

Overcoming seed dormancy in *Parthenium hysterophorus*: Seasonal dynamics and standardized approaches for synchronized germination

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Title

Overcoming seed dormancy in *Parthenium hysterophorus*: Seasonal dynamics and standardized approaches for synchronized germination.

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Abstract

Parthenium hysterophorus is a highly invasive weed species that exhibits remarkable adaptability to abiotic stresses, including drought, salinity, and temperature extremes. However, strong and season-dependent seed dormancy restricts uniform and synchronized germination under controlled conditions, posing a major limitation for reproducible laboratory experiments. Developing reliable approaches to overcome this dormancy is therefore essential for generating uniform plant material for physiological and molecular studies. In this study, we evaluated the influence of seasonal seed collection (summer and winter), storage duration, and multiple dormancy-breaking approaches, including water immersion, cold stratification, gibberellic acid (GA₃) treatment, and polyethylene glycol (PEG) priming, to identify effective strategies for improving seed germination in *P. hysterophorus*. Freshly harvested winter-collected seeds showed high and relatively uniform germination following the standard heat treatment protocol, whereas summer-collected seeds showed markedly lower germination. Storage at room temperature markedly reduced germination in both seasonal seed lots. Water immersion substantially improved germination in dormant seed lots. Among the additional dormancy-breaking treatments evaluated, GA₃ significantly enhanced germination and seedling vigour, with 20, 200 and 500 ppm producing the highest germination responses, while PEG priming showed only moderate, concentration-dependent effects. Independently, cold stratification effectively alleviated dormancy and promoted rapid, synchronized, and healthy seedling establishment under controlled laboratory conditions.

The optimized dormancy-breaking approaches generated more uniform germination and seedling establishment across the dormant seed lots tested. These results provide practical approaches for obtaining more uniform *P. hysterophorus* seedlings, facilitating reproducible studies on weed biology, stress physiology, and weed management-related research.

Keywords: *Parthenium hysterophorus*; Seed dormancy; Dormancy breaking; Cold stratification; Gibberellic acid (GA₃); Germination synchronization; Weed biology.

Introduction

In recent years, *Parthenium hysterophorus* (L.) has gained attention not only as an invasive weed but also as an experimental system for studying plant adaptation to environmental stresses such as drought, salinity, and temperature extremes (Ahmad et al. 2017; Shabbir et al. 2024). Its ability to survive and proliferate under harsh conditions makes it an attractive system for understanding mechanisms of stress tolerance and climate resilience. Despite its growing utility in physiological and molecular research, the species exhibits strong and variable seed dormancy, which frequently restricts uniform and synchronized germination under controlled laboratory conditions. Achieving reliable germination is therefore a prerequisite for generating relatively uniform plant populations required for reproducible experimental studies.

P. hysterophorus (Fig. 1), belonging to the family Asteraceae is an aggressive annual weed native to the American tropics that has achieved widespread distribution across Asia, Africa, and Australia (Joshi et al. 2016; Bagchi et al. 2016).



Fig. 1. *P. hysterophorus* in its natural habitat

Representative photograph showing the growth habit, dense branching, and prolific flowering of mature *P. hysterophorus* plant growing naturally at the collection site (JSS AHER campus, Mysuru, India).

Its invasive success is largely attributed to prolific seed production and efficient dispersal of small, lightweight cypselsae, enabling rapid colonization of disturbed habitats (Lalita and Kumar. 2018) (Fig. 2).



Fig. 2. Morphology of *P. hysterophorus* inflorescences and mature cypselae

Detailed morphological view showing (i) Intact mature cypselae with the protective outer husk (Pericarp) that facilitates wind dispersal and physical protection, and (ii) Manually dehusked seeds with the outer husk removed, exposing the thick black wall protecting the inner embryo and endosperm tissues prepared for viability testing and germination assays.

The seeds possess protective structural characteristics that may contribute to their persistence under adverse environmental conditions and influence germination behaviour (Nguyen et al. 2010) (Fig. 3).

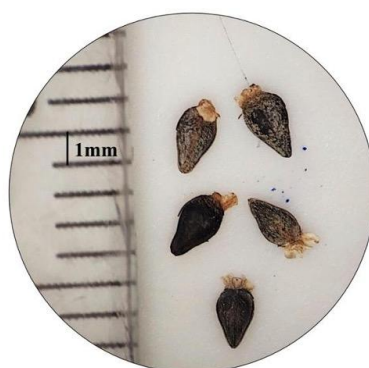


Fig. 3. Microscopic analysis of dehusked *P. hysterophorus* seeds showing maturity-dependent morphological variation

Microscopic view of manually dehusked *P. hysterophorus* seeds (Objective: 10X magnification). Dark brown to black seeds represent fully matured, viable seeds possessing well-developed internal embryo and endosperm tissues, whereas pale grey-colored seeds correspond to immature, or underdeveloped seed fractions.

Although *P. hysterophorus* has traditionally been studied primarily as a target for weed management and eradication (Adkins and Shabbir 2014; Kaur et al. 2014), increasing interest in its remarkable stress-adaptive traits has expanded its use as a model species for investigating physiological and molecular responses to environmental stresses (Ahmad et al. 2017; Ahmad et al. 2018; Dubey et al. 2020; Bashar et al. 2023).

Seed dormancy is an adaptive trait that prevents germination under unfavourable conditions and enhances long-term species survival (Finch-Savage and Leubner-Metzger. 2006; Mousavi et al. 2011). In *P. hysterophorus*, dormancy is influenced by storage duration, environmental conditions during seed maturation, and seasonal factors (Javaid et al. 2010; Akram et al. 2022). While ecologically advantageous, dormancy presents a major challenge for laboratory-based investigations by causing delayed, asynchronous, and inconsistent germination. Such variability can affect the reproducibility of physiological, biochemical, molecular, and stress-response studies that require relatively uniform plant material.

A range of physical, physiological, and chemical approaches have been employed to alleviate seed dormancy in different plant species. Physical approaches such as heat treatment and water immersion can improve germination in dormant seeds. Physiological approaches such as cold stratification mimic natural environmental cues associated with dormancy release and are widely used to improve germination in dormant seeds (Naithani et al. 2008; Mousavi et al. 2011). Chemical approaches, including gibberellic acid (GA₃) application and osmopriming treatments, can influence physiological and metabolic processes associated with germination. GA₃ promotes seed germination and seedling growth and has been associated with the induction of hydrolytic enzymes involved in the mobilization of stored reserves during germination (Gupta and Chakrabarty. 2013). In *P. hysterophorus*, dry heat treatment has previously been reported to improve germination (Ahlawat et al. 1979), while the role of GA₃ in promoting germination provides a basis for evaluating its potential for improving germination in dormant seed lots. However, comparative information regarding the effectiveness of these approaches across different dormant seed lots of *P. hysterophorus* remains limited. Polyethylene glycol (PEG) is widely used in seed research both as an osmotic agent for simulating water-deficit stress and as a priming agent to improve germination performance (Salah et al. 2015). Previous studies have employed PEG to evaluate drought responses in *P. hysterophorus* (Ahmad et al. 2017) however, its potential role in improving germination of dormant seeds in this species remains poorly understood. Similarly, the effectiveness of cold stratification in alleviating dormancy in *P. hysterophorus* has received limited attention despite its widespread use in dormancy studies of other plant species. Although *P. hysterophorus* generally germinates under favorable environmental conditions, the contribution of cold stratification to dormancy alleviation has not been systematically investigated.

