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Effects of Temperature on Asymbiotic Seed Germination of *Himantoglossum robertianum* (Loisel.) P.Delforge

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Abstract

Aim of study: Despite protection by international agreements, millions of orchid tubers are harvested from their natural distribution areas each year. Of these species, *Himantoglossum robertianum* is locally threatened due to overharvesting and requires precautionary measures to ensure its protection. Reproduction of the species in an asymbiotic environment is imperative for providing ex-situ protection. There are no studies on optimum germination temperature in *H. robertianum*. This study aimed to germinate *H. robertianum* seeds in-vitro under asymbiotic conditions.

Area of study: The study was carried out at the Silviculture Laboratories of Bursa Technical University, Faculty of Forestry, Department of Forestry Engineering.

Material and methods: H. robertianum seeds were used in the study. Seeds were germinated in five replications at four different temperatures (10, 15, 20, and 25°C (± 0.5°C). The study was conducted for 275 days under dark conditions with Sıgma-Phytamax P-6668 used as the medium.

Main results: The highest germination was 23.8% at 20°C and germination was not obtained at 10°C. While germination was faster at 25°C in the first 18 weeks, germination accelerated at 20°C after 18 weeks.

Highlights: These results indicate that temperature is an important factor in the germination of *H. robertainum* seeds.

Keywords: *Himantoglossum robertianum,* Asymbiotic Germination, Temperature

Himantoglossum robertianum (Loisel.) P.Delforge'un Asimbiyotik Tohum Çimlendirilmesinde Sıcaklığın Etkisi

Öz

Çalışmanın amacı: Uluslararası sözleşmeler tarafından korunmasına rağmen, her yıl doğadan milyonlarca orkide yumrusu sökülmekte ve doğal yayılış alanları yok edilmektedir. Bu türlerden biri olan *Himantoglossum robertianum*, aşırı toplama nedeniyle yok olma tehdidi altındadır ve korunması için önlemler alınmalıdır. Ex-situ korumanın sağlanması için türlerin asimbiyotik ortamda çoğaltılması zorunludur. *H. robertianum*'da optimum çimlenme sıcaklığı ile ilgili bir çalışma bulunmamaktadır. Bu çalışmanın amacı, türün asimbiyotik koşullarda doku kültürü ortamında ve farklı sıcaklıklarda çimlenme koşullarını belirlemektir.

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Materyal ve yöntem: Çalışmada *H. robertianum* tohumları kullanılmıştır. Tohumlar dört farklı sıcaklıkta beş tekerrürlü olarak çimlendirilmiştir. Çimlendirme ortamı olarak Sığma-Phytamax P-6668 kullanılmış ve karanlık koşullarda 275 gün süreyle yürütülmüştür.

Temel sonuçlar: En yüksek çimlenme 20°C'de %23.8 olarak gerçekleşirken, 10°C'de çimlenme elde edilememiştir. İlk 18 haftada 25°C'de çimlenme hızı yüksek iken daha sonra 20°C'de çimlenme hızlanmıştır.

Araştırma vurguları: Bu sonuçlar, *H. robertainum* tohumlarının çimlenmesinde sıcaklığın önemli bir faktör olduğunu göstermektedir.

Anahtar Kelimeler: *Himantoglossum robertianum*, Asimbiyotik Çimlenme, Sıcaklık

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Introduction

Orchidaceae is one of the largest families of flowering plants, with more than 28,000 species spanning 763 genera (Christenhusz & Byng, 2016). Seed production is high in orchids, with each capsule containing thousands or millions of small seeds, depending on the species (Arditti, 1967; Arditti & Ghani, 2000). Germination is generally difficult in temperate terrestrial orchids (Arditti et al., 1982 Rasmussen, 1995; Miyoshi & Mii, 1998), with only 0.2-0.3% of the millions of seeds in a capsule germinating in nature (Citil & Tekinşen, 2011). Since the tiny seeds have a small embryo with no endosperm, they require a mycorrhizal relationship for germination and development in nature, receiving water, nitrogen, carbohydrates, vitamins and organic compounds via mycobionts (Kauth et al., 2008). Low germination is also attributed to morphological and morphophysiological dormancy (Baskin & Baskin, 2014). Environmental conditions, such as light quality, quantity and temperature, can change the seed germination rate and duration (Lee et al., 2018).

