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EFFECT OF ULTRASONIC AND VACUUM TREATMENTS ON THE GERMINATION OF *HIMANTOGLOSSUM ROBERTIANUM* (LOISELEUR) P. DELFORGEI (ORCHIDACEAE) SEEDS UNDER *IN VITRO* CONDITIONS

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Abstract

Every year millions of orchid tubers are destroyed despite their conservation status as international conventions. Orchid species are consumed more frequently because of their tubers used for food production. *H. robertianum* is one of the main species utilized for food purposes due to its large tuber size and glucomannan content. As orchid seeds do not contain endosperms, there are challenges associated with their asymbiotic germination and reproduction. Seeds should be treated with appropriate chemical treatment with the right amount and duration for their asymbiotic germination, and thus seed dormancy could be eliminated. In these treatments, the aim is to weaken seed coat and facilitate water penetration. To eliminate seed dormancy appeared due to seed testa, physical methods such as ultrasonic and vacuum treatments can also be used. Sometimes these methods may be more reliable and effective than chemical methods. In this study, the effects of ultrasonic and vacuum treatments on the germination of *Himantoglossum robertianum* seeds, which have dormancy, were investigated. For this purpose, ultrasonic and vacuum treatments were performed at different durations, and their effects were assessed. Both treatments were found to increase the germination rate of the seeds significantly compared to the control groups. The highest germination rate was found in seeds treated with ultrasound for 3 minutes (20.5%) and with vacuum treated for 10 minutes (15.2%) when compared to controls' germination, which was 1.9% and 0.8%, respectively. Ultrasound and vacuum treatments increased the germination of *H. robertianum* seeds by promoting water uptake. According to these results, the methods can be used safely for the germination of orchid seeds.

Key words: *Himantoglossum robertianum*, Ultrasonic and vacuum treatments, Germination.

Introduction

Orchidaceae is one of the largest group in flowering plants, with more than 28000 species spanning 763 genera (Christenhusz & Byng, 2016). However, orchidaceae family is also among the most threatened species of all flowering plants and at the front-line of extinction (Swarts & Dixon, 2009; Huang *et al*., 2018). It is well known that orchid seeds are generally difficult to germinate in comparison with other taxa, since they contain no endosperm and few nutrient reserves into the embryo to support the development and growth of the plant in the earliest stages (Baskin & Baskin, 2001; Slaviero, 2016).

Terrestrial orchid seeds can have high levels of innate dormancy (Swarts & Dixon, 2009). One of the most important exogenous seed dormancy barriers is the hydrophobicity of the seed coat surrounding the embryo. (Weston *et al*., 2005). In terrestrial orchids, the seed coat surrounding the embryo has been reported to contain phenolic compounds, cutin, lignin, suberin, polyphenols, nutrient reserves, lipids and cuticle materials (Yeung *et al*., 1996; Eriksson & Kainulainen, 2011; Yeung *et al*., 2017; Barsberg *et al*., 2018). In addition, the seed coat shows a hydrophobic structure (Slaviero, 2016; Miura *et al*., 2019). Despite the lack of endosperm in mature orchid seeds, their embryo contains few nutrient reserves. Weakening or removal of the seed coat using chemical scarification have proven to prompt germination in several orchid species highlighting the presence of physical dormacy (Slaviero, 2016). The first step in the germination process is imbibition, which means that the seeds absorb water from the surrounding environment (Weston *et al*., 2005). Treatments such as soaking the seeds into hypochlorite can be used to weaken the testa, and improves permeability and germination. These treatments are often used to eliminate physical or physiological dormancy (Miyoshi & Mii, 1998; Bae *et al*., 2010).

The use of sahlep has a long history in Turkey (Zillioğlu, 1985; Kasparek & Grimm, 1999). Sahlep is made by grinding the dried tubers of some natural Orchidaceae species. It is consumed in powder form as a hot drink in winter, and it is also the main component of Kahramanmaraş type ice cream and is used to add consistency and flavor to ice cream (Citil & Tekinsen, 2011). Sahlep harvesting is considered a major threat to Turkey's diverse and unique orchid flora (Sezik, 2002, Sezik, 2006; Tecimen *et al*., 2010; Molnár *et al*., 2017). *H. robertianum* is the most damaged species for the production of sahlep. The plants attract collectors because they are valuable for the production of sahlep and it is easy to see their large flowers (Korkut, 1986). The plants have 20-60 cm flower length and stems can grow up to 100 cm (Davis, 1984; Rossi, 2002). It was revealed that the plant height and attractiveness, combined with an early flowering (february to april) made the plant easier to recognize (Aedo & Herrero, 2005; Gutiérrez *et al*., 2018). Molnár *et al*. (2017) reported that there is a considerable relation between tuber size and harvesting density, and that large tuberous species may have a greater risk of extinction. It was determined that the most harvested species was *H. robertianum* individuals with 39.5%. *H. robertianum* has been overharvested because of increasing demand in the market (Teoh, 2019). The threat is even greater due to the destruction of natural habitats. This orchid is under threat of extinction because of habitat chance and overharvesting for edible use. Therefore, it is important to establish an efficient regeneration method.

It is known that orchid seeds have dormancy originating from the seed coat. It is known that the main reason for this is the difficulty of water penetration through the seed coat (Arditti & Ghani, 2000; Katsalirou *et al*., 2019). Because it may sometimes take months for water to penetrate into the testa, reach the embryo and start germination. Different treatments are used to facilitate water penetration into seeds and germination. To shorten the germination time, water is pushed into seeds through vacuum and pressure treatments (Miyoshi & Mii, 1998; Loveys & Jusaitis, 1994; Custódio *et al*., 2016). One technique utilised for degrading the testa is sonication (Lauzer *et al*., 1994). Sonication and chemical treatments cause cracks in the testa, facilitating the uptake of water and nutrients by the embryo (Miyoshi & Mii, 1988).

