Dormancy-breaking and germination requirements for seeds of the threatened Austral papaya (Carica chilensis)

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Article

Keywords: After-ripening, Atacama Desert, desiccation tolerance, Mediterranean, physiological dormancy

Posted Date: July 20th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3088216/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Scientific Reports on October 13th, 2023.
See the published version at https://doi.org/10.1038/s41598-023-44386-y.

Abstract

Seed dormancy is one of the most important adaptive mechanisms in plants, which optimizes germination, seedling emergence, and establishment so that these processes occur when environmental conditions are favorable for plant survival and growth. Endemic to rocky environments of the southern Atacama Desert, the Austral papaya (*Carica chilensis*) is the papaya species with the southernmost distribution of the Caricaceae and the species growing in the most extreme environmental conditions. This threatened plant has minimal natural regeneration, attributed to low germination rates, but no information regarding seed dormancy release exists. Here we investigated the dormancy-breaking requirements and germination of *C. chilensis* by assessing the combined effects of desiccation with nine pre-sowing treatments. We hypothesized that, as in other members of the family, *C. chilensis* seeds would possess physiological dormancy. Our results confirmed this hypothesis and revealed that ultra-drying (< 3% moisture content) and treating seeds with sulfuric acid, gibberellic acid, or potassium nitrate are the most effective methods for germinating *C. chilensis*. These treatments are thus recommended to propagate this threatened papaya species.

Introduction

Plants rely on seeds for reproduction, genetic continuity, and colonization [1]. However, the successful establishment of seedlings depends on the timing of seed germination; consequently, seeds must possess strategies to perceive their surroundings and trigger germination accordingly [2]. Seed dormancy is a plant strategy that optimizes germination, seedling emergence, and establishment so that these processes occur when environmental conditions are favorable for plant survival and growth [2–4]. It refers to a state of inhibited germination in mature seeds even under favorable environmental conditions for germination [5]. Seed dormancy can be particularly important for plants in harsh and highly unpredictable environments (e.g., in arid ecosystems), where windows for recruitment are few and sparse; hence, it is more prevalent in these environments than in those with more benign conditions [6].

Seed dormancy is classified into four general classes [5]: 1) morphological dormancy (MD), where a seed is immature when the fruit falls and requires a period of growth and embryo differentiation for germination to occur; 2) physical dormancy (PY), where the seed coat is impermeable and prevents water from entering the seed, thus requiring mechanical or chemical scarification for germination to occur; 3) physiological dormancy (PD), where a physiological inhibiting mechanism in the embryo results in low growth potential, which prevents the emergence of the radicle through covering layers, hence seeds require a specific set of conditions (i.e., often a combination of temperature, moisture, and light) to initiate germination and; 4) combinational dormancy (PY + PD). Most seeds have PD, and depending on the strength of the physiological inhibitory mechanism, they can exhibit one of three levels of PD: 1) non-deep (dormancy can be broken with chemical reagents, gibberellic acid (GA_3), warm or cold stratification, after-ripening in dry storage, and mechanical or chemical scarification), 2) intermediate (dormancy can be broken after a long period of cold stratification, and GA_3 may or may not break dormancy), or 3) deep

(dormancy can be broken after a long period of cold or warm stratification, and GA_3 does not break dormancy) [2, 3, 7–9].

Carica chilensis (Planch. ex A.DC.) Solms., commonly known as the Austral Papaya, is an endemic papaya species of Chile distributed along the southern limit of the Atacama Desert [10–12]. This species belongs to the Caricaceae, a predominantly tropical plant family, which includes the common papaya (*Carica papaya*). The family has 35 species distributed across six genera [13], most of which have seed dormancy [3]. Although the Caricaceae is an economically important plant family, there are very few published reports of the germination requirements and desiccation tolerance among its species, and most studies have focused on *C. papaya*. Nonetheless, the available information shows that seeds within this plant family exhibit various types of dormancy and that the low seed germination rate is caused by inhibitory substances found in both the sarcotesta (the gel-like coating around the seeds) and the sclerotesta (the tough central layer of the testa) [8, 9, 14]. However, most species in the family possess seeds with PD [3].

