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Morpho-physiological studies on *in vitro* germination of *Berberis empetrifolia* Lam. (Berberidaceae)

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Abstract

Berberis empetrifolia is an endemic species from Chile that produces some bioactive compounds, highlighting the alkaloid berberine, which demonstrated antitumor properties. Taking into account the constant plant supply demand required for medicinal use, the establishment of *in vitro* propagation protocols allows plant multiplication, without affecting natural populations. Moreover, the *in vitro* germination is an efficient strategy to obtain axenic plants. The aim of this study was to establish the *in vitro* germination and to characterize seeds morphology and seedling development of *B. empetrifolia*. After decontamination of seeds, pre-germinative treatments were applied. The seed viability ranged from 75-90% and the embedding curve revealed permeable integument. The germination was characterized as epigeal phanerocotylar type. Seeds not submitted to pre-germinative treatments reached 10-30% germination rate (GR) and showed asynchronous germination. The most efficient pre-germinative treatment was the storage of seeds at 3˚C for 12 days followed by overnight soaking in GA_3 solution associated to seed coat removal. This treatment allowed the seed dormancy to be overcome, reaching GR above 95% and resulting in synchronous germination. The *in vitro* germination protocol established for *B.empetrifolia* was efficient to produce phenotypically normal axenic seedlings, allowing their use as source of plant material for biotechnological, phytochemical and pharmacological studies.

Keywords: Dormancy. Gibberellic acid. Medicinal plant. Post-seminal development. Pre-germinative treatments.

Abbreviations

TTC - 2,3,5-triphenyl tetrazolium chloride; WPM - Wood Plant medium; WPM½ - WPM medium with half salt concentration; GA_3 - gibberellic acid; GR - germination rate; GT - mean germination time; CVG - coefficient of velocity of germination; RF - relative frequency of germination

Introduction

The family Berberidaceae is composed of approximately 14 genera and 700 species of perennial herbs and shrubs. The genus *Berberis* has attracted interest due to its bioactive substances as alkaloids, steroids, glycosides, flavonoids, saponins, terpenoids and reducing sugars. Root extracts of some species contain quinine, a potent anti-malarial agent[1].

The species of genus *Berberis* present a broad spectrum of pharmacological activities as antitumor, cardiovascular protection, anti-hyperglycemic, antipyretic, anti-viral, antimicrobial, hepatoprotective, anti-inflammatory, antioxidant, anti-amoebic, anti-allergic, immune-modulator[1,2].

Berberis empetrifolia Lam. (Berberidaceae), known as uva de la cordillera, zarcilla or monte negro, is a sub-shrub perennial, endemic of the Andes (Argentina and Chile). Hermaphroditic flowers occurring in individual or in small umbels, of bright yellow color. Its fruits are dark purple sub-globular berries, containing 3 - 7 seeds. The plants had been used in the Mapuche culture as an infusion for treatment of various diseases, such as fever, inflammation, stomach pains and colitis[3,4]. The *B. empetrifolia* medicinal properties are mainly attributed to alkaloids, highlighting the berberine, an isoquinoline alkaloid, found in roots, barks and rhizomes, used in Ayurvedic and traditional Chinese medicine, as well as in homeopathic drugs. The berberine has antitumor properties, with effects on human's malignant brain tumor, esophageal cancer and leukemia and colon cancer cell lines[5].

Taking into account the constant plant supply demand required for the medicinal use of *B. empetrifolia* and for pharmacological and phytochemical studies with the species, the establishment of *in vitro* propagation protocols allows massal plant multiplication, without impacting natural populations. Moreover, *in vitro* cultures can also be used for continuous and homogeneous production of bioactive compounds. Therefore, the establishment of *in vitro* germination is considered the first step to achieve an efficient *in vitro* propagation protocol, since the seedlings can be used as source of young and axenic plant material[6].

The germination process may be affected by environmental and internal seed factors and, frequently, ideal conditions are species-specific and need to be determined through experimentation[6].Some species have a long germination period because of the dormancy, which consists in a protection to natural conditions. To ensure successful perpetuation of the species, the seeds will germinate in the most favorable environmental conditions for seedling development[7].