The inner and outer integuments may be thickened by lignin or cellulose, or contain waxy substances that make the seed impermeable. In the seeds that have developed a relatively impermeable seed coat, the entry of water and nutrients is prevented (Barsberg *et al*., 2013; Antonetti *et al*., 2021). Seeds with hard coats have been reported to require scarification with hypochlorite solutions to improve its effectiveness (Van Waes & Debergh, 1986; Sawma & Mohler, 2002). This is believed to occur through the breakdown and improved permeability of the seed coat, affecting both dormancy and germination (Rasmussen, 1995) and can be achieved using either NaOCl or Ca $(OCl)_2$ solutions (Miyoshi & Mii, 1998). In addition to these chemical treatments, physical treatments that mechanically facilitate water penetration through seed testa can break dormancy and increase seed germination.

The purpose of this study was to assess the effects of ultrasonic and vacuum treatments for different durations on the germination of *H. robertianum* seeds that are more difficult to germinate compared to other orchid species. For this purpose, seeds were subjected to ultrasonic and vacuum treatments for different durations. Following a 210-day incubation period, germination rates were determined, and the statistical differences between the treatments were identified.

Materials and Methods

Materials

Seed material, equipments and chemicals: *H. robertianum* seeds used in the study were collected from the seeds formed by open pollination at the Menemen Agricultural Research Institute at the time of full ripening in Spring 2019 and before the matured capsules opened. They were then air dried, and the capsules were cut in a controlled way. The seeds were placed in Eppendorf tubes and stored at + 4°C until the study was carried out.

Sigma-Phytamax P-6668 medium was used as germination medium. Seeds were disinfected with hydrogen peroxide (Sigma-Aldrich (Nord., 34.5-36.5%). Bandelin Sonorex (Typ DL 510 H, Berlin Germany) device was used for ultrasonic treatment of *H. robertianum* seeds. The vacuum device (Binder VD 23, Tuttlingen, Germany) was used in vacuum treatment. A vibrating laboratory shaker (Heidolph Unimax 2010/Schwabach, Germany) was used to ensure the penetration of hydrogen peroxide into the seeds. Purified water device (Elga DV 35-ELGA LabWater/UK) was used to obtain pure water. Medium pH adjusted with Hanna pH meter (H1991002) and autoclaved (Tomy SX-700e, Tokyo, Japan). Seeds were sown in biosafety cabinet (class II). The cultivated petri dishes were placed in a Digitally controlled cultuvation chamber (Lovibond TC 140 G-Liebherr, Dortmund, Austria). A stereo microscope (Irmeco, IM SZ550-B-ST5-H, Geesthacht, Germany) was used to monitor the germination of the seeds and for counting. Pictures were taken with a Nikon (Coolpix 990) camera.

Methods

Disinfection and pretreatments of seeds: *H. robertianum* seeds were placed in 2 ml Eppendorf tubes and subjected to superficial disinfection with 10% hydrogen peroxide for 20 minutes. Then the seeds were agitated in hydrogen peroxide for 40 minutes at a speed of 145 rpm. After that, they were rinsed three times with ultra-pure water.

Ultrasonic treatments: One of the methods used to ensure the penetration of water and nutrients through the impermeable seed coat is the application of sound waves to the seeds. Ultrasonic application has positive effects on promoting seed germination and breaking dormancy. Ultrasound duration can be an important factor in creating these effects (Nazari *et al*., 2014).

Ultrasound is a new physical method involving the application of sound frequencies to materials. When applied to solid materials, mechanical effects such as expansion and contraction are expected. This technique differs from other seed pretreatment methods in that it is simple, inexpensive, environmentally friendly and multifunctional (Aladjadjiyan, 2007; Goussous *et al*., 2010; Ghafoor *et al*., 2014; Guimarães *et al*., 2020).

The ultrasound bath tank was first filled with two liters of distilled water. The seeds soaked in pure water in 2 ml Eppendorf tubes with the tube platform and inserted into the ultrasound tank. To facilitate seeds' water intake, they were subjected to ultrasonic treatment for 0, 3, 6, 12, 24, 48 and 96 minutes at 35 kHz. During ultrasonic treatment, water temperature was kept at 25±2°C by adding ice from time to time.

Vacuum treatments: There are different theories that vacuum applied seeds eliminate substances that prevent wetting and germination (Hicks *et al.,* 2007). It was reported that intercellular spaces can create a short-term thermodynamic instability by maintaining a pressure difference between the interior of the tissues and the outer solution (Elmoazzen *et al*., 2005). When vacuum is applied, the air will be removed from the intercellular spaces, eliminating the pressure difference in the tissue, thus allowing the tissues to expand rapidly with the flow of the solution into the seed (Kazutaka *et al*., 2012; Nadarajan & Pritchard, 2014).

This technique was applied to impermeable orchid seeds (Antonetti *et al*., 2021) to allow water penetration. The seeds soaked in pure water in Eppendorf tubes were placed on the platform and inserted into the vacuum device. They were treated with 380 mm/Hg vacuum at a temperature of 27.3°C

in the vacuum device for 0, 5, 10, 20, 40 and 60 minutes. The vacuum was released slowly after treatments. