aging did not impact DPPH activity, seedlings from aged seeds treated with ultrasound led to a notable 14% increase compared to aged control (Fig. 5C). In addition, growth-related traits, photosynthetic pigments, and non-enzymatic antioxidant capacity (DPPH, ABTS, FRAP) are positively correlated with each other, suggesting an integrated physiological response to the treatment. Overall, these correlation patterns support the existence of a coordinated regulatory network underlying the effects of ultrasound priming, thereby strengthening the interpretation of the independently presented results (Fig. S1).

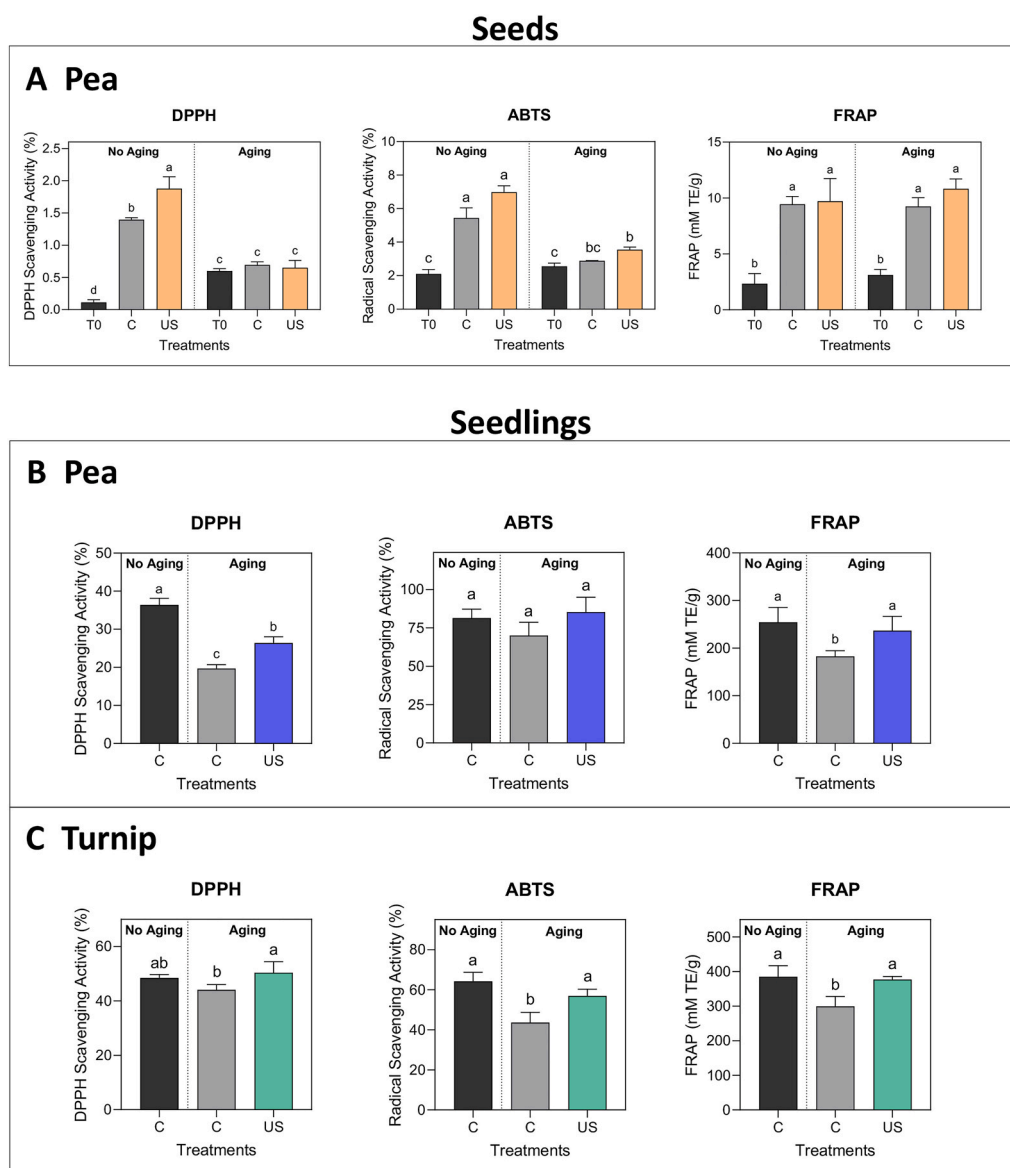


Fig. 5. Antioxidant activity in 2-day germinated pea seeds (A) and seedlings of pea (B) and turnip (C). Three antioxidant assays were performed: DPPH (radical scavenging activity, %), ABTS (radical scavenging activity, %), and FRAP (mM TE/g). Measurements were performed at the initial time (no germination, T0), under control conditions (C), and after ultrasound treatment (US), both without aging (No Aging) and with aging (Aging). Data are presented as means $\pm$ SE ($n = 3$). Data are presented as means $\pm$ SE ($n = 3$). Different lowercase letters indicate significant differences between treatments as determined by one-way ANOVA followed by Tukey's post-hoc tests.

4. Discussion

4.1. Ultrasound treatment effectively restores germination potential and seedling vigor in aged seeds

Seed vigor is essential for optimal crop growth and yield, a fundamental requirement for agricultural production. However, seed germinability decline is influenced by storage duration and conditions, and seed intrinsic characteristics (Thirusendura Selvi and Saraswathy, 2018; Reed et al., 2022). Despite advances in seed storage and the use of chemical or hormonal treatments to enhance germination capacity, there remains a need for eco-friendly methods to restore germination and seedling emergence in fast-decaying