This study investigates the seed dormancy-breaking requirements of the Austral papaya, the southernmost species of the Caricaceae. Knowledge of the mechanisms and factors regulating seed germination and dormancy in *C. chilensis* is crucial for several reasons. First, due to the papain content in its fruit, this wild papaya is a plant of interest for cultivation [15]. Secondly, *C. chilensis* is classified as Vulnerable due to its limited presence in a few fragmented, low-density populations and to the decline of natural populations due to habitat destruction [11, 12, 15]; therefore, studies examining its propagation have been recommended as a conservation strategy [16]. Finally, the lack of knowledge of germination requirements for the majority of non-tree native species in Chile is considered one of the major bottlenecks for the fulfillment of the country's restoration commitments [17]; hence, there is an urgent need for studies assessing the dormancy-breaking requirements of these species. Considering that most Caricaceae exhibit PD dormancy, and the aridity of the environment in which *C. chilensis* grows, we hypothesized that its seeds have non-deep PD and that reducing moisture content would promote dormancy break.

Results

Most essays successfully released the dormancy of *C. chilensis* seeds; however, germination was generally low (Table 1). Germination rate varied across treatments ($\chi^2 = 243.6$, gl = 9, $P < 0.0001$; Fig. 1). Specifically, compared to the reference germination rate of control seeds, germination was 200% higher for seeds treated with sulfuric acid (H_2SO_4) and 176% higher for seeds immersed in solutions of either gibberellic acid (GA_3) or potassium nitrate (KNO_3) (Table 2). Similarly, compared to the germination probability of fresh seeds, the germination of ultra-dry seeds was 77% higher (Table 2). Results from the GLM analysis showed significant interactive effects between moisture content (MC) and pre-sowing treatments (Deviance = 29.04, df = 162, Pr(> Chi) < 0.001). As germination probability curves revealed (Fig. 2), ultra-dry seeds treated with H_2SO_4, GA_3, or KNO_3 had the highest germination.

Table 1
Germination percentage of *Carica chilensis* according to seed moisture content (MC) and pre-sowing treatment (mean ± standard error).

MC	Treatment	Germination%
Fresh	Control	5.0 ± 2.24
	Cold stratified	0.00
	GA_3	8.0 ± 3.27
	H_2O_2	2.0 ± 1.33
	H_2SO_4	17.0 ± 2.60
	Hydro-conditioning	9.0 ± 3.40
	KNO_3	13.0 ± 3.0
	Percussion	1.0 ± 1.00
	Temperature shock	10.0 ± 2.58
Ultra-dry	Control	4.0 ± 1.63
	Cold stratified	1.0 ± 1.00
	GA_3	37.0 ± 4.48
	H_2O_2	0.00
	H_2SO_4	40.0 ± 7.30
	Hydro-conditioning	3.0 ± 1.53
	KNO_3	34.0 ± 8.06
	Percussion	0.00
	Temperature shock	7.0 ± 3.00

Table 2
Results of the Cox proportional hazards model of *Carica chilensis* seed germination with different moisture content (MC) and treated with different pre-sowing treatments.

	Treatment	BETA (SE)	HR (95% CI)	P
MC	Fresh seeds (ref)	-	-	-
	Ultra-dry seeds	0.77 (0.15)	2.15 (1.59, 2.91)	< 0.001
	Control (ref)	-	-	-
Pre-sowing treatment	Cold stratified	-2.21 (1.05)	0.11 (0.01, 0.98)	0.05
	GA_3	1.76 (0.37)	5.81 (2.78, 12.14)	< 0.001
	H_2O_2	-1.52 (0.78)	0.22 (0.08, 0.64)	0.006
	H_2SO_4	2.00 (0.36)	7.37 (3.18, 17.07)	< 0.001
	Hydro-conditioning	0.29 (0.44)	1.34 (0.58, 3.11)	0.49
	KNO_3	1.76 (0.36)	5.83 (2.24, 15.18)	< 0.001
	Percussion	-2.21 (1.05)	0.11 (0.03, 0.48)	0.003
	Temperature shock	0.70 (0.41)	2.02 (0.91, 4.46)	0.08

Discussion

Seed dormancy is an adaptive trait that allows plants to delay germination until environmental conditions become favorable for seedling establishment and growth [1, 18]; in arid ecosystems, such conditions are generally associated with rainfall events, which are highly variable and largely unpredictable [19, 20]. In the plant family Caricaceae, seed dormancy -particularly in the form of PD- is a common characteristic [3]. Our findings reveal that over 95% of *C. chilensis* seeds are dormant at maturity and that this dormancy can be partially alleviated by reducing seed MC or treating the seeds H_2SO_4, GA_3, or KNO_3. These results suggest that, as predicted, seeds of the Austral papaya have non-deep PD.

