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Scarification with a Low Concentration of Acid Facilitates Water Acquisition and Minimizes Cold Stratification Duration, Improving the Seed Germination of Canadian Buffaloberry (*Shepherdia canadensis* (L.) Nutt.)

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Abstract: Canadian buffaloberry (*Shepherdia canadensis* (L.) Nutt.) is a perennial shrub known for its drought tolerance, nitrogen-fixing ability, and suitability for land reclamation and vegetation, particularly on nutrient-poor industrially disturbed soils in Alberta, Canada. Despite its ecological importance, commercial nurseries and greenhouses face challenges due to limited knowledge of optimizing seed germination and maintaining genetic diversity in cultivated seedlings. In this study, we investigated the interactive effect of cold stratification duration (0, 2, 4, 8, 12, and 16 weeks) and scarification time (control (no scarification), 1, and 4 h) on buffaloberry seed germination. The seed germination rate was tested using a factorial experiment with two factors arranged in a completely randomized design with four replications. Our findings indicate that scarification with a low concentration of acid for 1 h significantly enhances germination outcomes and shortens the required stratification period from 16 weeks to 12 weeks, achieving an optimal germination rate of 82%. Our novel approach using low-concentration acid to scarify Canadian buffaloberry seeds will help commercial greenhouses and forest nurseries improve seed germination, which utilizes this species for land reclamation and reforestation. Furthermore, this method can be adapted to improve germination in other native species with similar dormancy challenges, broadening its applications in ecological restoration efforts.

Keywords: seed germination rate; synchronization index; mean germination time; seed pre-treatments; low-concentration acid

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1. Introduction

The successful regeneration of plant species in disturbed ecosystems is essential for ecological restoration, particularly in regions experiencing soil degradation, biodiversity loss, and climate-induced stress. Canadian buffaloberry (*Shepherdia canadensis* (L.) Nutt.), a deciduous shrub native to North America, has gained increasing attention for its potential in land reclamation use. This species is valued for improving soil fertility and organic matter status and tolerating inhospitable conditions [1–3]. It is particularly well suited for revegetating disturbed sites, including reclaimed mining areas in Alberta, Canada [4], where it contributes to nutrient cycling, improves soil quality, and creates favorable conditions for the establishment of other plant species. Additionally, buffaloberry plays an important role as a food resource for Indigenous people [5] and wild animals, such as

grouse, black bears, grizzly bears, and snowshoe hares [6–8]. These ecological and cultural benefits make Canadian buffaloberry a valuable species for successful restoration efforts.

Despite its ecological and cultural significance, Canadian buffaloberry has not been fully utilized for land reclamation and revegetation due to several practical challenges. This species has primarily grown in the wild and has only recently been cultivated in greenhouses, largely in response to changes in regulatory standards in Alberta, Canada. These new standards require resource extraction companies to restore disturbed land to "equivalent land capability", meaning the restored land must support forest goods and services comparable to those provided prior to the disturbance [9]. Achieving this goal requires the establishment of fully functioning plant communities, including herbaceous plants, low shrubs, tall shrubs, and trees [9]. However, cultivating many boreal native shrub species, including Canadian buffaloberry seedlings, presents significant obstacles, mainly due to two factors: (1) seeds are exclusively collected from the wild, which is a labor-intensive process, and (2) seed dormancy impacts germination, reducing the efficiency of seed use. At present, the cost of a single Canadian buffaloberry seedling is approximately CAD 3, largely due to inefficient propagation methods. Furthermore, commercial greenhouses and forest nurseries lack the necessary knowledge to optimize germination rates and maintain genetic diversity in seedlings. To address these challenges, it is essential to develop cost-effective and reliable germination and propagation techniques to enable the widespread use of this beneficial species in reforestation and land reclamation efforts. Many native boreal species, including Canadian buffaloberry, face similar germination challenges, posing difficulties for timely and efficient restoration efforts.