aged seeds, which holds significant economic value. This is particularly impactful, as seed aging significantly reduces the vigor of resulting seedlings (De Vitis et al., 2020; Pirredda et al., 2020, 2024). The present results demonstrated that ultrasound treatment effectively restored germination performance, enhanced antioxidant activity, and increased photosynthetic pigment content in pea (*Pisum sativum*) and turnip (*Brassica rapa* L.), revealing its potential as an innovative seed priming technology to counter seed

deterioration. The role of ultrasound in mitigating the effects of artificial aging on seeds, as well as its lasting impact on seedlings, was clearly demonstrated by PCA for both species studied. It should be noted that while the artificial aging protocol used in this study successfully simulated the biochemical and physiological degradation of seeds, further research using naturally aged seed lots is recommended to confirm the replicability of the ultrasound method. Preliminary data from our laboratory using naturally aged seeds suggests that the restorative effects of ultrasound are consistent across both artificial and natural aging processes.

Previous studies reported positive results of ultrasound treatment in improving seed germination and seedling vigor of tomato and maize seeds (Nogueira et al., 2024), particularly in drought stress conditions, but its effects on aged seeds and the physiological mechanisms underlying its recovery remains limited. Studies on aged tall fescue, Russian wild rye, and milk thistle, reported beneficial effects on germination following ultrasound treatment (Liu et al., 2016; Moosavi et al., 2018). The remarkable 340% increase in the germination of aged pea seeds compared to control, and the more modest but still significant improvement (38%) of aged turnip seeds after ultrasound treatment, reflect the importance of environmentally friendly methods to counteract seed aging decay.

A study on maize, bean and pepper seeds treated with ultrasound showed the downregulation of genes coding for enzymes involved in the biosynthesis of precursors of abscisic acid (ABA) (Nogueira et al., 2025). Furthermore, ultrasonic treatment appears to regulate hormone balance, including indole-3-acetic acid, gibberellic acid, and ABA, which are crucial for triggering germination (Gong et al., 2024; Huang et al., 2024). These ultrasound-induced biochemical changes likely contribute to improved germination and the observed reduction in T50, facilitating faster and more uniform seedling emergence. Ultrasound treatment was also shown to stimulate α -amylase and β -amylase activities that break down starch into metabolizable sugars, increasing nutrient bioavailability and providing carbon for enhanced germination (Silva et al., 2020; Chen et al., 2023; Gong et al., 2024). These improvements suggest that ultrasound treatment may enhance membrane permeability (by micro-fissures or “sponge effect”) caused by acoustic cavitation, generating mechanical pressure that facilitates water uptake and oxygen availability (Nogueira et al., 2023). It should be noted that the present experimental design does not include a non-aged + ultrasound treatment. Therefore, the conclusions of this study specifically address the capacity of ultrasound to restore physiological and biochemical performance in aged seeds, rather than its general stimulatory effects on high-quality seed lots. Nonetheless, evidence from prior studies on these species, suggests that the benefits of ultrasound treatment appear most significant in low-vigor seed lots, whereas high-quality seeds tend to show more limited improvement (Chiu and Sung, 2014; Nogueira et al., 2024). Seedling vigor is an important trait in establishing healthy plants, resulting in higher crop productivity. Studies conducted under different stresses (e.g., heavy metals and NaCl-stress) have associated the decrease in photosynthetic activity to oxidative damage of chloroplast structures and disruption of chlorophyll biosynthesis, especially in early enzymatic stages (Sidhu et al., 2017; Hamani et al., 2020). Two reports in soybean and quinoa seedlings from natural and artificial aged seeds showed inhibition of photosynthesis (Nazari et al., 2020; Mansouri and Omid, 2022), which is in agreement with our observation of lower concentration of photosynthetic pigments. However, ultrasound treatment restored chlorophyll and maintained the carotenoid levels of pea and turnip seedlings that supported the hypothesis that ultrasound enhanced the repair of aging-induced damage, potentially by stimulating enzymatic pathways involved in pigment biosynthesis. Hormonal or salicylic acid priming of seeds has previously been conducted with similar results by mediating an increase in stomatal conductance, upregulating the activity and concentration of enzymes involved in CO₂ fixation, which improved the photosynthetic rate, growth and biomass (Lotfi et al., 2020; Nazari et al., 2020; Mansouri and Omid, 2022). To our knowledge, this is the first study demonstrating that ultrasound treatment of aged seeds enhances chlorophyll concentration in seedlings, highlighting its potential for improving post-germination vigor and early plant establishment.

4.2. Ultrasound treatment alleviates oxidative damage by improving antioxidant and enzymatic defenses

Although direct molecular analyses (e.g., gene expression or hormone quantification) were beyond the scope of this study, enzymatic and biochemical parameters were used as functional proxies of redox homeostasis and metabolic restoration. Aging of seeds can disrupt intercellular homeostasis by increasing ROS production, which can impair normal cellular metabolism by promoting the oxidation of nucleic acids and proteins and peroxidation of lipids. MDA, a byproduct of lipid peroxidation, was directly associated with seed deterioration and reduced vigor. It destabilizes cell membranes, damages functional molecules, and disrupts metabolic processes, all of which contribute to the decline in seed quality (Osborne, 1986; Lin et al., 2022; Lv et al., 2024; Ye et al., 2024). Our results demonstrated that pea seeds treated with ultrasound showed a significant reduction in MDA levels compared to untreated aged seeds. Additionally, MDA levels were found to reflect the germination performance; high MDA levels were associated with poor germination performance, while lower MDA levels corresponded to improved germination. Similar results were reported in naturally aged soybean seeds, where ultrasound treatment reduced MDA accumulation on the first and third days of incubation. Additionally, the study pointed out a decrease in ROS content (O₂⁻ and H₂O₂) following ultrasound treatment, indicating enhanced ROS scavenging capacity and improved homeostasis during early germination (Huang et al., 2022). Moreover, ultrasound treatment effectively reduced MDA accumulation in seedlings from aged pea and turnip seeds, which is consistent with a study that reported a decrease in MDA levels on naturally aged Russian wild rye after ultrasound treatment, highlighting sonication's ability to restore membrane integrity and inactivate oxidative enzymes such as peroxidase and lipoxygenase (Liu et al., 2016).

Oxidative damage and impaired metabolic functions caused by ROS can overwhelm antioxidant defenses, ultimately disrupting germination and plant development (Li et al., 2022). Enzymatic antioxidants such as SOD and CAT serve as primary defenses against ROS. SOD catalyzes the conversion of ^{*}O₂ into H₂O₂, which is then detoxified by CAT into water and molecular oxygen. The activity of these enzymes is closely linked to a seed's ability to prevent cellular damage. However, artificial aging can impair these defense enzymes and inhibit their synthesis to variable degrees (Ighodaro and Akinloye, 2018). The decline in enzymatic activity results from cellular homeostasis disruption, evidenced by increased electrolyte leakage and lipid peroxidation, which may degrade or inactivate

these enzymes (Silva et al., 2020).