Asymbiotic germination is ideal for studying the germination and development of orchid seeds and seedlings. It is widely used since it is simpler than symbiotic germination and is an excellent technique for studying the effects of abiotic and biotic factors on seed biology (Kauth et al., 2008). More precise results can be obtained from asymbiotic germination studies carried out under controlled conditions in the laboratory compared to symbiotic germination studies.

For many plant species, temperature is a major factor in breaking physiological seed dormancy (Rasmussen, 1995; Baskin & Baskin, 2004). However, little research has explored the effects of temperature on the germination of orchid seeds (Arditti, 1967; Kauth et al., 2008; Johnson, 2011; Calevo & Bazzicalupo, 2020). Additional research is needed to reveal the effects of temperature on germination in terrestrial temperate orchid species (Johnson, 2011).

Orchid seeds can germinate over a wide temperature range (Arditti, 1967), but maximum germination is achieved only within a narrow range between 20 and 25°C (Rasmussen et al., 1990; Marić, 1995; Kauth et al., 2008; Lee et al., 2018). Although orchid seeds generally germinate *in-vitro* at constant temperatures, applying alternating temperature regimes is recommended to study germination ecology (Baskin et al. (2006; Kauth et al., 2008; McCormick et al., 2021). In a study of Western European orchids, the best germination occurred at 23°C in continuous darkness (Van Waes & Debergh, 1986; Baskin & Baskin, 2001). These studies provide valuable information regarding the role of temperature in the germination of orchid seeds (Kauth et al., 2008).

Orchid tubers can be ground into salep, a powder widely used for making hot drinks and ice cream. Salep is obtained from 30 species of terrestrial orchids belonging to *Orchis, Anacamptis, Himantoglossum, Ophrys, Serapias* and *Dactylorhiza* genera (Sezik, 1967; 1990; Tekinsen & Guner, 2009). Glucomannan and starch, as the major components used to make salep powder, are the primary indicators of the yield and quality of salep powder (Acemi et al., 2019 Teoh, 2019). Those who collect orchid tubers prefer to collect large tuberous species first. Therefore, species with large tubers tend to be harvested frequently. With a harvest rate of 39.5%, *Himantoglossum robertianum* is the most harvested species for salep production due to its large tubers (Molnár et al., 2017).

Himantoglossum is a genus of orchids native to the Canary Islands, Europe, Southwest Asia and Northern Africa that is found in dry, calcareous soils (Rasmussen, 1995; Rossi 2002; Parlak & Tutar, 2012). The plants grow 60-80 cm tall (Davis, 1984; Delfolge, 2006; Rossi, 2002; Teoh, 2016), are easy to find, and are valuable for salep production. Unfortunately, due to the overharvesting of their tubers for salep production, many species in this genus are locally threatened in Türkiye (Dulić et al, 2018; Teoh, 2019).

In-situ or *ex-situ* protection measures should be taken, but are limited by a lack of research on *H. robertianum* (Szendrák, 1997; Aybeke, 2013a; 2013b; 2013c; Katsalirou et al., 2017; Katsalirou et al., 2019; Calevo et al., 2020). The reproductive physiology of *H. robertianum* and germination protocols under asymbiotic conditions must be determined to

ensure its *ex-situ* protection. Although the effects of temperature on germination have been studied in some orchid species (e.g., *Dactylorhiza majalis* (Rasmussen et al., 1990; Rasmussen & Rasmussen, 1991) and *Psygmorchis pusilla* (Vaz et al., 2004)), no studies on the optimal germination temperature of *H. robertianum* have been reported. Therefore, this study aims to reveal the effects of temperature on the germination of *H. robertianum*.

Materials and Methods

Materials

Himantoglossum robertianum seeds were collected from plants grown by open pollination in 2019 and donated by the Ministry of Agriculture's Agricultural Research Centre in Menemen, İzmir, Türkiye. The seeds were air-dried for 2 weeks at room temperature and stored in Eppendorf® tubes at 4°C until use.

A digitally controlled germination cabinet (Lovibond TC 140 G-Liebherr, Dortmund/Austria), stereomicroscope (Irmeco, IM SZ550-B-ST5-H, Geesthacht/Germany), flow cabinet (biosafety cabinet class II), autoclave (Tomy SX-700e, Tokyo/Japan), pure water device (Elga DV 35-ELGA LabWater/UK) and hydrogen peroxide for seed sterilization (Sigma-Aldrich (Nord., 34.5-36.5%) were used. Sigma-Phytamax P-6668 medium was used for germinating the seeds in sterile 90 mm plastic petri dishes (İsolab). No modifications were made to the medium and 27.3 g/L powder were used to prepare the medium.