Native boreal species typically have lower germination rates compared to agronomic species [10], posing challenges to the efficient and timely revegetation of native plant communities. Canadian buffaloberry seeds exhibit physiological and physical dormancy, preventing germination under unfavorable environmental conditions. Seed physiological dormancy is primarily caused by metabolic inhibition within the embryo, which can be overcome through cold stratification [11]. Cold stratification involves moistening the seeds and storing them at low temperatures (~5 °C) for several weeks or months [12]. The duration of cold stratification required to break dormancy varies among native shrub species, with some species taking weeks or months to achieve an optimal germination rate, often in combination with other treatments. For example, the challenges of overcoming dormancy in native species are illustrated by studies on *Rubus occidentalis*, where seeds subjected to sulfuric acid scarification for an extended duration (up to 3 h) followed by cold stratification exhibited significant improvements in germination rates, reaching up to 74% after 12 months [13]. In the case of Canadian buffaloberry, a stratification period ranging from 8 to 14 weeks, when combined with scarification, has been shown to improve germination rates [11,14]. For instance, when seeds were exposed to 14 weeks of stratification combined with scarification, a maximum seed germination rate (~40%) was achieved [14]. However, these efforts have been limited by relatively low success rates and challenges such as reduced seedling uniformity and the risk of overexposure to high-concentration acid. Research into alternative approaches, such as using reduced acid concentrations and shorter stratification periods, remains sparse, indicating an urgent need for improvement. Despite evidence that seed germination linearly improved with increasing cold stratification duration, the overall germination rate of this species remains low, even after the recommended treatment applications. This highlights the need to investigate whether extending the cold stratification period could further enhance germination rates and to determine the limits of this improvement.

Canadian buffaloberry also exhibits physical dormancy due to the impermeability of the seed coat to water [11,14,15]. Water uptake in seeds is a key process in overcoming

dormancy and initiating germination. This process, known as water acquisition, consists of three distinct phases [16–18]. Phase I, referred to as imbibition, involves rapid physical water uptake driven by the matrix potential of the seed coat and internal tissues. During this phase, water absorption occurs without metabolic activity. Phase II is marked by metabolic activation, where water uptake stabilizes, and enzymes essential for germination are activated. Phase III involves radicle elongation and active growth, signaling the completion of germination, and only viable seeds can reach this phase [19]. Scarification treatments primarily affect Phase I by improving seed coat permeability and facilitating rapid water uptake during imbibition [17,18]. Acid scarification with sulfuric acid (H_2SO_4) is a highly effective scarification method for breaking physical dormancy in seeds, making them permeable [20]. This technique is widely recognized for its efficiency, precision, and reliability in promoting the germination of hard-coated seeds [11,20]. However, selecting the appropriate acid concentration with soaking time is essential to avoid damaging the embryo, which can cause a low germination rate [21]. Notably, only one study investigates the effect of acid scarification using concentrated sulfuric acid (98%) to improve buffaloberry seed germination [14]. Using concentrated sulfuric acid may cause the risk of overtreatment, such as acid damage and losing significant portions of a population that may or may not be connected to critical genetic factors. Losing a portion of the genetic diversity of a population would make it less resilient in the future. It is also a concern that a long seed exposure time to strong acids may excessively weaken the seed coat and reduce survival during cold stratification [22,23]. Nevertheless, cold stratification in combination with acid scarification is recommended for seed germination in some species [24–27]. Although seed scarification in combination with cold stratification has resulted in improvements in germination in Canadian buffaloberry [14], there is still a need to investigate low acid concentrations and a minimum length of stratification, which provide maximum germination, increased uniformity, and less germination time. Our study uniquely explores the use of low-concentration sulfuric acid as a practical and safer alternative for nurseries, coupled with optimized stratification durations to maximize germination rates while maintaining seed viability.