Our study found a decrease of SOD and CAT activities (47% and 32%, respectively) in seedlings from aged pea seeds, however, ultrasound treatment notably restored enzymatic, particularly SOD, to levels comparable to control seedlings, underscores its potential to reactivate metabolic pathways disrupted by aging. A study on naturally aged soybean seeds supports the above hypothesis showing significant increases in SOD, CAT, and acetaldehyde dehydrogenase activity after ultrasound treatment, consistent with elevated expression of genes such GmSOD1, GmSOD3, GmCAT1, GmAPX2, and GmALDH1 during germination (Huang et al., 2022). The contrasting responses observed in turnip seedlings from artificially aged seeds compared to pea seedlings may reflect species-specific adaptations likely reflecting a compensatory upregulation of enzymatic defenses to counter ROS damage. Consistently, correlation analysis revealed that turnip exhibited a more tightly coordinated physiological response, with a higher number of strong negative correlations between oxidative damage and antioxidant and metabolic traits, whereas pea showed fewer and weaker correlations, indicating a less integrated regulation of oxidative stress.

The significantly reduced antioxidant capacity of pea and turnip seeds and seedlings caused by aging and the notorious ability of ultrasound to restore antioxidant activity may explain the improved germination and enhanced seedling performance observed in treated seeds. To our knowledge, this is the first report analyzing the non-enzymatic antioxidant capacity of aged seeds treated with ultrasound. In a study on soybean species, the authors suggested that the improvement in antioxidant activity could be due to changes in the quantity and structure of free phenols (Chen et al., 2023), while a study on oat seeds suggested that ultrasound acts as an abiotic stressor, triggering plant defense mechanisms and stimulating the synthesis of secondary metabolites, including phenolic compounds (Ding et al., 2019). Indeed, ultrasound increased the total phenolic and flavonoid content in soybean and black highland barley, enhancing DPPH and ABTS scavenging activity (Zhang et al., 2023; Qiao et al., 2024). From an applied perspective, these responses support the potential of ultrasound-assisted priming as a non-chemical pre-sowing strategy to improve the quality of aged or sub-optimal horticultural seed lots. This technology could be investigated for integration into large-scale seed conditioning or hydration steps in industrial processing lines, either as a preparatory step prior to drying and coating or as a short, continuous treatment applied immediately prior to germination in bulk handling or continuous-flow systems for seeds intended for immediate sowing. Such integration would require assessing compatibility with existing operations (e.g., drying, coating, pelleting) and evaluating treatment uniformity at scale. Consequently, further analyses under field and industrial-scale conditions are essential to validate the agronomic relevance and technical of these effects in practical agricultural scenarios.

5. Conclusion

In conclusion, this study demonstrated that ultrasound technology offers a promising, eco-friendly strategy to counteract the physiological and biochemical deterioration associated with seed aging. Ultrasound treatment effectively restored the germination potential in artificially aged seeds and promoted early seedling development. These improvements are associated with reinforced antioxidant and enzymatic defense systems, mitigation of lipid peroxidation and the restoration of photosynthetic pigments. Importantly, the persistence of beneficial effects during early seedling development highlights the practical relevance of ultrasound treatment beyond germination. Furthermore, the species-specific responses observed highlight the adaptability of ultrasound treatment across different crops, positioning it as a scalable and cost-effective method for sustainable agriculture with strong potential to improve seed quality. Future research should explore its efficacy on naturally aged seeds and long-term field performance to fully realize the potential of this technology in global crop production. Also, integrating transcriptomic or targeted gene-expression approaches will be required to validate the molecular mechanisms underlying ultrasound-mediated seed invigoration.

CRedit authorship contribution statement

António Nogueira: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Hélder Puga:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Joana T. Martins:** Writing – review & editing, Supervision, Methodology. **Hernâni Gerós:** Methodology, Data curation, Conceptualization. **António Teixeira:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Funding

This work was supported by Fundação para a Ciência e Tecnologia (FCT), Portugal, under the strategic program UIDB/04436/2020 and UIDB/BIA/04050/2020. This project was supported by an FCT doctoral grant (UI/BD/150938/2021).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2026.104010>.

Data availability

Data will be made available on request.

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