Germination was 77% higher in ultra-dry than in fresh seeds. This result reveals that *C. chilensis* seeds can be classified as orthodox, as a reduction of 97% of their MC did not reduce survival. There are conflicting reports in the literature about the effect of reducing MC on seed viability and germination among Caricaceae. For example, Ellis and collaborators [21] found a loss of seed viability when the seed MC of *Carica papaya* was reduced to < 7%. However, in another experiment with the same species, a reduction of MC to 5% either improved germination or did not affect it, depending on the seed lot [22]. Similar to our results, ultra-drying promoted germination in *Vasconcellea quercifolia* [7]. Reducing seed MC can promote germination via several mechanisms. For example, it can alter seed water potential [23]. In this regard, seeds with lower MC have more negative matric water potentials than seeds with higher

MC; as a result, they absorb water more quickly [24]. In contrast, solution uptake by fresh or fully imbibed seeds is driven by differences in osmotic potential between the seed and the surrounding solution [25]. Decreasing MC can also alter the hormonal balance in seeds, reducing levels of abscisic acid (ABA) and increasing gibberellins (GA) [26–28]. These two hormones regulate seed germination in opposite manners: ABA promotes seed dormancy and inhibition of germination, whereas GA promotes germination [29].

A reduction in seed MC is likely to occur naturally in *C. chilensis* habitat because the species produces mature fruits between August and October, prior to the onset of summer. Since both temperature and the gradual loss of MC are integrated over time to alter the depth of the dormancy [2], the hot and dry conditions of the Mediterranean summer in central Chile likely contribute to the gradual release of dormancy release throughout the season. Consequently, seeds can germinate in the field at the onset of the winter rain pulses [30] and take advantage of the moister soils and warmer temperatures during the spring to promote seedling establishment. Nonetheless, given that germination patterns and seed dormancy can vary geographically [31] and that we collected seeds from a population located towards the southern end of *C. chilensis*' distribution in Chile, which has drier and warmer summers, it will be informative to confirm if seeds from other localities show similar behavior.

Dormancy was released by treating *C. chilensis* seeds with H_2SO_4, GA_3, or KNO_3. The seed coat of Caricaceae is multiplicative, consisting of a fleshy outer layer known as the sarcotesta, enclosing a hard, lignified portion of the testa [32]. Hence, scarification with H_2SO_4 can enhance water absorption and uptake, essential for initiating the germination process, by breaking down the seed coat [33]. Gibberellins are a family of plant hormones that control many aspects of plant growth, including germination [2]. GA_3 stimulates germination through several mechanisms that include the production of hydrolytic enzymes that weaken the seed coat, mobilization of nutrient reserves, and stimulation of plant embryo expansion and hypocotyl elongation [28, 34]. Numerous studies have documented enhanced germination rates in *Carica papaya* with the application of GA_3 [35]. Similarly, GA_3 promotes germination in a few species of *Vasconcellea* [7, 8]. However, apart from these findings, there is currently a lack of information regarding the effects of GA_3 in other Caricaceae species. Finally, KNO_3 can break seed dormancy and promote germination in at least two ways. First, it has hygroscopic properties; when seeds are imbibed in a KNO_3 solution, they absorb moisture from the surrounding environment, weakening the seed coat. Second, it inhibits ABA synthesis while stimulating the synthesis of GA, which promotes germination by enhancing the growth potential of the embryo and overcoming the mechanical barriers imposed by the testa [2].

One of the major impediments to the potential use of wild species germplasm for habitat restoration is the need for more knowledge about techniques for breaking seed dormancy and caring for germinating seeds [36]. Thus, improving our understanding of seed biology is crucial to restoring a broader and more representative range of species [37]. Chile is one of 115 countries that has subscribed to restoration commitments [38]. However, germination requirements and dormancy breaking of seeds of most non-tree species in Chile remain poorly known. This lack of knowledge hinders the propagation of threatened

plants and is recognized as a bottleneck for fulfilling Chile's restoration commitments [17]. This study contributes to filling this gap with the aim of promoting more extensive use of species diversity in ecological restoration and helping the conservation of endangered plant species.