Given the ecological and practical importance of Canadian buffaloberry for restoration, this study aims to (i) determine the required cold stratification duration to achieve an optimum germination rate; (ii) evaluate the effect of a low concentration of sulfuric acid to overcome seed physical dormancy and improve germination; and (iii) assess the interactive effect of cold stratification duration and scarification time on buffaloberry seed germination using a low concentration of sulfuric acid. By addressing these challenges, this study aims to develop effective propagation strategies for buffaloberry, ultimately supporting its use in ecological restoration programs under changing environmental conditions. Furthermore, the findings of this study will provide new insights into propagation techniques that could be adapted for other native species with similar germination challenges, facilitating broader applications in land reclamation and restoration efforts.

2. Materials and Methods

2.1. Fruit Collection and Seed Extraction

Matured buffaloberry fruits were collected near the town of Peace River, Alberta, Canada (altitude: 571 m, latitude: 56.23° N, and longtitude: 117.27° W), between June and July 2019. To ensure genetic diversity, seeds from four different seed lots were evenly mixed. The locations of the seed lots are listed in Table 1. Following the collection, fruits were stored at 4 °C (39.2 °F) for up to 7 days before seeds were extracted, according to the procedures described by [28]. The dry-cleaned seeds, with a moisture content of 4–8%, were then stored in a sealable bag at −20 °C (−4 °F).

Table 1. Seedlot number, collecting year, and geographical coordinates of Canadian buffaloberry seeds.

Seedlot No.	Year of Collection	Latitude	Longitude
NAIT 952	2019	N-56.33477	W-117.34823
NAIT 953	2019	N-56.33469	W-117.34814
NAIT 954	2019	N-56.54013	W-116.92723
NAIT 955	2019	N-56.59835	W-116.94783

2.2. Experiment Design

The experiment followed a factorial design with two factors, arranged in a completely randomized design with four replications. The first factor included six levels of cold stratification duration (0, 2, 4, 8, 12, and 16 weeks), while the second factor comprised three scarification treatments (1 h scarification, 4 h scarification, and a control with no scarification). A total of 1800 seeds were used for the germination test, with 25 seeds per Petri dish × 6 stratification durations × 3 scarification treatments × 4 replications (25 × 6 × 3 × 4 = 1800).

2.3. Seed Treatments

The experiment was conducted in the Laboratory of the Centre for Boreal Research, Northern Alberta Institute of Technology, AB, Canada. The seeds were divided into three groups and subjected to different scarification times: 1 h, 4 h, and a control group (soaked in deionized water for 1 h). For the scarification treatments, seeds were soaked in 5% sulfuric acid (98%, VWR International, Edmonton, AB, Canada) for either 1 h or 4 h. This acid concentration was chosen based on preliminary trials, which indicated no significant difference in seed germination rates between 5% and 10% sulfuric acid. After the scarification process, the seeds were thoroughly rinsed with deionized water and placed on double layers of filter paper (Whatman No.1) in 9 cm plastic Petri dishes moistened with sterilized distilled water. Following scarification, the seeds were stratified at 5 °C for either 0 (control), 2, 4, 8, 12, or 16 weeks. The Petri dishes were sealed with parafilm to maintain moisture throughout the cold stratification period.

2.4. Visible Germination Test

After each cold stratification period, the seeds in the Petri dish treated with different scarification time treatments were placed in a germination chamber. The germination chamber was set to a daily photoperiod of 12:12 day–night, a temperature of 25 ± 2 °C, and 75% relative humidity. Visible germination was monitored every 2 days for 50 days and recorded when emerged radicles (3 mm) were observed [29].

2.5. Water Acquisition Tests

To evaluate the impact of scarification time on seed water uptake during the water acquisition process, a water acquisition test was conducted. Ten seeds were placed in each Petri dish, with five replications for each scarification treatment. The seeds were weighed initially (dry mass) and then watered with deionized water. Subsequent weights were recorded at 24 h intervals over four days. Seeds were removed from the Petri dish for each replication, quickly surface-dried using filter paper, and weighed. The percentage of *water acquisition* (%) was calculated using the following equation:

$$Water\ acquisition\ (\%) = \frac{(W2 - W1)}{W1} * 100$$

where *W1* is the mass of dry seeds in each replication, and *W2* is the mass of the seeds after *water acquisition* at the specified time interval.