Methods

Medium preparation

The natural distribution of *H. robertianum* occurs mostly on limestone bedrock and calcareous soils (Davis, 1984; Rossi, 2002). Rasmussen (1995) reported that media with a neutral or slightly alkaline pH, which resembles the conditions in their natural habitat, may be suitable for many of the European orchid species. Therefore, the media pH was adjusted to 7 with the use of 0.1 N sodium hydroxide (NaOH). After adding 7 g/L agar, the medium was autoclaved at 121°C for 60 min. Approximately 20 ml of medium was poured into each petri dish after cooling to 65-70°C.

Sterilization, sowing and incubation of seeds

The *H. robertianum* seeds were placed in 2-ml Eppendorf® tubes and treated with 10% hydrogen peroxide for 60 min before being rinsed three times with pure sterile water. The seeds were sown into sterile plastic petri dishes with a 90 mm diameter using a curved paper clip that was disinfected in alcohol and flamed. The closure of the culture dishes affects germination (Rasmussen, 1995), so the petri dishes were wrapped with a double layer of transparent cling film. Since high germination occurs when seeds of many orchid species are incubated at constant temperatures (Arditti, 1967; Baskin & Baskin, 2014), the petri dishes were placed in a climate cabinet set at 10, 15, 20, and 25°C (± 0.5°C). The glass surface of the climate cabinet was covered with aluminium foil to provide a dark environment. There were five replicates and the average number of full seeds in the petri dishes varied between 105 and 185. The seeds were sown on May 30, 2019, and observations of protocorm development were recorded every 15-40 d for 275 d.

Seed count and statistical analyses

Seeds were observed and counted according to Szendrák (1997) using a stereomicroscope with a magnification of 3.35 to 180x during the incubation period of 275 d. The germination stages (Figure 1) of the seeds were evaluated according to a modified method by Stewart & Kane (2006). At each counting time the germination rates were calculated using the following formula for each temperature (Eq. 1):

$$\text{Germination percentage } (\%) = \frac{\text{seed number (stages } 3-5)}{\text{total seed number (stages } 0-5)} \times 100 \quad (1)$$

An arc-sin transformation was applied to the germination percentage values. The transformed data were subjected to analysis of variance (ANOVA) and the means were compared with Duncan's multiple range test using SPSS ver. 22. (IBM Corp., Armonk, NY, USA). Significance was defined at the 0.05 level.