The seeds of *B. empetrifolia* have showed a low germination percentage under field conditions. Studies conducted by Celedón-Neghme*et al.*[8] considered the presence of dormancy in the species. Seeds dormancy can be imposed by the embryo, the envelopes (seed coat, endosperm), or a combination of both factors. Seeds dormancy may also occur due to the presence of germination inhibitors, which can be removed by leaching and/or partial removal of the seed husk on the side of the root protrusion[9].

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The present study characterizes both seeds morphology and post seminal development of *B. empetrifolia* and evaluate the use of pre-germinative treatments to overcome dormancy in order to establish the *in vitro* germination, allowing the supply of plant material for biotechnological, phytochemical and pharmacological studies.

Material and Methods

Plant material

Plants of *B.empetrifolia* were collected in Trakura, 20 km from Icalma, Cautín province, La Araucania Region, Chile (38° 50' 52.1" S, 71° 29' 15.5" W), 1298 m altitude, between the months of January and March. The taxonomic identification was conducted by the botanist Rubén Carrillo, from the Facultad de Ciencias Agropecuarias y Forestales of the Universidad de La Frontera. Exsiccate was deposited in the Herbarium of the Facultad de Ciencias Agropecuarias y Forestales of the Universidad de La Frontera (UFRO-Be301).

The fruits were transported at 6˚C in the dark. They were washed with distilled water and decontaminated with 75% ethanol. The seeds were manually extracted from the fruits, rinsed with sterile distilled water, dried and conditioned in zip lock plastic bags with hermetic closure and maintained at room temperature. The seeds were sent to the Laboratório de Biotecnologia de Plantas (Labplan/UERJ) for analysis.

Morphological and biometric characterization of seeds

The physical characteristics, such as the length, width and thickness of the seeds were measured with an analogical pachymeter (Mitutoyo 200mm 0,05mm mod.530-321). The morphological description of seeds was carried out using a stereomicroscope (Nikon SMZ745T), following the terminology described in Barroso *et al*.[10].

Evaluation of seeds viability and coat permeability

Seed viability was assessed by topographical tetrazolium test[11]. Seeds were preconditioning in distilled water at room temperature for 24 h. Then, they were cut longitudinally, immersed in 1% (w/v) of 2,3,5 - triphenyl tetrazolium chloride solution (TTC) for 24 h at 30˚C, in the dark. After this period, the seeds were rinsed with distilled water and observed under the stereomicroscope to determine the location and the staining intensity of embryos. The results were expressed as percentage of viable seeds.

To evaluate the integument permeability seeds were immersing in distilled water for 48 h at 26 ± 1˚C. The seed fresh weight was measured at regular time intervals and data were plotted to establish the imbibition curve.

In vitro germination

Seeds were soaked with 10% neutral detergent for 5min, and rinsed in running tap water. Then, they were decontaminated with 2% NaOCl (sodium hypochlorite) solution plus Tween 80 (0.05% v/v) for 20 min, and rinsed three times in sterile distilled water. The seeds were inoculated in test tubes containing 10 mL of WPM medium[12] with half-salt concentration (WPM½) added with 3% sucrose. The pH was adjusted to 5.8 and the medium solidified with 0.8% agar (Merck) before autoclaving (121˚C for 15 min). The tubes were closed with double aluminum foil caps and maintained in a growth chamber at 26 ± 1˚C, photoperiod of 16 h with light intensity of 50 μmol $m^{-2}s^{-1}$.

Pre-germinative treatments

Seeds were subjected to the following treatments: mechanical scarification with sandpaper (n˚ 120); storage at 3˚C for 5, 10 or 15 days; storage at 3˚C under immersion in 50µM solution of gibberellic acid (GA_3) for 7 days; storage at 3˚C for 12 or 15 days followed by overnight soaking in 50µM GA_3 followed or not by seed coat removal; storage at 3˚C for 12 days followed by removal of the seed coat; removes only the seed coat. After pre-germinative treatments, seeds were decontaminated, inoculated under *in vitro* conditions and maintained in a growth chamber as described above.

The post-seminal development was monitored daily to determine the following parameters[13]: germination rate (GR), mean germination time (GT), coefficient of velocity of germination (CVG) and relative frequency of germination (RF), defined as:

Germination rate: GR (%) = $\frac{N.100}{A}$

where: N = total number of germinated seeds; A = total number of seeds;

Mean germination time: GT (days) = $\frac{\sum \text{niti}}{\sum \text{ni}}$

where: n_i= total number of germinated seeds per day; t_i = incubation time (days); Σn_i= total number of germinated seeds.