Preliminary observations in our laboratory revealed substantial seasonal variation in germination behaviour. Seeds collected during the winter (Dec–Feb) germinated readily within two weeks of harvest, whereas seeds collected during summer (Mar–May) or subjected to prolonged storage exhibited markedly reduced and inconsistent germination, consistent with earlier reports (Javaid et al. 2010; Shrestha et al. 2024). These observations highlighted the need for reliable approaches to overcome dormancy and generate synchronized seedling populations for experimental studies.

Based on these observations, we hypothesized that different dormancy-breaking approaches, including baseline heat treatment combined with water immersion, hormonal stimulation through GA₃ application, osmopriming using PEG, and independently applied cold stratification, would differentially alleviate dormancy in seasonally variable seed lots of *P. hysterophorus*. Therefore, the objectives of this study were to: (i) evaluate the influence of seasonal seed collection and storage on seed germination behaviour, (ii) assess the effectiveness of multiple

dormancy-breaking approaches for improving germination and seedling establishment, and (iii) identify reliable strategies for generating synchronized and uniform plant populations suitable for downstream physiological, molecular, stress biology, and weed research studies.

Materials and Methods

Plant growth conditions and seed sterilization:

The study was carried out in the Plant Molecular Biology Laboratory at JSS Academy of Higher Education and Research (JSS AHER), Mysuru, Karnataka, India. Mature, brown-coloured inflorescences bearing fully matured seeds of *P. hysterophorus* were collected from its natural habitat within the JSS AHER campus (12.3362703° N, 76.6530339° E) during Jan 2026 (Winter) and Apr 2026 (Summer). The harvested inflorescences were dried in an incubator at 37°C for 24 h and stored in clean, moisture-free paper bags at room temperature (25 ± 2°C) until use. Heat treatment at 45°C for 24 h was performed as a default pre-treatment step for all seed lots prior to sterilization, except for seeds subjected to the cold stratification protocol. Following heat treatment, seeds were surface sterilized by immersion in 2mL of 70% (v/v) ethanol for 2 min, followed by treatment with 2mL of 3% (v/v) sodium hypochlorite (NaOCl) supplemented with 1.5% (v/v) Tween-20 for 10 min. The seeds were then rinsed 4–5 times with autoclaved ultrapure water to remove residual NaOCl and Tween-20.

Sterilized seeds were placed in autoclaved glass Petri plates containing 1X Johnson's medium containing 6mM KNO₃, 4mM Ca (NO₃)₂·4H₂O, 0.5mM KH₂PO₄, 1mM MgSO₄·7H₂O, 0.004mM FeSO₄·7H₂O-Na₂EDTA·2H₂O, 0.05mM KCl, 0.025mM H₃BO₃, 0.005mM MnSO₄·H₂O, 0.002mM ZnSO₄·7H₂O, 0.0005mM CuSO₄·5H₂O, 0.0001mM H₂MoO₄ prepared according to (Johnson et al. 1957), supplemented with 1% (w/v) sucrose, 2.5mM MES, 0.55mM Mio-inositol and solidified with 0.8% agar. The pH was adjusted to 5.8 with KOH before adding agar and autoclaving. This medium was used for all germination experiments except the GA₃ and PEG treatments. The plates were sealed with cling film and incubated in a CONVIRON plant growth chamber maintained at 27°C, 60% relative humidity, and approximately 7100 lux light intensity under a 16/8 h light/dark photoperiod.

Experimental design and replicates

Mature seeds were harvested from 4-5 independent *P. hysterophorus* plants growing within the study site. Approximately similar quantities of mature seeds collected from these plants were pooled to generate a composite seed lot, thoroughly mixed, and used for all subsequent experiments. For each treatment, randomly selected pooled viable seed lots were placed in independently prepared Petri plates with approximately 150 seeds per plate for all experiments except the GA₃ and PEG assays, in which 35-40 seeds were used per plate. Three independently prepared Petri plates were used as experimental replicates for each treatment.

Seed viability test

Seed viability was assessed using the Blue Tetrazolium Chloride (BTC) assay following (Grabe et al. 1959) method. To obtain uniform seeds for the assay, freshly harvested seeds were first soaked in autoclaved ultrapure

water for 2 h. Floating seeds were discarded as they were presumed to be immature or potentially non-viable, while seeds that settled at the bottom were selected for further testing.

The outer black pericarp/cypsela wall of selected seeds was carefully removed using a scalpel. The dehusked seeds, consisting of the white endosperm and embryo, were placed in sterile Petri plates lined with tissue paper soaked in 1% BTC solution. The plates were incubated in the dark at $25 \pm 1^\circ\text{C}$ for 24 h to allow staining. Because the assay is destructive, a separate aliquot of seeds representing each lot was used. Following incubation, seeds exhibiting distinct purple-blue staining in embryo and endosperm tissues were categorized as metabolically active and viable. Viability percentage was calculated as

$$\text{Viability (\%)} = \left(\frac{\text{Number of stained seeds}}{\text{Total number of seeds tested}} \right) \times 100$$

Seed germination experiments

Seasonal variation in germination behaviour of freshly collected seeds

Seasonal variation in germination behaviour of *P. hysterophorus* was evaluated through controlled germination assays. Freshly collected seeds from winter and summer seasons were manually dehusked, and BTC viability testing was performed on an aliquot as described above. Viable seed batches were subjected to default heat treatment (45°C for 24 h) and surface sterilization before being placed in sterile Petri plates containing 1X Johnson's medium prepared according to (Johnson et al. 1957). The plates were sealed with cling film and incubated under the growth chamber conditions described above. Germination was recorded on day 4, with visible radicle emergence serving as the criterion for germination. Germination percentage was calculated as:

$$\text{Germination (\%)} = \left(\frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \right) \times 100$$

Assessment of storage-induced dormancy and baseline heat response

To evaluate the depth of secondary dormancy induced by storage and the baseline effect of dry heat pre-treatment, seed batches collected during winter and summer seasons were stored at room temperature ($25 \pm 2^\circ\text{C}$) for 3-4 months. Following storage, seeds from each seasonal cohort were divided into two evaluation groups: i) Untreated control seeds maintained at 25°C without heat treatment. ii) Seeds subjected to standard heat treatment (45°C for 24 h in a dry incubator), modifying the protocol by (Ahlawat et al. 1979) where the temperature was increased from 40°C to 45°C . Both groups were surface sterilized and placed on sterile Petri plates containing 1X Johnson's medium (Johnson et al. 1957) to evaluate baseline dormancy alleviation and seedling emergence uniformity.

Water immersion treatment

To evaluate the effect of water immersion on summer seed lots exhibiting deep physiological dormancy, summer-collected seeds were subjected to baseline heat treatment (45°C for 24 h) and surface sterilized as described above. Sterilized seeds were fully immersed in autoclaved ultrapure water in a sterile 5mL centrifuge tube, ensuring complete submersion of all seeds, and incubated at room temperature ($25 \pm 2^\circ\text{C}$) for 24 h under sterile conditions to facilitate hydration and imbibition. Following water immersion, seeds were transferred to sterile glass Petri

plates containing 1X Johnson's medium (Johnson et al. 1957) and incubated under growth chamber conditions as described above in *Plant growth conditions section*. Non-immersed heat-treated summer seeds served as controls. Germination was recorded on day 4.