Methods

Study species

Carica chilensis is an endemic shrub of northern Chile distributed along the southern limit of the Atacama Desert, between the Atacama (28°39'S; 71°42'W) and Valparaiso (33°09'S; 71°42'W) Regions. It is classified as threatened, and due to its slow growth and low germination rates, *C. chilensis* experiences minimal natural regeneration. Like most Caricaeae [13], this species is polygamous with male, female, and hermaphrodite plants. It produces mature fruits from July to October, with peak fruiting in September. Each fruit contains an average of six seeds enveloped by a gelatinous layer. As the southernmost species in the family, *C. chilensis* is subject to extreme environmental conditions, such as drought and saline soils, across its distribution range.

Fruit Collection site and procedure

We collected mature *C. chilensis* fruits from 40 individuals in the Pupío basin in North Central Chile (32°07S, 71°26W, 190 masl) between July and September 2021. The area has a Mediterranean climate with warm, dry summers and cool, wet winters [39]. The mean annual temperature is ~ 22°C; July is the coldest month (~ 9 – 8°C, mean coldest temperature), and February is the warmest (~ 22.7°C, mean warmest temperature). Mean annual precipitation is 227 mm, 86% of which falls from May to August (data from Los Vilos Weather Station, Dirección General de Aguas, 1982–2019).

We collected between five and eight fruits per plant and placed them in paper bags in a cooler until they were processed a maximum of two days later in the laboratory at Universidad de La Serena. We manually removed the seeds from the fruits and cleaned them with a toothbrush under running water to remove the sarcotesta. Then, we pooled the seeds together for the experiments.

Dormancy-breaking requirements

We determined the dormancy-breaking requirements of *C. chilensis* seeds by examining the combined effects of moisture content (MC) and pre-sowing treatments. Specifically, we assessed germination of fresh and ultra-dry seeds in each of nine pre-sowing treatments: 1) Control (no pre-sowing treatment); 2) seeds soaked in a 400 ppm solution of gibberellic acid (GA_3) for 24 h at room temperature; 3) seeds soaked in a 1M solution of potassium nitrate (KNO_3) for 30 min; 4) seeds soaked in distilled water changed daily for seven days at room temperature (hydro conditioning); 5) seeds soaked first in distilled water for 24 h and then in hot water (35ºC) for four h (Temperature shock); 6) seeds shaken in a glass jar for 10 min (percussion), 7) seeds cold (moist) stratified, kept at 5ºC for seven days; 8) seeds soaked in a 100% solution of hydrogen peroxide (H_2O_2) for 20 min and; 9) seeds soaked in a 10% solution of sulfuric

acid (H_2SO_4) for 30 min. To establish the MC treatment, we first assessed initial seed MC by weighing 100 seeds before and after drying at 130°C for three h [40]. Seeds for the ultra-dry treatment were then placed in Eppendorf tubes with blue silica gel and dried to < 3% of their initial MC.

We replicated each treatment combination in 10 Petri dishes and sowed each dish with ten seeds in sterilized sand (60 g), which was hydrated to carrying capacity (*ca.* 12 ml of water). Petri dishes were incubated in germination chambers with 16 h of light at 25°C, and germination was monitored periodically for 94 days. We considered a seed germinated when the radicle emerged (> 2mm).

Statistical Analyses

We first assessed the independent effects of MC and pre-sowing treatments on *C. chilensis* seed germination using a Cox Proportional hazards model ('survival' package [41]; nested within each petri dish to account for the non-independence of seeds. In these models, the dependent variable is the hazard function, which describes how the "risk" of a seed germinating (i.e., the hazard) changes over time with respect to a reference germination rate. We used the germination rates of fresh and control seeds as references because this is how seeds are naturally found. We then examined the combined effects of MC and pre-sowing treatments with a Generalized Linear Model in which the independent variables were MC and pre-sowing treatment, and the dependent variable was the number of germinated seeds per Petri dish (GLM, family: Poisson, link: log). All statistical analyses were performed using the R statistical environment [42].

Relevant legislations, permitting and consent

The collection of plant material was carried out in accordance with relevant institutional, national, and international guidelines and legislation, including the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. Seeds were obtained from plants on private lands, and permission was obtained from the landowners to access the areas and collect the seeds. Given that *C. chilensis* is the sole wild Caricacea species in Chile and easily identifiable in the field, there was no need to collect voucher specimens.