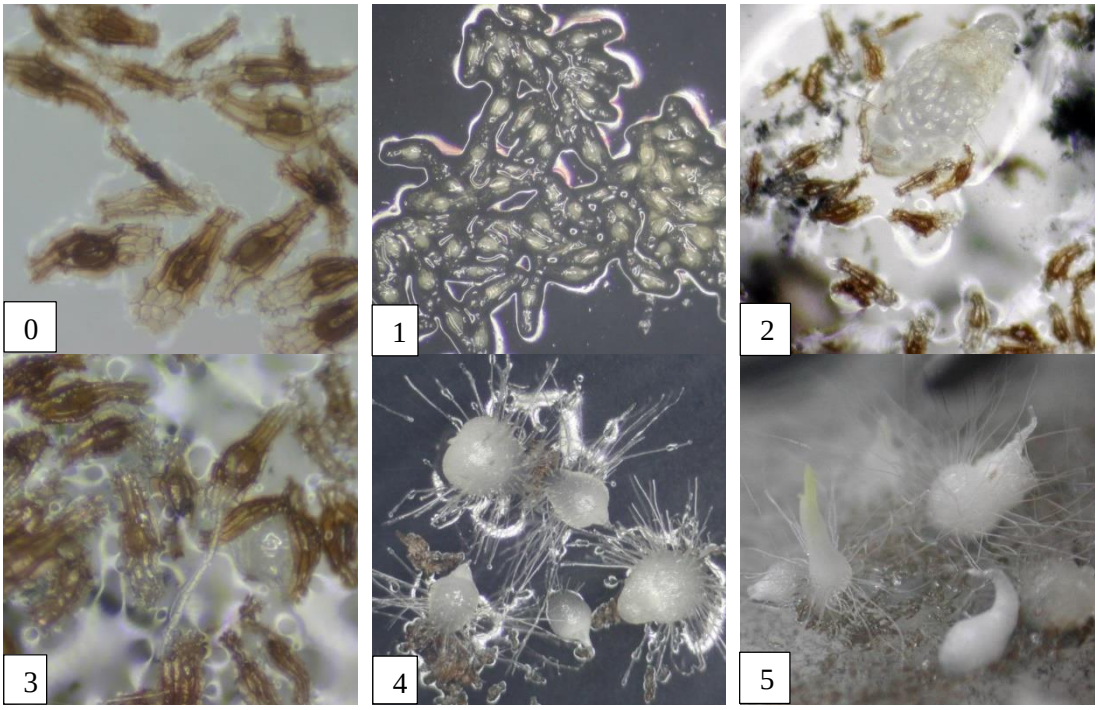

Figure 1. Germination stages of asymbiotically cultured *Himantoglossum robertianum in vitro* (adapted from Stewart & Kane, 2006). 0) Ungerminated seed, testa intact; 1) embryo whitening and swelling; 2) enlarged embryo, testa ruptured (35 days after sowing); 3) appearance of rhizoids indicates germination (at least 1 mm); 4) appearance of promeristem; and 5) emergence of the first leaf (seedling).

Results and Discussion

Seeds began to swell within 2 weeks (stage 1) of sowing and germinated to form protocorms within the 35 d after sowing, as shown in Figure 1, stage 2. The study was completed once germination ceased at 275 d. Homogeneous germination did not occur in *H. robertianum* seeds and some of the seeds did not germinate until the end of the study. Rasmussen (1995) reported that germination of *H. robertianum* is sporadic, even when seeds are sown from green capsules, and very slow, taking up to 10 months. At temperatures both above and below the optimal range, some viable seeds remain dormant.

Temperature was a significant factor in the germination of *H. robertianum* seeds (Table 1). Duncan's test revealed significant differences among all four temperatures (Table 2). The best germination rate was obtained at 20°C, followed by 25°C and 15°C (Table 2; Figure 2). At 10°C, germination was 0%.

Table 1. Analysis of variance for *Himantoglossum robertianum* germination.

	Sum of Squares	df	Mean Square	F	Sig.
Among groups	1964.032	3	654.677	103.252	0.000
Within groups	88.768	14	6.341		
Total	2052.800	17			

Table 2. Duncan test results for germination rates of *Himantoglossum robertianum* by temperature.

Temperature (°C)	N	Subset for alpha = 0.05
10	5	0.0000 ± 0.00000^{a}
15	5	11.7531 ± 1.72935^{b}
25	4	17.9284 ± 2.71872^{c}
20	4	28.9898 ± 4.26736^{d}
Sig.		1.000

Means followed by the same letter indicate no statistical difference at the 5% level.

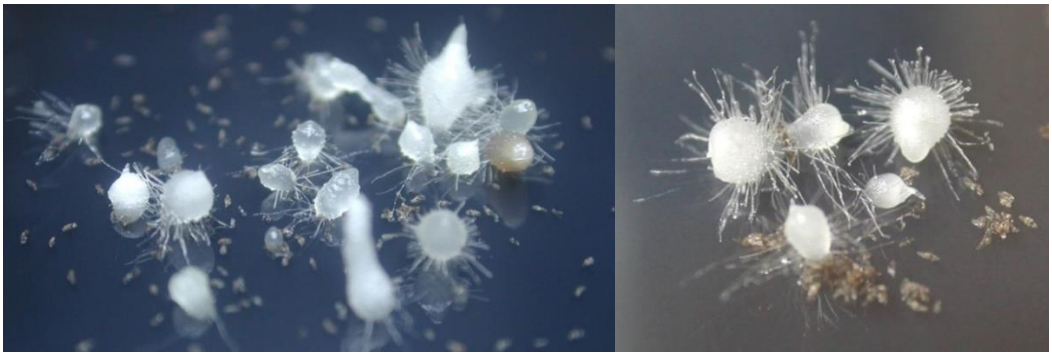

Figure 2. Seeds germinating at 20°C (left) and 25°C (right) 210 days after sowing.