Cold Stratification

Summer-collected *P. hystrophorus* seeds exhibiting low germination were manually dehusked and surface sterilized. Explicitly omitting the default 45°C heat treatment, seeds were subjected directly to cold stratification to evaluate cold treatment as an independent physical mechanism. Approximately 150 seeds per Petri plate were placed on sterile glass Petri plates lined with two layers of autoclaved tissue paper pads moistened with 10 mL of autoclaved ultrapure water. The seeds were covered with an additional two layers of autoclaved moistened tissue paper to maintain complete darkness during stratification. The plates were sealed with cling film and incubated at 4°C in complete darkness for 7 days. Following cold stratification, seeds were transferred aseptically using sterile forceps to sterile glass Petri plates containing 1X Johnson's medium prepared according to (Johnson et al. 1957). The plates were sealed with cling film and incubated in a CONVIRON plant growth chamber maintained at conditions as described above (*plant growth conditions section*). Untreated stored summer seeds maintained under identical conditions without stratification served as controls. Three independently prepared Petri plates (experimental replicates), each containing approximately 150 seeds, were used for each treatment. Germination was monitored until day 4, and seeds showing visible radicle emergence were recorded as germinated. Germination percentage was calculated as follows:

$$\text{Germination (\%)} = \left(\frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \right) \times 100$$

Germination experiment with Gibberellic acid (GA₃)

Summer-collected seed lots exhibiting reduced germination were selected for GA₃ treatment assays. Seeds were manually dehusked, subjected to default dry heat treatment (45°C for 24 h), and surface sterilized as described above. Sterilized seeds were placed in sterile glass Petri plates lined with autoclaved tissue paper pads moistened with 20 mL of freshly prepared GA₃ solution (≥99% purity; HIMEDIA Cat. No. RM9157) at concentrations of 0 ppm (Autoclaved ultrapure water control), 20, 50, 100, 200, and 500 ppm. Thirty-five seeds were used per plate, across three experimental replicates. Plates were incubated in the dark at 27°C for 48 h in the plant growth chamber to facilitate hormone imbibition. Unlike the water immersion treatment described above, in which seeds were fully submerged in water, seeds here were imbibed on moistened tissue paper without complete submersion. Following imbibition, seeds were transferred directly to fresh sterile Petri plates lined with tissue paper pads moistened with 20 mL autoclaved ultrapure water and incubated for an additional 5 days. Germination was monitored on day 5. Radicle and plumule lengths were quantified using ImageJ software. Germination (%) and seedling vigour index were calculated as follows:

$$\text{Germination (\%)} = \left(\frac{\text{Number of seeds germinated}}{\text{Total number of seeds sown}} \right) \times 100$$

$$\text{Seedling Vigour Index} = \text{Germination (\%)} \times \text{Mean Total Seedling Length (cm)}$$

Germination experiment with Polyethylene Glycol (PEG)

Polyethylene glycol (PEG 6000; HIMEDIA, Cat. No. GRM401) solutions were prepared at concentrations of 0% (control; autoclaved ultrapure water), 5, 10, 20 and 50% (w/v) following (Faijunnahar et al. 2017) method. Summer-collected seeds were subjected to default heat treatment (45°C for 24 h) and surface sterilized. Surface-sterilized seeds were immersed in respective PEG solutions for 24 h at room temperature ($25 \pm 2^\circ\text{C}$) under sterile conditions to induce osmopriming. Note: As with the GA₃ assay, the 0% PEG (Autoclaved ultrapure water) control involved seed imbibition on moistened tissue paper rather than full submersion, and is therefore not directly comparable to the fully submerged water immersion treatment described above.

After incubation, PEG-treated seeds were directly transferred to sterile glass Petri plates lined with autoclaved tissue paper moistened with 20 mL of autoclaved ultrapure water. Thirty-five seeds were used per plate, with three experimental replicates per treatment. Plates were incubated in the growth chamber, and germination percentage, radicle length, plumule length, and seedling vigour index were determined on day 5 as described above.

Statistical analysis

All experiments were performed using three independently prepared Petri plates (experimental replicates). Data are presented as mean $\pm$ standard error of the mean (SEM). Mean, standard deviation, and SEM were calculated using Microsoft Excel. For experiments comparing two groups (seasonal comparisons, storage/heat treatment comparisons, water immersion, and cold stratification assays), statistical significance was assessed using an unpaired two-tailed Student's t-test. For experiments involving more than two groups (GA₃ and PEG concentration series), one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test was performed. All statistical analyses and graphs were generated using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered at $P < 0.05$.

Results

Seed viability assessment

Microscopic observation of dehusked seeds revealed clear variation in seed colour and internal morphology among the seed fractions (Fig. 3). Dark brown to black-coloured seeds generally showed well-developed internal tissues and better germination, whereas pale, grey-coloured seeds exhibited poor germination in preliminary observations. Seed viability following floating selection was assessed using the BTC assay. Freshly harvested seeds that settled at the bottom of ultrapure water were selected for testing. More than 70% of the tested seeds exhibited intense purple-blue staining localized in embryo and endosperm tissues (Fig. 4), indicating active dehydrogenase enzyme activity and metabolically viable seed tissues. Viable seed batches identified through the BTC assay were subsequently used for the germination experiments.

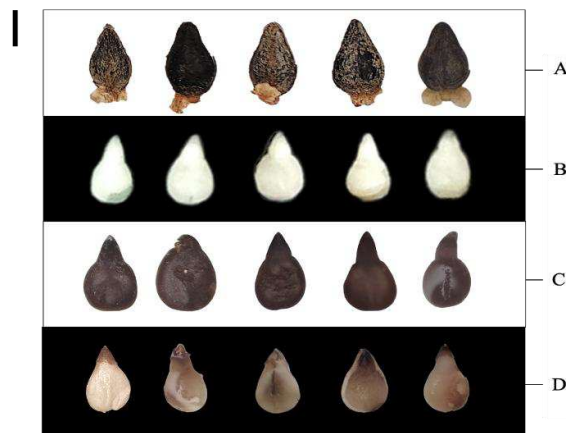


Fig. 4. Seed viability screening of *P. hysterophorus* using the Blue Tetrazolium Chloride (BTC) assay

Representative stereomicroscopic images illustrating the sequential preparation and viability assessment of *P. hysterophorus* seeds using the BTC assay. A) Intact mature cypselae with the protective outer wall (Pericarp). B) Dehusked seeds showing the exposed white endosperm and embryo tissues prior to staining. C) Metabolically active, viable seeds displaying distinct dark purple-blue staining localized in embryo and endosperm tissues following 24 h dark incubation in 1% BTC solution at 25 ± 1 °C, reflecting active respiratory dehydrogenase activity. D) Inadequately stained or pale seeds indicating non-viable or immature seed fractions. (Scale bar = 1 mm; $n=50$ seeds per batch across independent experimental replicates).