2.6. Data Analysis

Germination data were analyzed using the GerminaR R package [30], which provides tools for calculating key germination indices. Three primary germination variables were calculated:

1. **Germination rate:** it represents the proportion of seeds that successfully germinated out of the total number of seeds tested. It is expressed as follows:

$$\textbf{\textit{Germination Rate}} = \left(\frac{Number\ of\ seeds\ germinated}{Total\ number\ of\ seeds}\right) \times 100$$

2. **Mean gtermination ime (MGT):** MGT represents the average time required for seeds to germinate and was calculated using the following formula:

$$MGT = \frac{\sum ni{\cdot}ti}{\sum ni}$$

where *ni* is the number of seeds germinated at time *ti*, and *ti* represents the time elapsed from the start of the germination test.

3. **Synchronization index (SYN):** SYN quantifies the degree of synchronization in germination timing and was calculated using the following formula:

$$SYN = \frac{\sum C_{ni,\,2}}{N}$$

where $\sum C_{ni,2} = \frac{ni{\cdot}(ni-1)}{2}$ represents the number of pairwise combinations of germinated seeds at each time *i,* and N = $\sum ni{\cdot}(\sum ni-1)/2$ is the total number of possible pairwise combinations.

The data were examined graphically for the normality of the distribution (probability plots of residuals) and homogeneity of variance (scatter plots) using the R software (Version 4.2.0, R Development Core Team) before being subjected to an analysis of variance (ANOVA). An effect was considered significant if $p \leq 0.05$. When the ANOVA shows a significant ($p \leq 0.05$) stratification duration effect, scarification time effect, or a significant interaction, Tukey's post hoc test was used to compare individual means.

3. Results

3.1. Visible Germination Test

The interactive effects of stratification duration and scarification time significantly influenced the germination rate ($p < 0.05$, Table 2). Germination occurred rapidly within the first 10–15 days and plateaued thereafter (Figure 1). Overall, the germination rate improved with increasing stratification duration, though the rate of improvement diminished beyond 12 weeks (Figure 1). Seeds stratified for 12 weeks combined with a 1 h scarification demonstrated the highest germination rate of 88 ± 5.16%, significantly outperforming other treatments (Figure 2). Conversely, seeds treated with a 4 h scarification or no scarification achieved lower germination rates (66 ± 12% and 66 ± 5.16%, respectively) after 12 weeks of stratification.

Table 2. ANOVA results (*F* and *p* values) for the effects of stratification duration (Strat), scarification time (Scar), and their interactions on germination rate, mean germination time (MGT), and the synchronization index (SYN) of *S. canadensis*. The seeds were exposed to six levels of stratification duration (0, 2, 4, 8, 12, and 16 weeks) and three levels of scarification time (control (no scarification), 1 h, and 4 h). The numbers in bold are significant at <0.05.

	Strat (DF = 5)		**Scar (DF = 2)**		**Strat × Scar (DF = 10)**	
Variable	***F***	***p***	***F***	***p***	***F***	***p***
Germination rate	186.465	**<0.001**	2.345	0.106	2.206	**0.031**
MGT	9.852	**<0.001**	9.877	**<0.001**	1.785	0.093
SYN	4.346	**0.003**	0.076	0.927	1.270	0.284

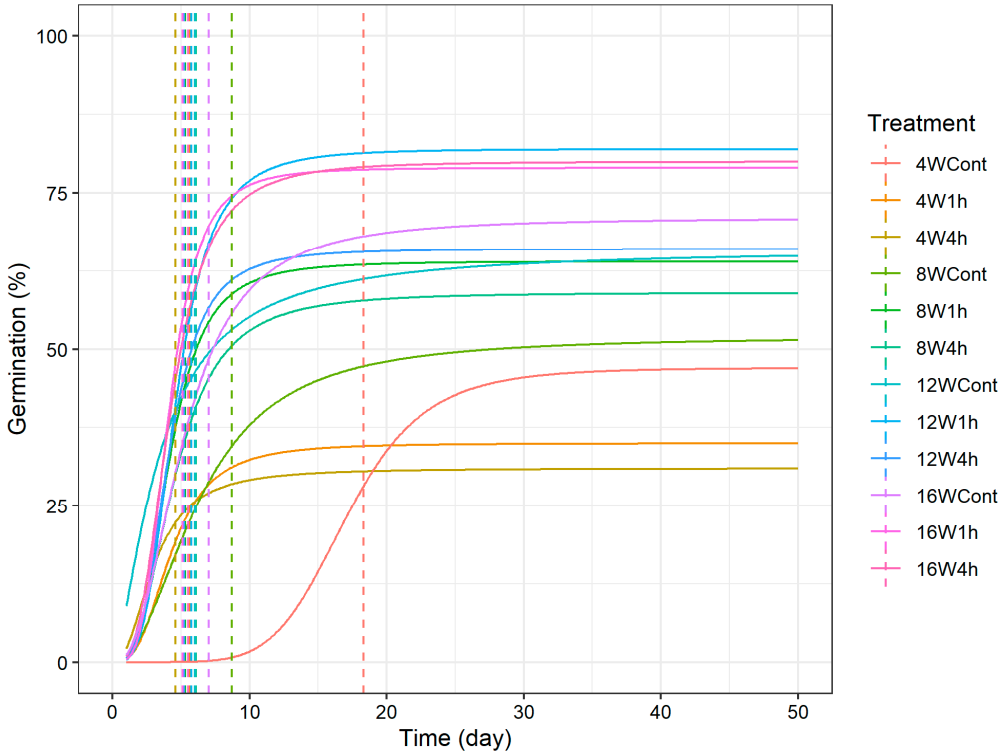

Figure 1. The cumulative percentage germination rate of *S. canadensis* seeds in response to four levels of cold stratification duration (4, 8, 12, and 16 weeks) and three levels of scarification time (control (no scarification), 1 h, and 4 h). Vertical dotted/dashed lines indicate mean germination time.

3.2. Mean Germination Time (MGT)

Cold stratification and scarification time significantly affected MGT ($p < 0.01$, Table 2). Longer stratification durations reduced MGT, with 12 and 16 weeks resulting in the shortest times (6.12 ± 1.47 and 6.07 ± 1.35 days, respectively). Similarly, a 1 h scarification significantly reduced the MGT to 7.74 ± 6.69 days compared to untreated seeds (14.09 ± 11.60 days; Figure 3a,b).

3.3. Synchronization Index (SYN)

While cold stratification marginally improved SYN, the differences were not statistically significant ($p > 0.05$, Table 2). Stratification durations exceeding 8 weeks showed an increasing trend in SYN, with 16 weeks achieving the highest SYN value of 0.41 ± 0.17 (Figure 3c). Shorter stratification durations (0–4 weeks) exhibited lower synchronization, suggesting that extended stratification may promote more uniform germination over time.