Coefficient of velocity of germination: CVG (days^{-1}) = $\frac{1}{GT}$

Relative frequency of germination related to the incubation time: RF (%) = $\frac{\text{ni}.100}{\sum ni}$

Seedlings were considered normal when essential structures were perfectly developed[11].

Statistical analysis

Each treatment was repeated three times with 20 seeds per replication. Data were submitted to analysis of variance (ANOVA) and the means were compared by Tukey test 5% level of significance. The statistical analysis was performed using the GraphPad Prism 5 statistical software package.

Results and Discussion

Morphological and biometric characterization of seeds

The seeds have dark brown color, smooth testa (seed coat), reniform shape, length 3.7 ± 0.3 mm, width 1.7 ± 0.2 mm and thickness 1.5 ± 0.3 mm (**FIGURE 1a**) and fresh weight of 4.65 ± 0.83 mg. The embryo is straight, having almost the full length of the seed and the fleshy endosperm (**FIGURE 1b**).

Evaluation of seed viability and seed coat permeability

According to the TTC test, 75 to 90% of the seeds were viable (**FIGURE 1c**). This value is higher than the results obtained by Celedón-Neghme *et al.*[8], evaluating seeds of *B. empetrifolia* collected in Valle Nevado area, Andes of Central Chile, at 2600m altitude.

The imbibition curve (**FIGURE 2**) showed a marked increase in fresh weight during the first 4 hours of immersion in distilled water, followed by stabilization after this period, indicating high seed-coat permeability.

In vitro germination

The decontamination of seeds was efficiently performed by immersion in 2% NaOCl solution for 20 min, achieving a minimum contamination after *in vitro* inoculation (up to 15%). This decontamination protocol was selected because it has already been successfully used with other species studied in Labplan/UERJ[6] and the efficacy of seed decontamination was observed. Moreover, the seedlings of *B. empetrifolia* developed on WPM½ medium showed normal phenotypical aspect (**FIGURE 1d-h**). The WPM basic culture medium was selected for our experiments due to the positive results for *in vitro* germination of *B. chitria* Lindl.[14]. The authors observed a higher germination percentage in WPM (78.89%) when compared to Murashigue and Skoog (MS) medium (50%), which has been the most used culture medium in *in vitro* studies. In addition, seeds germinated faster in WPM (± 6 days after inoculation) when compared to MS (± 15 days after inoculation). Positive results using the WPM medium were also found in the *in vitro* germination of other woody species[15,16].

FIGURE 1: Morphological characterization: a - intact seed; b - seed in cross section; c - viable seeds by tetrazolium test. *In vitro* post-seminal development of *Berberis empetrifolia* inoculated on WPM medium with half salt concentration (WPM½): d - radicle protrusion (1 day); e - primary root development (7 day); f - primary root elongation (9 day); g - tegument free cotyledons (15 day); h - elongated hypocotyl and primary root and visualization of leaves from cotyledonary node (20 day). Bars = 0.5 cm.