Seasonal variation in germination behaviour of *P. hysterophorus*

Seed collection season was associated with marked differences in germination performance. Freshly harvested, baseline heat-treated winter-collected seeds (45°C for 24 h) exhibited a high germination rate of $89.3 \pm 1.5\%$ on 1X Johnson's medium, accompanied by rapid and relatively uniform seedling emergence within 4 days. In contrast, freshly harvested summer-collected seeds subjected to identical baseline heat treatment displayed significantly lower germination ($23.0 \pm 1.2\%$) characterized by delayed and non-uniform emergence (Fig. 5; Table-1, Supplementary File).

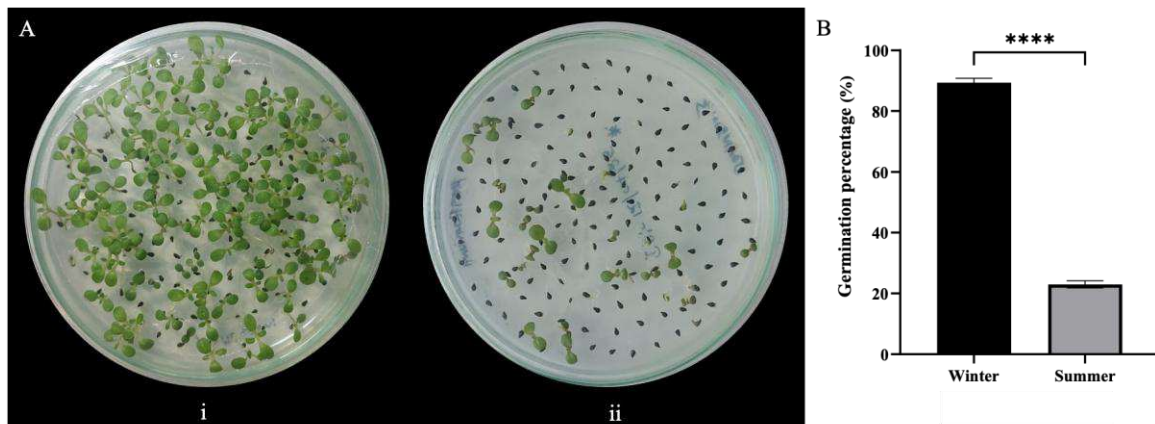


Fig. 5. Seasonal variation in germination behaviour of *P. hysterophorus* seeds

A) 45°C-24 h heat-treated, surface-sterilized seeds were germinated on 1X Johnson's medium under controlled growth chamber conditions. Germination was monitored, and representative images were captured at the end of the incubation period (Day-4). (i) Winter- collected seeds (without storage) exhibited higher germination: ~89%. (ii) Summer-collected seeds (without storage) exhibited ~23% germination. Images are representative of three independent experimental replicates. $n = \sim 150$ seeds per plate. B) Quantification of germination percentage. Bars represent mean $\pm$ SEM ($n = 3$ independent experimental replicates). **** Indicates $p < 0.0001$.

Storage-associated reduction in germination of *P. hysterophorus*

Storage at room temperature ($25 \pm 2^\circ\text{C}$) for 3–4 months markedly reduced germination in both seasonal seed lots. When tested without baseline heat treatment, stored winter-collected seeds showed very low germination, with a mean germination of 5%, while stored summer-collected seeds showed a higher but still limited mean germination of 13% (Fig. 6A; Table-2, Supplementary File).

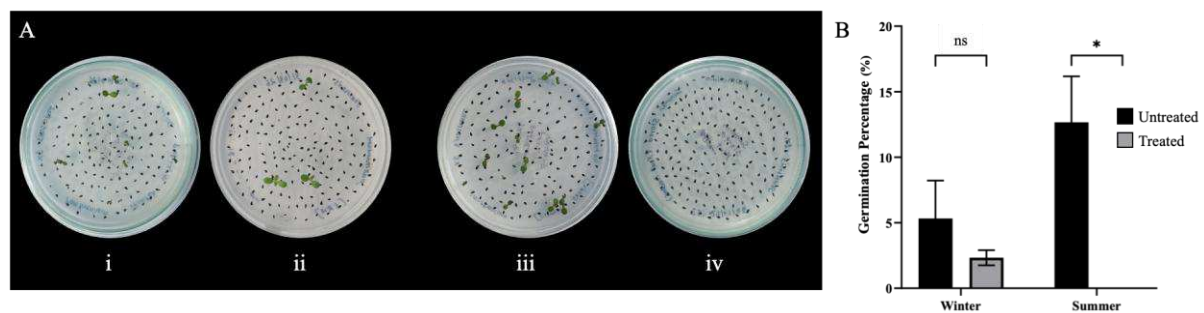


Fig. 6. Storage-induced dormancy and effect of heat treatment on germination of *P. hysterophorus* seeds

Seeds collected during winter and summer were stored at room temperature ($25 \pm 2^\circ\text{C}$) for 3–4 months and tested for germination with or without dry heat treatment (45°C for 24 h). Germination was recorded on day 4 after sowing. A) Representative images showing germination of stored seed lots under the different treatment conditions. (i) Winter-collected-stored seeds without heat treatment: ~ 5% germination. (ii) Winter-collected-stored seeds with heat treatment: ~ 2% germination. (iii) Summer-collected-stored seeds without heat treatment: ~13% germination. (iv) Summer-collected-stored seeds with heat treatment: ~0% germination. Images are

representative of three independent experimental replicates. $n = \sim 150$ seeds per plate. B) Quantitative comparison of germination percentage under the respective treatments. Bars represent mean $\pm$ SEM ($n = 3$ independent experimental replicates). * Indicates $P < 0.01$; ns indicates no statistically significant difference.

To determine whether the standard heat treatment could improve germination after storage, stored seeds from both seasonal lots were subjected to dry heat treatment at 45°C for 24 h before germination testing. Heat treatment did not improve germination in either seed lot. Winter-collected seeds showed a mean germination of approximately 2% after heat treatment compared with 5% without heat treatment. In summer-collected seeds, no germination was observed following heat treatment, compared with a mean germination of 13% in the untreated stored seeds (Fig. 6A; Table-2, Supplementary File). Thus, under the conditions tested, dry heat treatment alone was not effective in improving germination of stored *P. hysterophorus* seeds.

Alleviation of dormancy in summer seeds by water immersion and cold stratification

To improve germination of summer-collected seeds, two physical treatments were evaluated:

Heat treatment combined with water immersion: Seeds were pre-heated at 45°C for 24 h, surface sterilized, and subsequently immersed in autoclaved ultrapure water for 24 h at room temperature ($25 \pm 2^{\circ}\text{C}$). Heat treatment followed by 24 h water immersion markedly increased germination to $92.3 \pm 1.8\%$, compared with $23.0 \pm 1.2\%$ in the non-immersed heat-treated control (Fig. 7; Table-3, Supplementary File).