Declarations

Acknowledgments

We express our gratitude to Cristian Delpiano and José Reyes for assisting us in collecting seeds in the field. Likewise, we would like to acknowledge Enrique Ostria and Máximo Gonzalez for providing us with germination chambers for the experiments.

Contributions

A.P.L. designed experiments, analyzed the results, and wrote the first draft of the manuscript; G.C. set up the experiment and collected the data. P.G-G and D.E.C. provided a general overview, assisted with data

analysis, and contributed to preparing the final version of the manuscript.

Data availability

The datasets used in the current study are available in the Excel file included in the supplementary material section.

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Figures

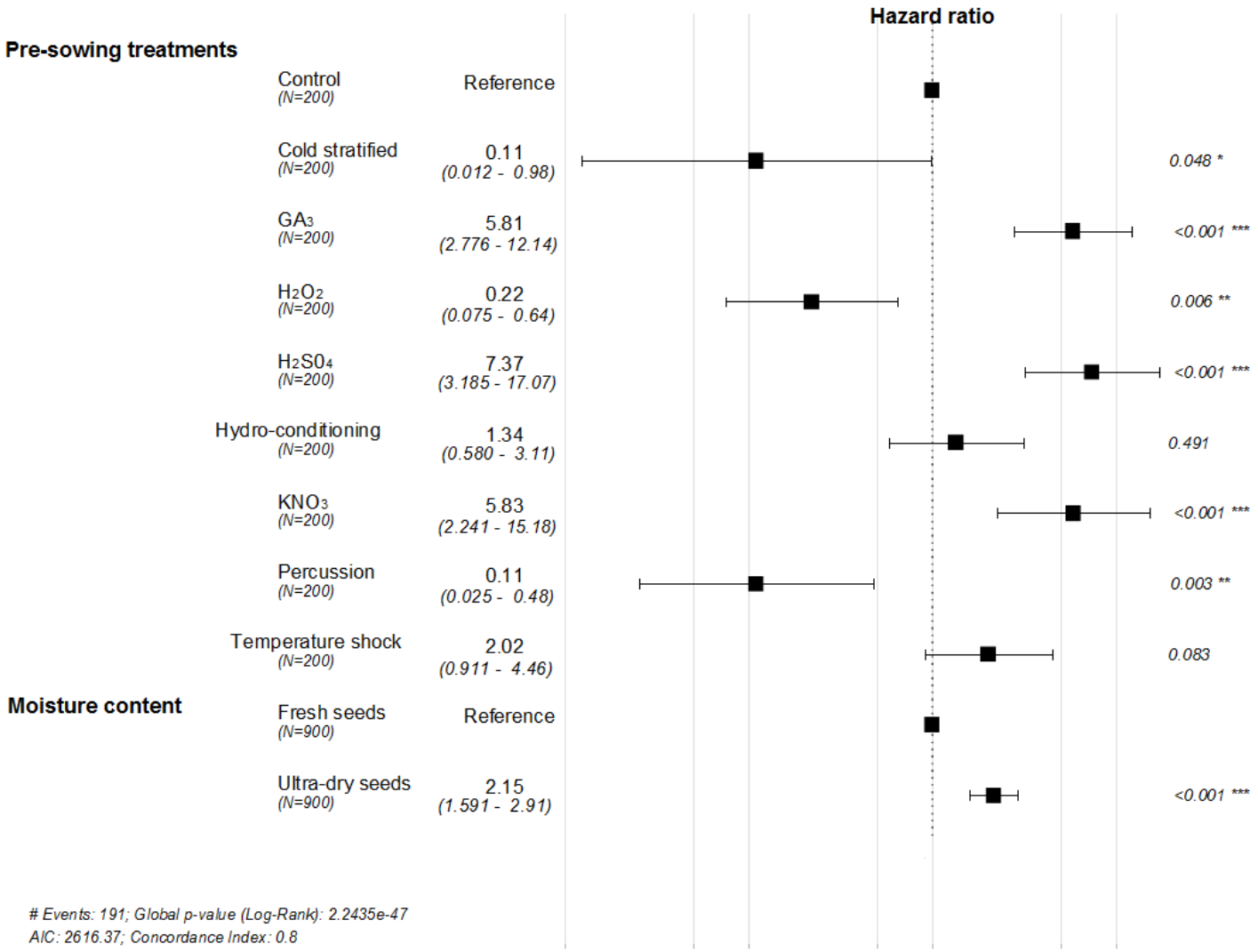

Figure 1

Forest plot of the Cox proportional hazards regressions of germination clustered by Petri dish replicate. The plot shows seed germination probabilities (hazard ratio, HR) and 95% CI from seeds (N) with different moisture content and treated with different pre-sowing treatments. The HR for control and fresh seeds is standardized to 1 and denoted by the dashed vertical line. An HR > 1 indicates an increased germination probability, whereas an HR < 1 indicates a decreased probability.