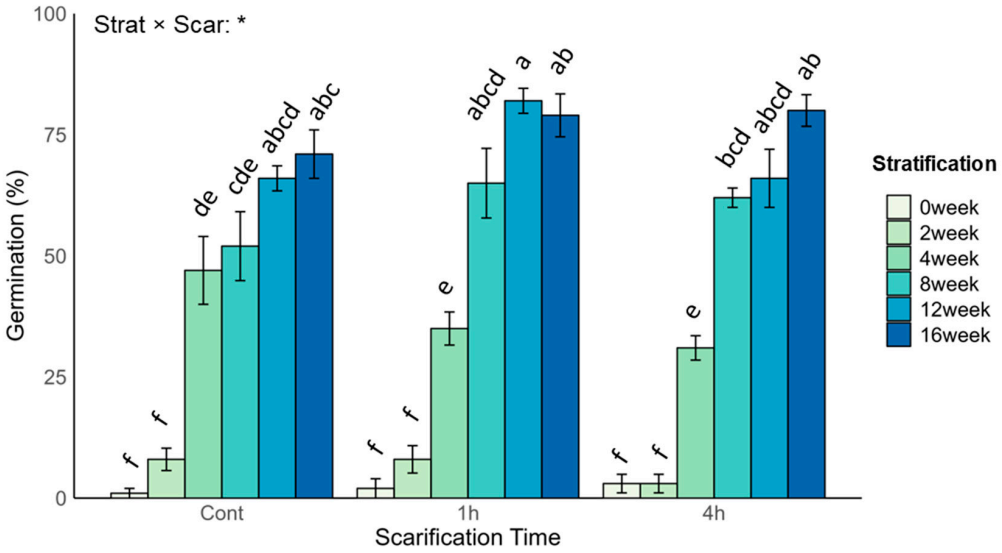

Figure 2. Interactive effect of stratification and scarification treatments on the germination percentage of seeds. Bars represent mean germination percentages (%), and error bars indicate the standard error. Different letters above the bars indicate statistically significant differences among treatments based on post hoc pairwise comparisons ($p < 0.05$). The *x*-axis shows the scarification time (control, 1 h, and 4 h), while the different colors represent the duration of stratification (0 to 16 weeks). Significance levels: * $p < 0.05$.

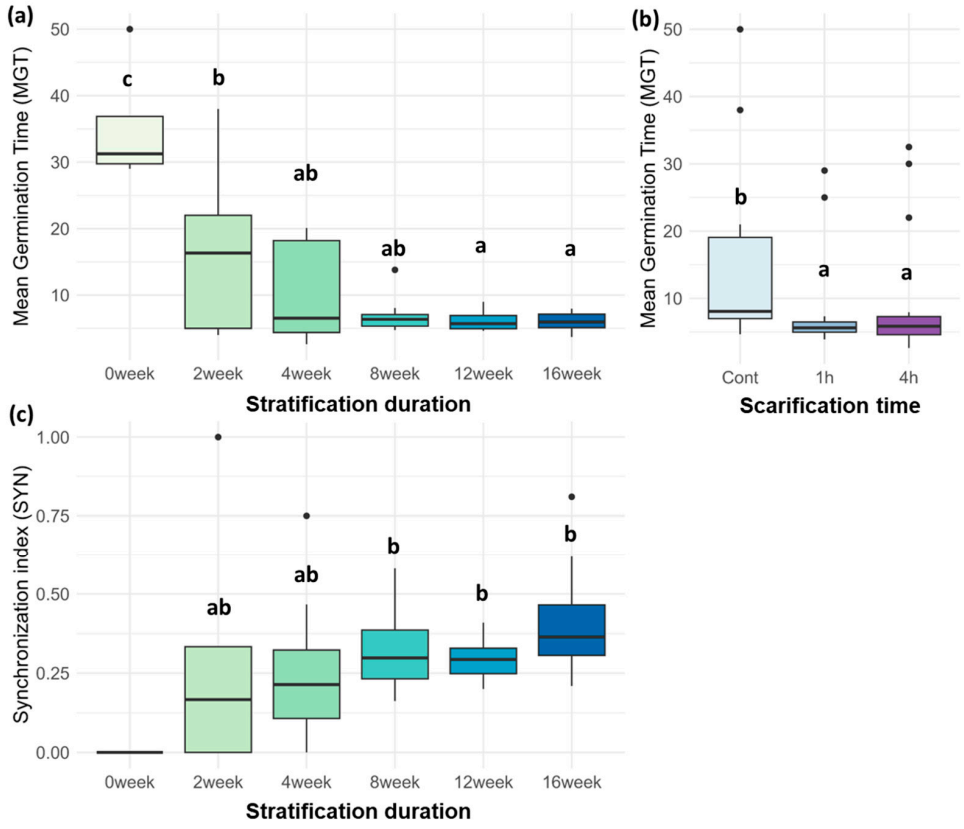

Figure 3. Boxplots showing the effects of stratification duration and scarification time on germination metrics of Canadian buffaloberry. (**a**) Stratification duration's and (**b**) scarification time's effect on mean germination time (MGT), and (**c**) the main effect of stratification duration on the synchronization index (SYN). Results were averaged over scarification time for (**a**,**c**) and over stratification duration for (**b**) due to the lack of a significant two-way interaction. The letters above boxplots indicate statistically significant differences ($p < 0.05$).