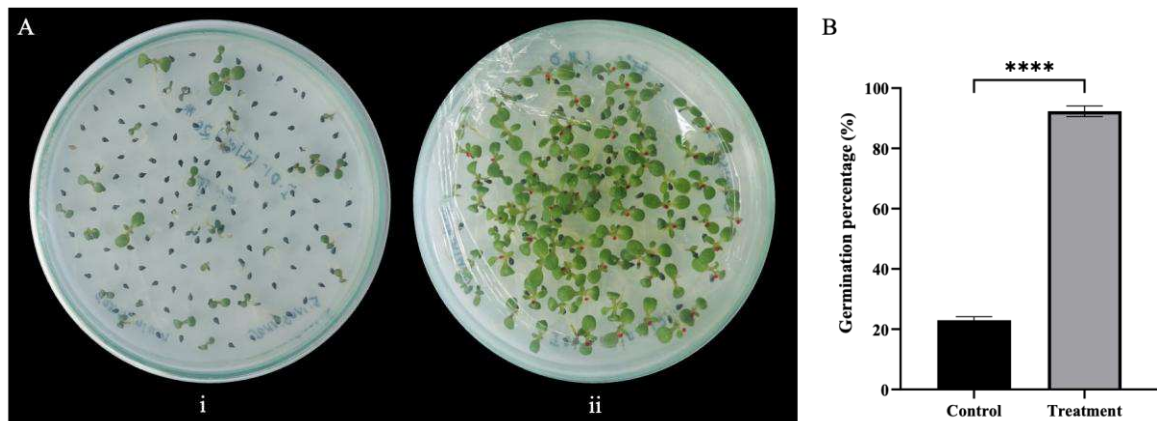


Fig. 7. Effect of water immersion following heat treatment on germination of *P. hysterophorus* seeds

A) Summer-collected seeds were subjected to heat treatment at 45°C for 24 h and subsequently evaluated with or without 24 h water immersion at room temperature ($25 \pm 2^{\circ}\text{C}$). (i) Representative images showing germination of heat-treated summer-collected seeds without water immersion (Control: $\sim 23\%$ germination). (ii) Seeds subjected to 24 h water immersion following heat treatment (water immersion: $\sim 92\%$ germination). Images are representative of three independent experimental replicates. $n = \sim 150$ seeds per plate. B) Quantitative comparison of germination percentage between the control and water-immersed seeds. Bars represent mean $\pm$ SEM ($n = 3$ independent experimental replicates). **** Indicates $p < 0.0001$.

Cold stratification: Surface-sterilized seeds were stratified at 4°C in complete darkness for 7 days before transfer to 1X Johnson’s medium. Cold stratification was performed independently without the 45°C heat treatment step and resulted in a higher germination of $94 \pm 1.0\%$ than the untreated stored seed-control (Fig. 8; Table-4, Supplementary File).

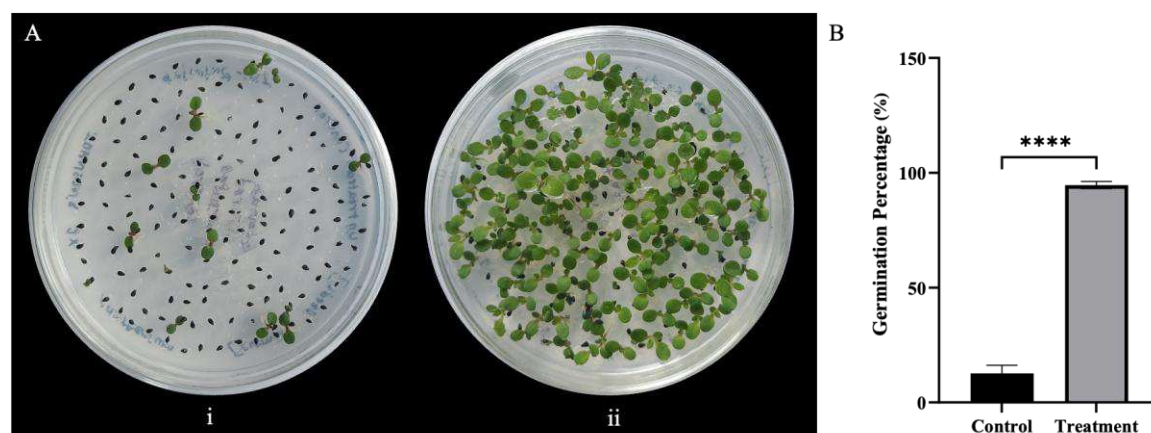


Fig. 8. Effect of cold stratification on germination of *P. hysterophorus* seeds

Summer-collected seeds were sterilized and subjected to cold stratified at 4°C in the dark for 7 days before germination testing. Germination was recorded on day 4 after sowing. A) Representative images showing germination of (i) summer-collected seeds without cold stratification (Control: ~13% germination) and (ii) after 7 days of cold stratification (Cold stratification: ~94% germination). Images are representative of three independent experimental replicates. $n = \sim 150$ seeds per plate. B) Quantitative comparison of germination percentage between non-stratified and cold-stratified seeds. Bars represent mean $\pm$ SEM ($n = 3$ independent experimental replicates). **** indicates $P < 0.0001$.

Chemical dormancy release using Gibberellic Acid (GA₃)

Treating pre-heated summer-collected seeds with GA₃ altered germination across the tested concentrations. Germination reached its highest and statistically comparable at 20, 200, and 500 ppm ($85.0 \pm 1.73\%$, $93.0 \pm 0.88\%$, and $85.0 \pm 1.7\%$ respectively), compared to $63.0 \pm 1.73\%$ in the water control (0 ppm). This water control involved tissue-paper imbibition rather than full submersion and is therefore not directly comparable to the fully submerged water immersion treatment reported above. Intermediate concentrations of 50 and 100 ppm resulted in moderate germination of 73.0 and 76.0%, respectively, indicating a non-linear response to GA₃ concentration (Fig. 9; Table-5, Supplementary File).

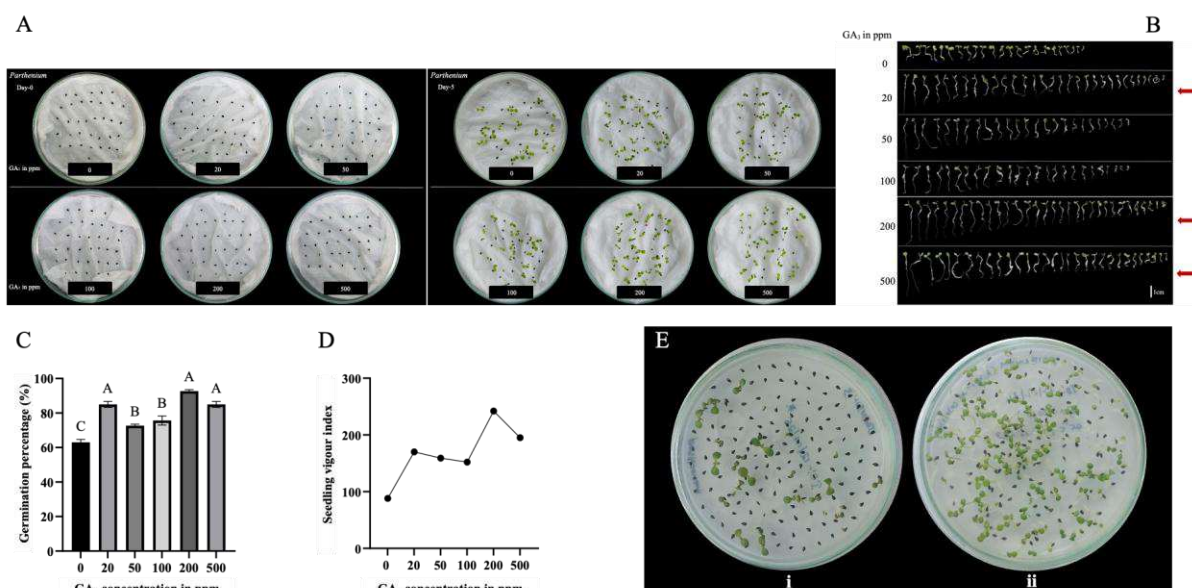


Fig. 9. Effect of GA₃ treatment on germination, seedling growth and vigour of heat-treated summer collected *P. hysterophorus* seeds