As with many other species, orchid seeds germinate over a wide temperature range, but maximum germination is achieved only within a narrow range (Kauth et al., 2008). Arditti (1967) reported that orchid seeds germinate best at 20-25°C. For most orchid species, the temperature yielding the highest germination rates is between 22 and 25°C, although some germinate best below 20°C. (Rasmussen, 1995). Although the first germination of *H. robertianum* (Szendrák, 1997; Calevo et al., 2017) was reported to occur 60 d after sowing, the first protocorm formation began after 35 d in our study. The highest germination rate (23.8%) was observed under dark conditions at 20°C after 275 d (Figure 2). The results of our study are consistent with those of previous studies. Calevo et al., (2017; 2020) found germination rates of 23% and 46.1% for *H. robertianum* after 180 days at 26±1°C. Tsutsumi et al. (2011) found the highest germination rate at 20°C under dark conditions for *Liparis fujisanensis*, *L. koreojaponica* and *L. kumokiri*. Roca (1984) determined that temperatures of ~20°C favoured *in vitro* development of alpine plants. Gümüş et al. (2017) found that at the lower temperature, the highest germination rates (20.34-24.41%) were observed for *Dactylorhiza nieschalkiorum*. On the other hand, Özkoç & Dalcı (1994) obtained the highest germination rate (25.1%) for *Orchis laxiflora* Lam in Knudson C medium, which does not contain inorganic nitrogen. The optimum temperature range for *Dactylorhiza majalis* seeds appears to be between 23 and 24.5°C. Germination rates for *D. majalis* decreased at temperatures below 15°C and above 27°C. These rates were 42% and 21%, respectively, under symbiotic and asymbiotic conditions at 23.6°C (Rasmussen et al., 1990; Rasmussen & Rasmussen, 1991). The best germination rate for *Cattleya purpurata* was 46.5±6.4% in ½ MS medium and 26.3±4.3% in KC medium (Bazzicalupo et al., 2021). In another study, Calevo & Bazzicalupo (2020) determined that temperature changes play an important role in the germination of *Orchis patens* and recommended the application of variable temperature instead of constant temperature for the germination of European orchid species. Arditti (1967) reported that seeds of many orchid species germinate at high rates when incubated at constant temperatures between 20 and 25°C. Van Waes & Debergh (1986) obtained the highest germination rate in 21 of 23 European orchids in a BM1 environment, at 23°C in constant darkness. These studies show that the parameters of orchid seed germination are species-specific (Kaut et al., 2008).

In our study, no seed germination was observed at 10°C (Figure 2). Low temperatures delayed germination and development in a study of *Bletia purpurea* (Johnson & Kane, 2011). Rasmussen (1995) also pointed out that low temperatures may prevent seeds from germinating immediately after dispersal. The seeds of *Liparis* spp. did not germinate at all at 5°C and had the lowest germination at 10°C (Tsutsumi et al., 2011).

In this study, although the germination of seeds was higher at 25°C for the first 18 weeks, the germination at 20°C continued to increase after 18 weeks (Figure 3).

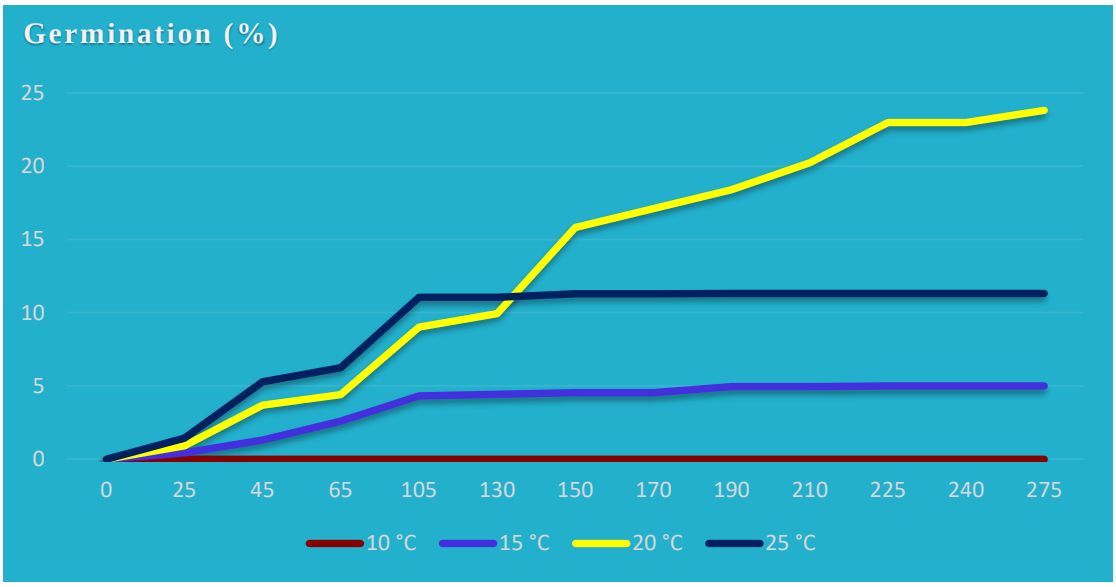

Figure 3. Germination rates of *Himantoglossum robertianum* at 10, 15, 20, and 25°C (± 0.5°C).