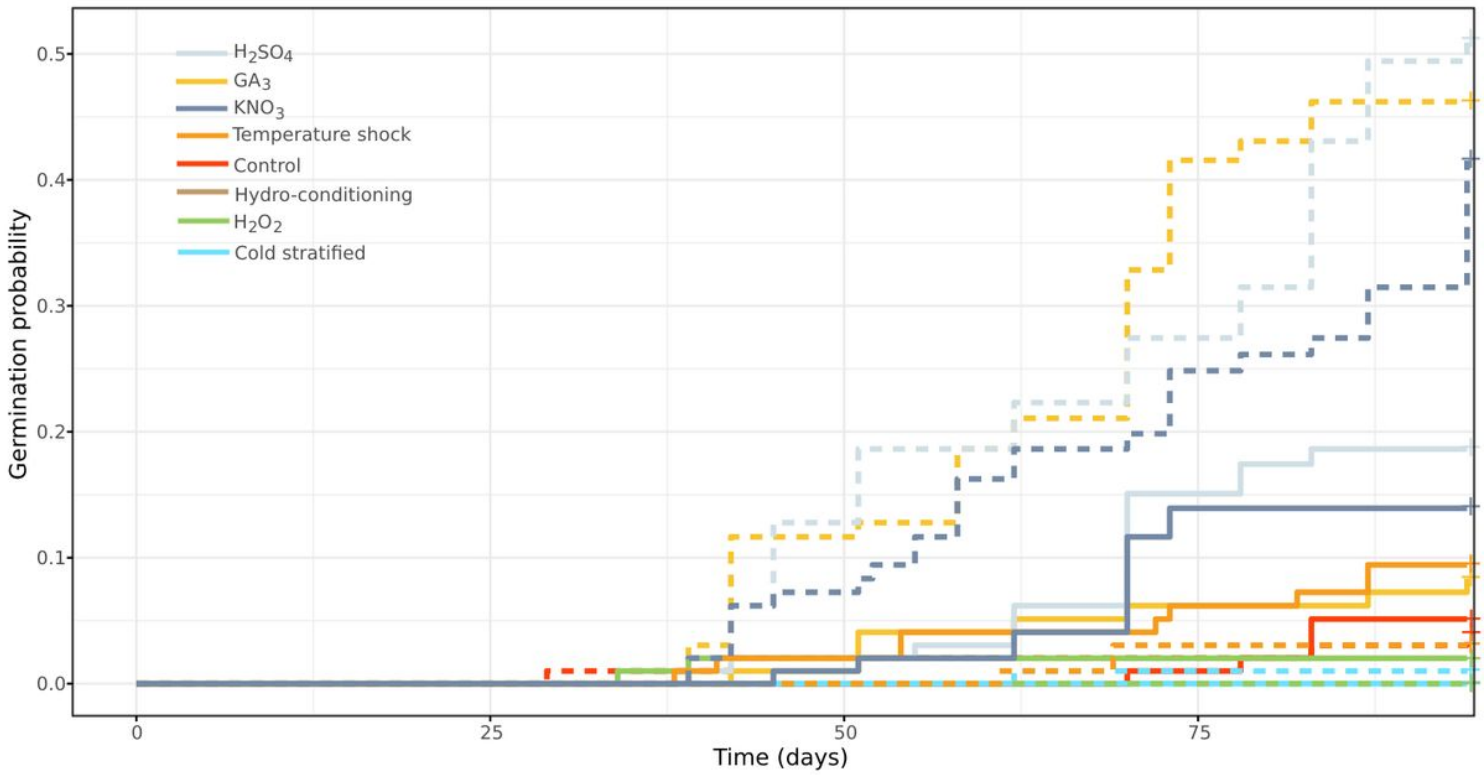

Figure 2

Germination probability curves based on an accelerated failure time model for the combined effects of pre-sowing treatments with moisture content (MC). Germination probabilities of fresh and ultra-dried seeds are shown in solid and dashed lines, respectively.

Supplementary Files

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