Heat treated summer-collected seeds were treated with different concentrations of GA₃ and evaluated for germination and seedling development. A) Representative images showing seed germination before treatment (Day-0) and on day 5 after transfer of GA₃-treated seeds to autoclaved ultrapure water. Seeds treated with 20, 200 & 500 ppm GA₃ showed the highest germination response. Images are representative of three independent experimental replicates. n= ~35 seeds per plate. B) Representative seedling morphology following treatment with different concentrations of GA₃. Concentrations are indicated in the respective panels. Red arrows indicate the GA₃ treatment showing the highest germination percentage. C) Effect of GA₃ concentration (0–500 ppm) on germination percentage. Maximum germination was observed at 20, 200 and 500 ppm GA₃. Values represent mean $\pm$ SEM (n = 3 independent experimental replicates). Different letters indicate statistically significant differences among treatments, whereas the same letters indicate no significant difference, based on one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$). D) Seedling vigour index (SVI) of GA₃-treated seeds. SVI was calculated as germination percentage $\times$ mean seedling length. E) Establishment of GA₃-treated seedlings on 1X Johnson's medium following treatment with 20 ppm GA₃, washing with autoclaved ultrapure water, and transfer to the solid medium, (i) Control: ~23% germination (ii) Treatment with 500ppm GA₃: ~82% germination.

Phenotypic evaluation showed that GA₃ treatment was associated with increased radicle elongation and seedling vigour. Seedlings treated with 200 ppm and 500 ppm GA₃ developed the longest radicles, with 53% of seedlings in the 200 ppm treatment forming robust roots exceeding 1.94 cm (Table-6, Supplementary File). The seedling vigour index peaked at 200 ppm (241.8 ± 0.15) compared to 88.2 ± 0.09 in the control (Fig. 9B, C; Table-7, Supplementary File). Although 200 ppm yielded the highest total vigour (Fig. 9D), the 20 ppm treatment also produced strong germination (85%) and healthy seedling morphology without excessive root elongation, making 20 ppm a practical choice for downstream assays where lower hormonal influence is desired.

Suitability of GA₃-treated seeds for subsequent seedling establishment

To further evaluate the suitability of GA₃-treated seeds for subsequent seedling establishment on solid medium, an additional assay was performed using 20 ppm GA₃. Seeds were treated with GA₃ for 24 h in the dark at room temperature, washed 5–6 times with autoclaved ultrapure water to remove residual GA₃, and subsequently transferred to 1X Johnson's medium. The GA₃-treated seeds germinated and produced visible seedling growth by day 4 after transfer to the medium (Fig. 9E). This approach allowed seedlings to develop directly on solid medium and facilitated clearer observation of root and shoot development.

Osmopriming with Polyethylene Glycol (PEG 6000)

Pre-heated seeds treated with PEG 6000 for 24 h showed a concentration-dependent variation in germination. Germination increased from $37 \pm 4.9\%$ in the control (0 % PEG) to a maximum of $67 \pm 3.7\%$ at 20% PEG. Lower concentrations (5% and 10% PEG) produced moderate increases ($44 \pm 3.4\%$ and $52 \pm 2.4\%$, respectively), whereas a high concentration (50% PEG) suppressed germination back to control levels ($38 \pm 6.7\%$) due to severe osmotic stress (Fig. 10A; Table-8, Supplementary File). Phenotypic evaluation on day 5 after sowing demonstrated that PEG treatment markedly influenced seedling length distribution and vigour. Seedlings were categorized based on total seedling length into long (4.4–8.1 cm), medium (2.7–4.3 cm), and short (0.8–2.6 cm) classes.

While the control group produced mostly short seedlings (≤ 2.6 cm, 75 %) with no long seedlings observed, treatment with 20% PEG generated 42% long seedlings and 37% medium seedlings, leading to the highest seedling vigour index (278.9 ± 0.32) among all PEG treatments (Fig. 10B; Table-9 & 10, Supplementary File).

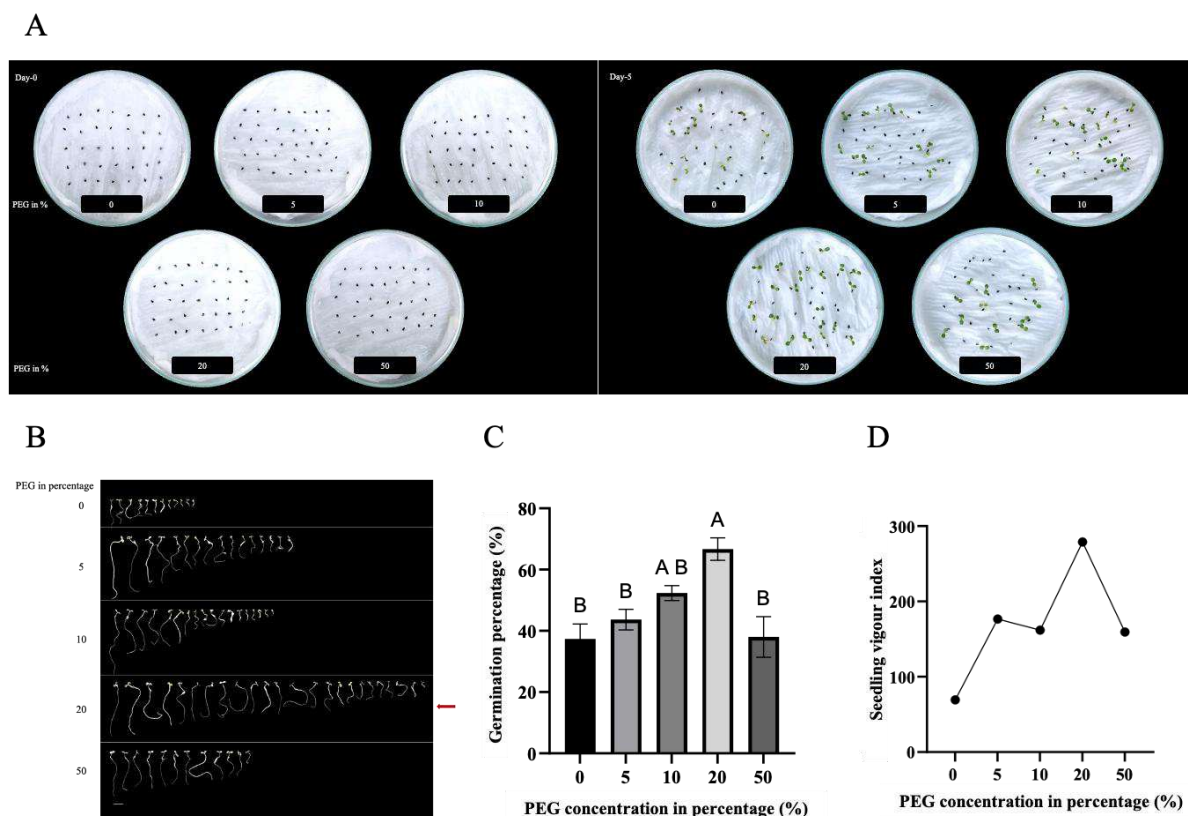


Fig. 10. Effect of polyethylene glycol (PEG) priming on seed germination, seedling growth and vigour of *P. hysterophorus*