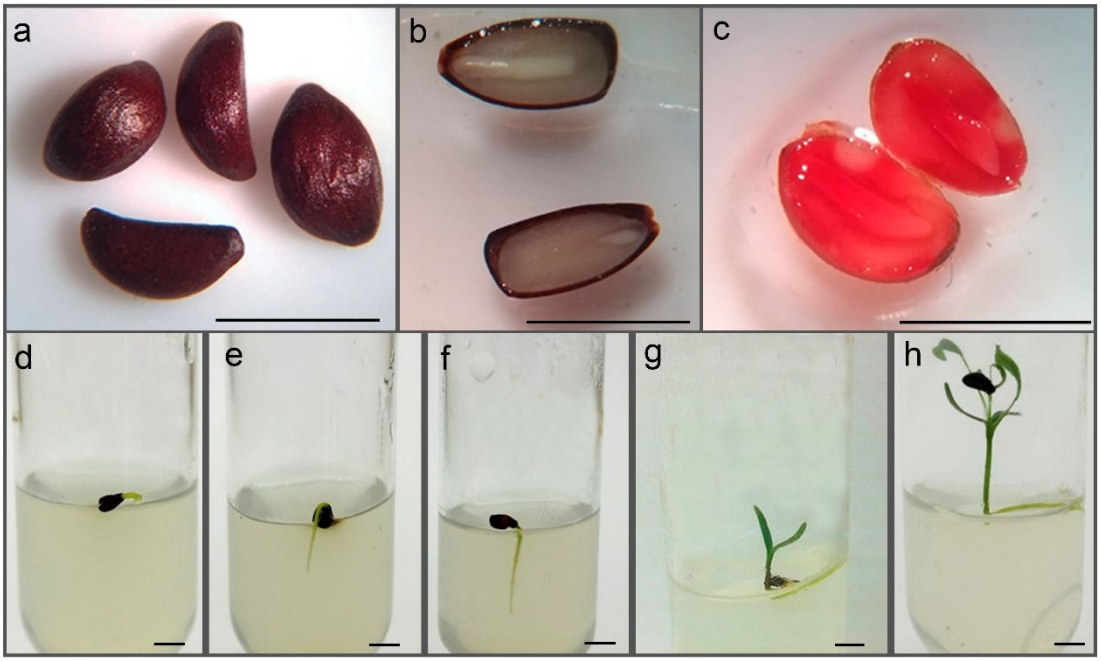
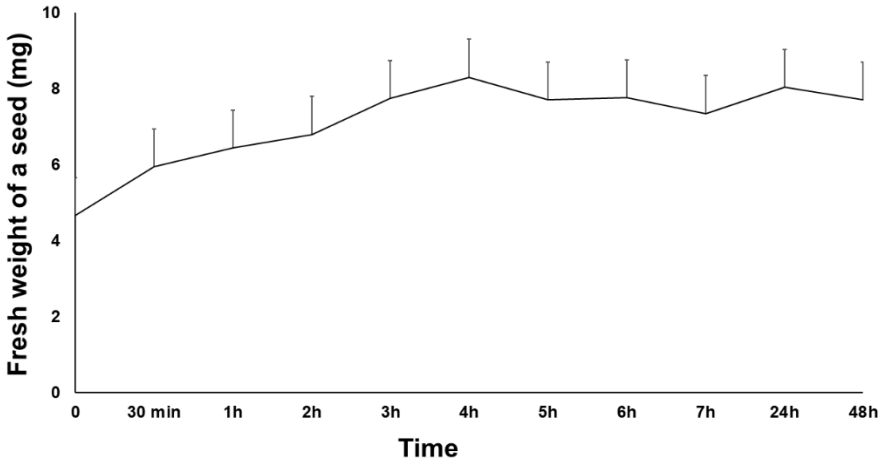


FIGURE 2: Imbibition curve of *Berberis empetrifolia* seeds.



The description of post-seminal development was carried out for the first time for the species. This aspect is very important once the characterization of the first stages of plant development allows the differentiation between species and provides information on natural regeneration, management and conservation. The germination of *B. empetrifolia* seeds was characterized as epigeal phanerocotylar type. Integral rupture and root protrusion occurred between 30 and 60 days after sowing (**FIGURE 1d**). Primary root is axial with positive geotropism. About seven days after germination, the primary root was about 1.0 cm in length, color is greenish-yellow, thick, smooth surfaced and it is cylindrical (**FIGURE 1e**). About ten days after germination (**FIGURE 1f**), the hypocotyl appears characterized by a curvature in relation to the root and the thickening of the axis delimiting the root portion. Then, the radicle emerges from the seed and on the 15th day the hypocotyl elongates in epigeal germination and releases the cotyledons from the integument (**FIGURE 1g**). Cotyledons are elliptic, opposite, persistent, thin and green, measuring about 0.8 cm long and 0.2 cm wide. The base of the cotyledon does not taper to a petiole-like structure. After 20 days (**FIGURE 1h**), the hypocotyl and primary root were elongated, and at least one pair of narrow leaves from the cotyledonary node. The petioles were long, measuring about 1 cm, and the limbus was 0.5 cm long and 0.2 cm wide approximately. At about 30 days, the seedling was complete, with developed epicotyl, containing leaves petiolate, narrow, linear, whole, slightly twisted at the tips, with rounded edges. The presence of secondary roots is observed in this period. Cotyledons remain on the seedling until the end of the observation period.