3.4. Water Acquisition Tests

Water acquisition tests revealed that a 1 h scarification facilitated the highest water uptake, with a mean acquisition of 20.97 ± 3.61% at 96 h (Figure 4). Seeds subjected to a 4 h scarification showed slightly lower acquisition (19.63 ± 2.5%), while non-scarified seeds exhibited significantly reduced water uptake. These results confirm the effectiveness of a 1 h scarification in overcoming physical dormancy.

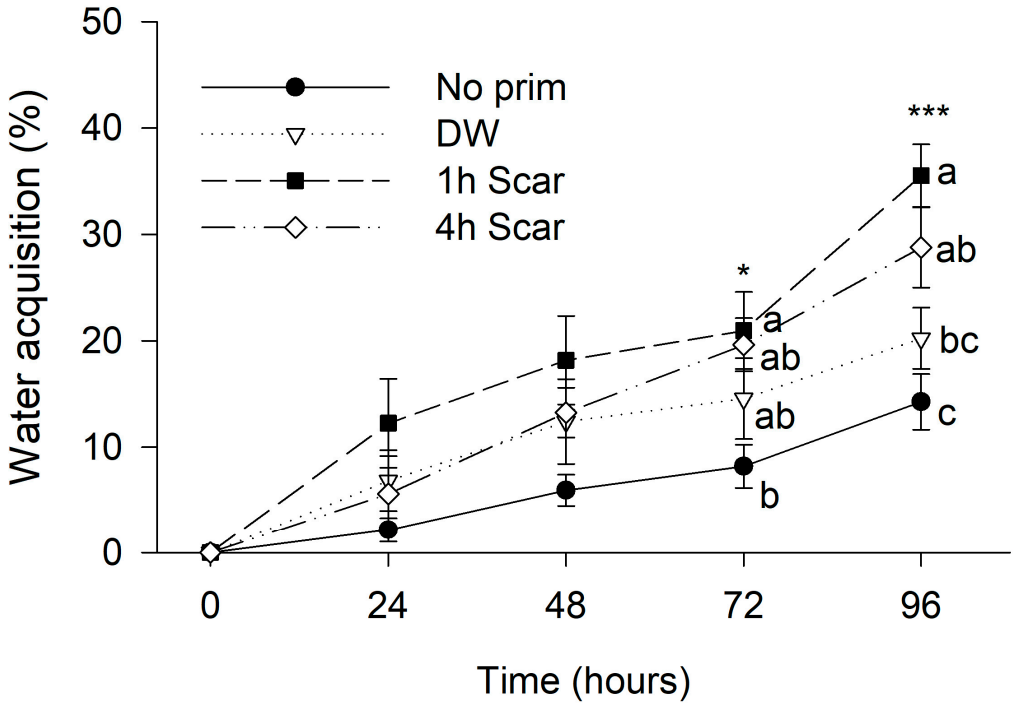

Figure 4. Mean (+SE) percentage of *water acquisition* (%) of buffaloberry seeds. The seeds were treated with four different treatments (deionized water (control), 1 h and 4 h acid, and no prim) (for a total of 200 seeds; 10 seeds × 5 replications × 4 levels of scarification treatments). The letters indicate statistically significant differences ($p < 0.05$). Significance levels: * $p < 0.05$; *** $p < 0.001$.

4. Discussion

Our findings align with previous research, confirming that a longer cold stratification period is the most effective method for enhancing buffaloberry seed germination [14,31]. As expected, the germination rate increased in correlation with the duration of cold stratification. However, the increase in germination rate diminished after 12 weeks of stratification. For instance, seeds stratified for 12 and 16 weeks germinated almost a day earlier than those stratified for 8 weeks, with a MGT of 6.92 days (8 weeks), 6.12 days (12 weeks), and 6.07 days (16 weeks). Although not statistically significant, the marginal increase in synchronization observed in seeds stratified for longer durations suggests a potential trend that warrants further investigation. This underscores the complexity of germination dynamics and highlights the need for more detailed studies to explore the relationship between cold stratification and synchronization of this species.