A) Germination of *P. hysterophorus* seeds treated with different concentrations of PEG (0–50%). Left: seeds immediately after PEG treatment; Right: germination on day 5 after transferring PEG-treated seeds to ultrapure water. Seeds treated with 20% PEG exhibited the highest germination percentage. Images are representative of three independent experimental replicates ($n = \sim 35$ seeds per plate). B) Seedling morphology of *P. hysterophorus*, following treatment with different PEG concentrations. PEG concentration is indicated on the left, and arrows indicate the concentration associated with the highest germination percentage. The red arrow indicates the PEG treatment associated with the highest germination percentage. Differences in seedling growth and radicle elongation demonstrate the concentration-dependent effects of PEG. Scale bar = 1 cm. C) Effect of PEG concentration (0–50%) on seed germination. Maximum germination was recorded at 20% PEG. Values represent mean $\pm$ SEM ($n = 3$ independent experimental replicates). Different letters indicate significant differences among treatments, whereas the same letters indicate no significant difference, as determined by one-way ANOVA followed by Tukey's multiple comparison test. D) Seedling vigour index (SVI) of heat-treated *P. hysterophorus* seeds in response to different PEG concentrations. SVI was calculated as germination percentage $\times$ mean seedling length.

Discussion

Establishing reliable and synchronized seed germination protocols is a fundamental prerequisite for plant biology research, particularly when utilizing stress-adapted invasive species as experimental model systems. While *P. hysterophorus* possesses remarkable adaptive plasticity to abiotic stresses such as drought, salinity, and extreme temperatures, its utility in laboratory-based physiological and molecular investigations has been hampered by strong, unpredictable seed dormancy. The present study systematically evaluated the season of seed collection and post-harvest storage on germination behaviour, followed by comparison of physical, hormonal, and osmotic approaches for alleviating dormancy and improving seedling establishment. The results demonstrate that germination behaviour was strongly dependent on the physiological history of the seed lot, with season of collection and storage duration influencing the response to subsequent dormancy-breaking treatments. Among the approaches evaluated, heat treatment followed by water immersion and direct cold stratification produced the most pronounced improvements in germination, whereas GA₃ provided a concentration-dependent enhancement and PEG 6000 produced a moderate improvement in germination and seedling vigour.

Seasonal variation in natural seed dormancy

Our findings demonstrate that season of seed collection was associated with substantial differences in germination behaviour of *P. hysterophorus*. Freshly harvested winter-collected seeds displayed high germination efficiency ($\sim 89\%$) after the standard heat treatment, with rapid, synchronized seedling emergence within 4 days. In contrast, freshly harvested summer-collected seeds exhibited pronounced primary dormancy, yielding only $\sim 23\%$ germination under the same conditions. This seasonal difference is consistent with previous reports indicating that the germination behaviour of *P. hysterophorus* can vary with the environmental conditions prevailing during seed

maturation and collection (Javaid et al. 2010; Shrestha et al. 2024). Such seasonal variation may reflect differences in the physiological status of seeds formed under contrasting environmental conditions.

The present study does not directly establish the physiological or hormonal basis of this seasonal difference; however, the contrasting germination responses suggest that environmental conditions associated with seed maturation may influence the depth or expression of dormancy.

From a practical laboratory perspective, these results indicate a crucial operational window: freshly collected winter seeds can be utilized directly for experimental studies without pre-treatment. However, summer-collected seed lots require additional dormancy-breaking treatments before they can be used for controlled experiments.

Ineffectiveness of heat treatment on storage-induced secondary dormancy

Prolonged storage at room temperature (3–4 months) markedly reduced germination in both winter- and summer-collected seed lots. When tested without additional heat treatment, stored winter-collected seeds showed approximately 5% germination, whereas stored summer-collected seeds showed approximately 13% germination. Importantly, the additional 45°C dry heat treatment did not restore germination in either stored seed lot. Germination remained very low in stored winter seeds and was completely suppressed in the stored summer seed lot following heat treatment under the conditions tested.

These observations indicate that the effectiveness of dry heat treatment is dependent on the physiological status of the seed lot and should not be considered a universally effective dormancy-breaking treatment for stored seeds. Dry heat has previously been reported to enhance germination in *P. hysterophorus* (Ahlawat et al. 1979), and its use as a pre-treatment in the present study was therefore retained as the baseline protocol. However, our results demonstrate that the same treatment was insufficient to overcome the pronounced dormancy associated with 3–4 months of room-temperature storage.

The reduction in germination following storage may reflect the development of secondary dormancy, loss of viability, or a combination of both; these possibilities were not distinguished in the present study. Nevertheless, the marked reduction in germination following storage highlights storage history as an important determinant of subsequent germination performance and should be considered when establishing reproducible *P. hysterophorus* experimental systems.

Mechanism of dormancy release by physical interventions: Water immersion vs. Cold stratification

To bypass intrinsic physiological barriers, two physical strategies demonstrated high efficiency in achieving synchronized germination:

Two-step physical approach (Heat + Water Immersion): Soaking baseline heat-treated seeds in autoclaved water for 24 h dramatically boosted germination to ~92%. The physiological rationale for this success lies in its two-step synergistic action: dry heat treatment may facilitate subsequent water uptake, while water immersion provides rapid hydration and reactivates metabolic processes required for germination. Thus, the

substantial increase in germination following water immersion indicates that hydration was an important component of dormancy alleviation in the summer seed lot tested.

One-step physiological approach (Cold Stratification): Subjecting dormant summer seeds directly to cold stratification (4°C for 7 days in complete darkness) yielded ~94% germination with exceptional seedling health and growth synchrony upon transfer to growth conditions. Remarkably, cold stratification achieved high germination without prior heat treatment. Cold stratification mimics winter environmental signals that induce endogenous hormonal shifts specifically upregulating gibberellin biosynthesis while downregulating abscisic acid (ABA) sensitivity (Tian et al. 2026). Such hormonal changes have been associated with dormancy release during cold stratification in various plant species; however, the underlying hormonal changes were not directly measured in the present study. This demonstrates that cold moist conditioning independently activates dormancy release pathways, offering a streamlined, single-step alternative that avoids multi-temperature thermal pre-processing. Notably, the degree of water contact also appeared to influence dormancy alleviation. Full submersion of seeds in water for 24 h (Fig. 7) produced substantially higher germination (~92%) than the tissue-paper-based imbibition used as the vehicle control in the GA₃ and PEG assays (63% and 37%, respectively), despite comparable or longer imbibition durations in the latter. This suggests that complete submersion may promote more thorough hydration indicating that the method of water delivery, not only its duration, is an important variable in dormancy alleviation protocols for *P. hysterophorus*.