Germination started early at 25°C, and the protocorms that were initially yellow in colour turned brown over time. As a result of the drying of 7 protocorms germinating at 15°C and 8 protocorms at 25°C, the germination graph shows a decreasing trend (Fig. 4). Pierce and Belotti (2011) reported that this occurs because protocorms are left in the medium for too long. Stoutamire (1974) and Neiland (1994) reported that this is common and possibly caused by inappropriate cultural conditions. Additionally, phenolic compounds may exudate during seedling growth and cause browning. This might be intensified by light, high temperatures and oxidizing substances, such as iron, but is reduced by transferring the cultures to a dark environment or by frequent subcultivation of new media (Harbeck, 1968; Haas, 1977; Ponert et al., 2011).

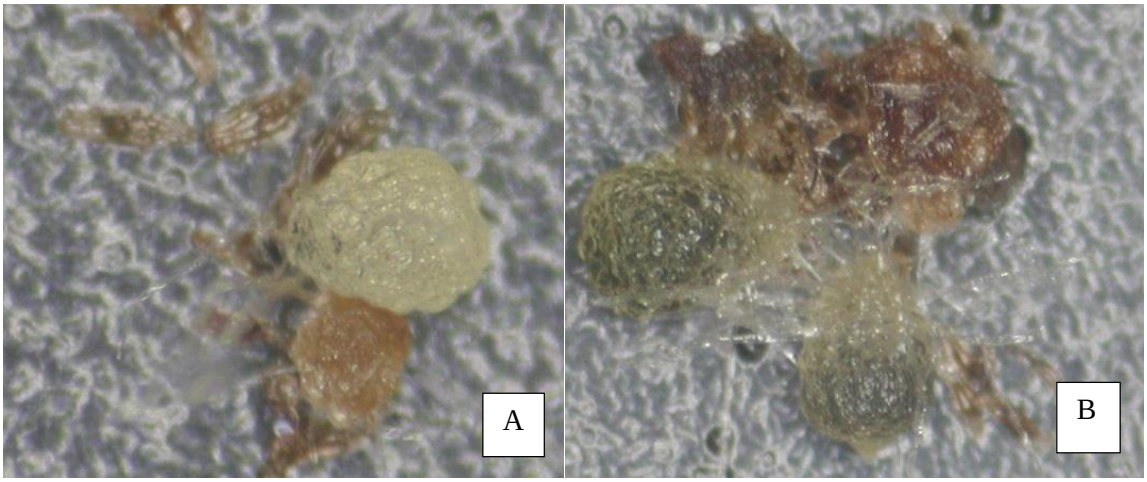

Figure 4. Protocorms that germinate early (A) and begin to turn brown (B)

Conclusion

Excessive harvesting of orchids tubers from their natural habitats for salep production threatens orchid species survival. Harvesters prefer *H. robertianum* because of its large tubers and easy identification, limiting and fragmenting its natural distribution areas. Isolation and habitat fragmentation can cause problems, such as insufficient pollination and seed formation. Therefore, it is important to be able to culture this species to prevent it from being overharvested from its natural environment. In this study, germination trials of *H. robertianum* were carried out at 10, 15, 20, and 25°C (± 0.5°C). The best germination was 23.8% at 20°C, while no germination was observed at 10°C. Temperature is significant in the germination of this endangered species. More germination trials should be conducted at temperatures of 20±3°C to narrow the optimal temperature range. Testing the effects of changing temperature regimes on seed germination would also benefit conservation efforts for *H. robertianum*.

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Ethics Committee Approval

N/A

Peer-review

Externally peer-reviewed.

Author Contributions

The author confirms sole responsibility for the following: study conception and design, laboratory studies, data collection, analysis and interpretation of results, and manuscript preparation.

Conflicts of interest

The author declares that they have no conflict of interest.

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