Many boreal shrub species exhibit deep dormancy to ensure seed germination occurs under favorable environmental conditions [11,32,33]. Buffaloberry, like other species in its range, relies on chilling temperatures to break the physiological dormancy of its seeds, aligning germination with the appropriate seasonal conditions [11]. Interestingly, although Baskin and Baskin (2014) suggested that cold stratification is not very effective in breaking the dormancy of this species unless exposing its seeds to warm stratification prior to cold stratification, the present study showed seed germination improvements up to 71%,

followed by 16 weeks of stratification without warm stratification. When inducing the germination of native plant seeds, it is essential to consider the phenology of the seed phase of the life cycle in the natural habitat, such as the temperature between seed maturation and germination [11,34]. The requirement of warm stratification for this species could depend on the timing of seed collection. For instance, fruiting buffaloberry occurs in late June and ripens in mid–late July, depending on summer temperatures [35–37]. In our case, we collected only ripened seeds; therefore, we assume that the seeds met the requirement of warm temperature and completed seed maturation before the seed collection.

In addition to cold stratification, water availability is another crucial factor influencing seed germination [11,12,38]. Buffaloberry seeds exhibit physical dormancy due to an impermeable seed coat, which prevents water uptake (Phase I: imbibition) and delays germination. Acid scarification has been widely used to overcome this barrier by making the seed coat more permeable [15]. Sulfuric acid (H_2SO_4) has been recommended for scarifying seeds to make the seed coat more permeable and to enhance water uptake during imbibition. This process possibly facilitates the progression to Phase II, where metabolic activation occurs, and eventually to Phase III, characterized by radicle elongation and germination [17,18]. Previous work by Rosner and Harrington (2003) examined the interactive effect of scarification time of concentrated sulfuric acid and cold stratification lengths on the seed germination of buffaloberry. The authors found the optimal treatment combination was a 5 min acid soak and 14 weeks of stratification resulting in a germination improvement from 27% to 38%.

In our study, we used a lower concentration of sulfuric acid (5%) to avoid embryo damage while still enhancing seed germination. The effectiveness of acid scarification depends on the acid concentration, species, and exposure duration [11,39]. Generally, high sulfuric acid concentrations are used in species that possess impermeable seed coats [40–42]. Our water acquisition test showed that water uptake in non-scarified seeds was gradual and significantly slower than in scarified seeds. Low-concentration sulfuric acid (5%) effectively improved water acquisition, confirming that it successfully breaks physical dormancy in buffaloberry seeds. Scarification for 1 h was particularly effective, reducing the optimal stratification duration from 16 to 12 weeks while achieving the highest germination rate (82%). Seeds scarified for 4 h, however, exhibited a lower germination rate (66%) after 12 weeks of stratification, though this increased to 80% after 16 weeks. These results suggest that a 1 h scarification with 5% sulfuric acid is an optimal treatment for improving seed germination while minimizing stratification time.

5. Conclusions

Cold stratification duration plays a critical role in enhancing buffaloberry seed germination. Both with and without acid scarification, extending the stratification period leads to higher germination rates. However, seeds scarified with a low concentration of sulfuric acid (5%) for 1 h not only exhibited improved germination results but also reduced stratification length requirements from 16 to 12 weeks, achieving an optimal germination rate of 82%. This novel approach of using low-concentration acid for seed scarification offers a practical solution for commercial greenhouses and forest nurseries seeking to improve germination rates of buffaloberry, thereby supporting land reclamation and reforestation efforts. Additionally, future studies could explore how genetic diversity is affected by these treatments and assess the environmental dependencies of stratification and scarification outcomes, such as temperature fluctuations or other treatments.

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