Hormonal regulation of dormancy release by Gibberellic Acid (GA₃)

Exogenous GA₃ application successfully restored germination in dormant seed lots in a concentration-dependent manner, consistent with a role for GA/ABA hormonal dynamics in regulating physiological dormancy in *P. hysterophorus*. Interestingly, germination responded in a non-linear, multiphasic pattern: high and statistically equivalent germination was observed at 20, 200, and 500 ppm (~85–93%), with an intermediate decline at 50–100 ppm (~73–76%). This non-linear response is characteristic of hormetic signaling dynamics commonly observed in plant hormone perception (Ma et al. 2018; Agathokleous et al. 2019). As endogenous GA and ABA levels were not directly quantified in this study, the receptor-level mechanisms proposed below remain a plausible interpretation rather than a confirmed mode of action. Lower concentrations (20 ppm) provide sufficient hormonal stimulus to activate reserve-mobilizing hydrolases (such as α -amylase) and radicle emergence without saturating receptors. Intermediate concentrations may trigger transient negative feedback regulation or receptor desensitization, whereas higher concentrations (200 ppm) override receptor thresholds to maximum physiological output. From an experimental standpoint, while 200 ppm yielded the highest overall seedling vigour index (241.8), the 20 ppm GA₃ treatment is recommended as the practical optimum for downstream molecular and physiological studies. The 20 ppm concentration achieves rapid, uniform germination (~85%) while maintaining balanced seedling morphology without the excessive radicle hyper-elongation or carry-over hormonal stress induced by higher concentrations.

Osmopriming dynamics with Polyethylene Glycol (PEG 6000)

Osmopriming using PEG 6000 demonstrated a distinct osmotic threshold for germination enhancement. While mild concentrations (5% and 10%) showed only moderate numerical increases, 20% PEG emerged as the

statistically significant threshold, increasing germination from ~37% in controls to ~67% with the highest seedling vigour index (278.9). Controlled osmotic stress at 20% PEG creates a low water potential environment that allows pre-germinative metabolic processes and tissue repair processes during imbibition, while physically holding back radicle protrusion until seeds are transferred to water. Upon transfer, primed seeds exhibit rapid, synchronized hydration and uniform emergence. However, raising the concentration to 50% PEG imposed severe osmotic restriction, suppressing overall germination (~38%). Interestingly, surviving seedlings under 50% PEG developed relatively long radicles, suggesting that severe osmotic stress selectively promotes root elongation in desiccation-tolerant individuals. Thus, 20% PEG serves as an effective osmopriming agent for establishing synchronized plant populations on a fixed experimental timeline.

Practical implications for stress resilience research and weed control

The standardized protocols validated in this study hold valuable practical applications for both fundamental plant biology and weed management research:

Enabling Stress Tolerance & Climate Resilience Studies: As a climate-resilient species capable of surviving drought, salinity, and heat, *P. hysterophorus* offers a rich genetic repository for stress-tolerance mechanisms. Our optimized protocols summarized in Fig. 11 overcome the long-standing bottleneck of asynchronous germination, enabling researchers to generate highly uniform seedling cohorts on defined days. This synchrony is essential for reproducible transcriptome, proteome, and biochemical profiling during early stress exposure.

Implications for Weed Control & Seedbank Ecology: Understanding that summer seeds possess deeper primary dormancy and that prolonged storage substantially reduces germination explains one aspect of the persistence and variable emergence of *P. hysterophorus* seed populations.

Conclusion and Recommended Protocol Framework

In summary, seed dormancy in *P. hysterophorus* is dictated by seasonal collection timing and storage history. Freshly collected winter seeds showed high germination following the standard 45°C heat treatment. For dormant summer seed lots or stored winter/summer seed batches, dry heat treatment (45°C for 24 h) alone is insufficient. To achieve reproducible, high-efficiency (~85–92%) synchronized germination, we recommend the following treatment options based on the seed lot and intended downstream application (Fig. 11):

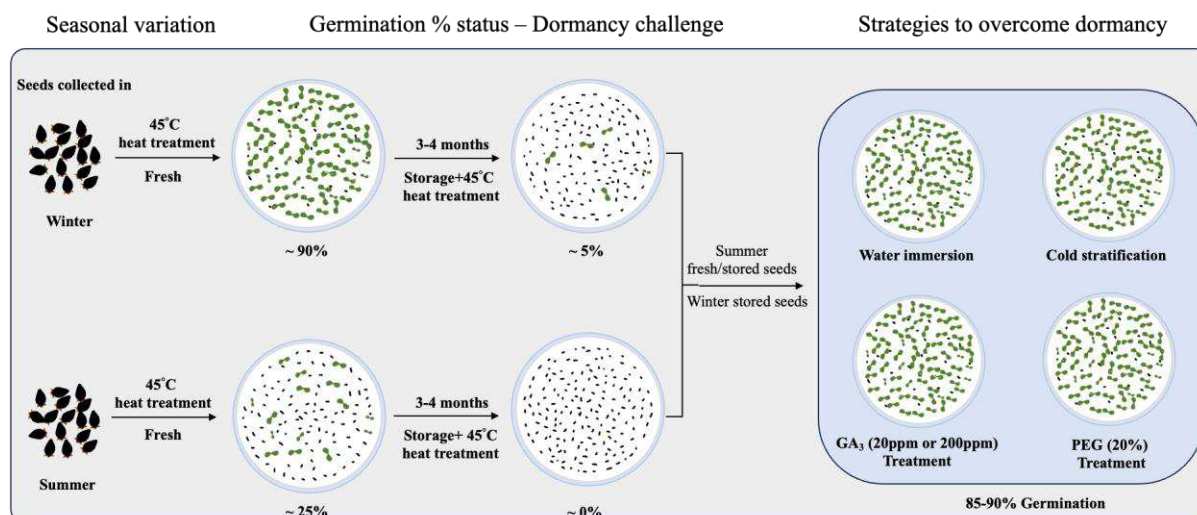


Fig. 11. Graphical overview of seasonal variation in seed germination, storage effects, and evaluation of different strategies for overcoming seed dormancy.

Option A (Physical Hydration): Baseline heat treatment (45°C for 24 h) followed by 24 h water immersion.

Option B (Hormonal/Physiological): Direct 7-day cold stratification at 4°C (without heat treatment) or baseline heat treatment followed by GA₃ treatment. Among the concentrations tested, 200 ppm produced the highest germination and seedling vigour, whereas 20 ppm provided a practical lower-concentration treatment with relatively balanced seedling growth.

Option C (Osmopriming): 20% PEG 6000 treatment may be considered when osmopriming is desired, as it produced the highest germination and seedling vigour among the PEG concentrations tested, although its germination-promoting effect was lower than that of the most effective physical and GA₃ treatments.

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Declarations

Conflict of Interest: The authors declare no competing interests.

Ethical Approval: Not applicable. This study did not involve human participants or animals.

Use of AI tools: The authors used AI tools (ChatGPT and Gemini) solely for improving English language clarity and grammar. The scientific design of experiments, data interpretation, and conclusions are entirely the responsibility of the authors.

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