Previous studies developed by Celedón-Neghme*et et al.*[8] evaluating the endozoochoric dispersion of *B. empetrifolia* seeds, demonstrated that the germination percentage of the species, without any pre-germinative treatment, reached only 5%, after 60 days. This low germination percentage, without synchronism, was also observed in the present study. Seeds not submitted to any pre-germinative treatment showed asynchronous germination, reaching GR between 10% and 30%, with root protrusion observed between 30 and 120 days after inoculation, characterizing the presence of dormancy in these seeds.

Seed dormancy is a genetic characteristic, strongly influenced by the environment, during seed development. Seed dormancy varies between species due to the wide variety of climates, resulting in different classes and levels of dormancy[17]. Considering the dormancy classification, an extensive system was developed by Nikolaeva and Vorobèva[18], who divided endogenous dormancy (embryo dormancy) into: physiological, morphological and morphophysiological; and exogenous dormancy (dormancy imposed by

the envelopes) in: mechanical (mechanical resistance), physical (water impermeability) and chemical (presence of chemical inhibitors in extra-seminal tissues)[19]. However, a more recent proposal by Baskin and Baskin[20] considers combined dormancy, which associates physical and physiological dormancy.

Pre-germinative treatments

In order to increase the germination percentage and achieve a more synchronous germination process, different pre-germinative treatments were used. **TABLE 1** describes the effect of these treatments under the germination parameters.

Mechanical scarification with sandpaper resulted in low germination percentage (GR = 23.67%), but with a high mean germination time (GT = 57.33days) (**TABLE 1**). The distribution of the polygon of relative frequency showed the heterogeneity of the germination (**FIGURE 3**). Celedón-Neghme *et al.*[8] verified that the germination percentage of *B. empetrifolia* lizard-ingested seeds presents significant difference in relation to control. The values reach 14% after about 60 days of sowing, compared to 5% for control group over the same period. Moreover, the germination of seeds ingested by lizards began in the first week after sowing, while control seeds germinated after the sixth week. The mechanical and/or chemical action of the lizard digestive tract promotes the removal of the outer layers of the seed coat by thinning the cuticle and palisade sclerenchyma, as observed in the histological cuts, allowing the entry of water necessary for the development of the embryo. However, the authors concluded that this mechanical and/or chemical scarification did not positively affect the germination percentage, which remained low. These results corroborate the present study, in which the mechanical scarification with sandpaper did not increase the germination rate. In addition, the imbibition curve of the seeds (**FIGURE 2**) indicated full permeability of the integument to water, confirming that there is no physical dormancy in the species.

The use of pretreatment with abscisic acid (ABA) imbibition alone was not effective in the present study for *B. empetrifolia* (GR = 7.50% e 12.0%) (**TABLE 1**). On the other hand, for seeds of *B. aristate* DC an increase in germination rate was observed when seeds were soaked in 50 µM gibberellin (GA_3) for six hours, reaching a maximum frequency of 70.4%[21]. The plant phytohormones ABA and gibberellins (GA) are involved in several plant mechanisms like dormancy and germination. ABA is involved in dormancy induction during seed development and GA is involved in germination promotion by inducing cell wall hydrolases, which cause endosperm weakening and embryonic growth. This ABA/GA hormonal balance regulates the maintenance or termination of dormancy and the promotion of germination[7].

The storage pre-treatments at 3˚C for different times with or without soaking in GA_3 solution were not effective for breaking the dormancy of *B. empetrifolia* seeds (GR = 5.50% to GR = 23.33%) (**TABLE 1**) (**FIGURE 3**). However, seeds of other species of Berberidaceae have dormancy overcoming after pre-germinative treatment of stratification followed by soaking in GA_3. *Jeffersonia dubia* (Maxim.) Benth. & Hook. F. ex Baker & S. Moore has the morpho-physiological dormancy broken using hot stratification (25/15˚C or 25˚C for 8 weeks) followed by cold stratification (5˚C for 8 weeks) or treatment with 1000 $mg.L^{-1}$ GA_3 followed by incubation at 15/6˚C[22]. Moreover, moist cold stratification is often used to break dormancy and promote subsequent germination of different species. Cold stratification is associated with increased GA content and a decline in ABA content[23]. Additionally, a lower degree of physiological dormancy can be overcome with weeks or months of storage in dry conditions[24].

TABLE1: Effect of pre-germinative treatments on Germination Rate (GR), Mean Germination Time (GT), and Coefficient of Velocity of Germination (CVG) of *Berberis empetrifolia* seeds inoculated under *in vitro* conditions.

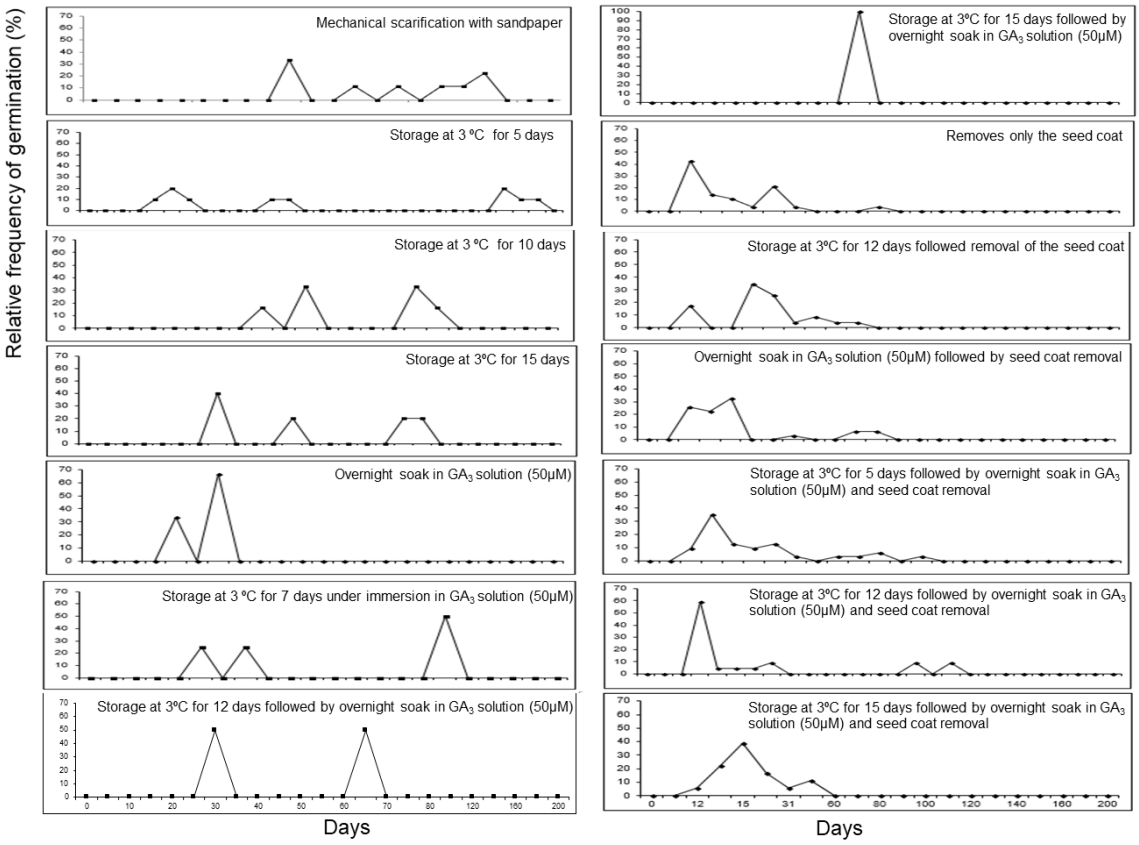
Pre-treatments	GR (%)	GT (days)	CVG (days^{-1})
Mechanical scarification with sandpaper	23.67±3.84^{d}	57.33±1.45^{bc}	0.0175±0.0004^{b}
Storage at 3˚C for 5 days	23.33±6.00^{d}	83.00±6.24^{c}	0.0120±0.0002^{b}
Storage at 3˚C for 10 days	12.00±3.05^{d}	62.67±1.45^{bc}	0.0160±0.0004^{b}
Storage at 3˚C for 15 days	11.67±1.20^{d}	62.33±1.45^{bc}	0.0160±0.0004^{b}
Overnight soak in GA_3 solution	7.50±0.29^{d}	25.33±2.60^{a}	0.0400±0.0041^{ab}
Storage at 3˚C for 7 days under immersion in GA_3 solution	12.00±2.31^{d}	75.33±2.91^{c}	0.0133±0.0005^{b}
Storage at 3˚C for 12 days followed by overnight soak in GA_3 solution	8.67±0.67^{d}	49.33±5.81^{b}	0.0208±0.0024^{b}
Storage at 3˚C for 15 days followed by overnight soak in GA_3 solution	5.50±0.29^{d}	50.00±5.77^{b}	0.0205±0.0024^{b}
Removes only the seed coat	60.00±2.89^{c}	16.33±2.03^{a}	0.0631±0.0078^{a}
Storage at 3˚C for 12 days followed removal of the seed coat	56.00±9.45^{c}	29.67±4.91^{a}	0.0358±0.0063^{ab}
Overnight soak in GA_3 solution followed by seed coat removal	77.98±1.52^{b}	37.00±3.61^{a}	0.0276±0.0029^{ab}
Storage at 3˚C for 5 days followed by overnight soak in GA_3 solution and seed coat removal	77.33±4.67^{b}	25.33±3.84^{a}	0,0411±0.0054^{ab}
Storage at 3˚C for 12 days followed by overnight soak in GA_3 solution and seed coat removal	95.33±2.91^{a}	34.33±4.33^{a}	0.0300±0.0034^{ab}
Storage at 3˚C for 15 days followed by overnight soak in GA_3 solution and seed coat removal	94.67±2.91^{a}	23.33±3.30^{a}	0.0444±0.0055^{a}

Mean ± standard error in columns followed by the same letter do not differ by Tukey test ($p \leq 0.05$). GA_3 - Gibberellic acid(50µM)

In many species, seed coating prevents germination by interfering with water uptake, restricting oxygen diffusion primarily at higher temperatures, imposing mechanical restrictions, containing chemical inhibitors, and modifying the light that reaches the embryo[25]. Seeds that have integuments that act as physical barriers to embryo expansion often contain non-dormant embryos, which germinate easily after integument removal[26]. Seed coat of *Fraxinus* species proved to be a more influence factor that the high ABA concentration in the embryonic axis, since the removal of the coat allowed the embryo to grow[18]. Manual removal of seed coat from *Prunus cerasoides* D. Don[27], *P. yedoensis* Matsum[26] and mango varieties[28], also promoted increased and accelerated the germination process.

The presence of inhibitors in the integument is also a possibility associated to dormancy. Germination inhibitors may be present in the seed envelope, characterizing a chemical dormancy, as well as these inhibitors may be present in the embryo, characterizing a physiological dormancy. However, some authors have considered as physiological dormancy all dormancy caused by the presence of growth inhibitors[19]. Many inhibitors are water soluble and must be leached, while others must be degraded for germination promotion to occur. Chemical inhibition of seed coat can be reversed by the use of exogenous growth promoters such as gibberellins, as well as the removal of the integument [29,30].

FIGURE 3: Distribution of the relative frequency (RF) of germination of *Berberis empetrifolia* seeds, inoculated under *in vitro* conditions.



The most efficient pre-germinative treatment applied to B. *empetrifolia* seeds under *in vitro* conditions were the storage at 3˚C for 12 days followed by overnight soaking in GA_3 solution (50µM) associated to seed coat removal. This treatment resulted in a significant increase in the parameters evaluated (**TABLE 1**), as well as it promoted a more synchronous germination process (**FIGURE 3**). These results showed that the removal of the B. *empetrifolia* seeds integument was fundamental for the germination process. Possibly the integument acts as a physical barrier to embryonic expansion, characterizing a mechanical dormancy. Moreover, *B. empetrifolia* seeds germination mechanical and physiological dormancy aspects were observed, as low-temperature pretreatment and GA_3 imbibition associated with tegument removal significantly increased the germination percentage, when compared to the removal of the integument alone (**TABLE 1**). These features indicate the combined dormancy in the species.

Conclusion

In conclusion, this study describes an efficient protocol to *in vitro* germination of *B. empetrifolia* and demonstrates that the process is optimized with the use of pre-germinative treatments. The establishment of axenic and vigorous seedlings is the first step for *in vitro* cultivation of the species, aiming at mass production of plants and metabolites under *in vitro* conditions, for future phytochemical and pharmacological studies and, consequently, without harvesting natural populations.

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Conflict of Interests

There is no conflict of interests.

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Contributors

Study design: TCC; CS-G; NA
Data curation: TCC; DSS
Data collect: TCC; CS-G; NA
Data analysis: EM-E; MPH
Writing of the original manuscript: EM-E; MPH
Proofreading and Editing: TCC; DSS; CS-G; EM-E; MPH; NA.

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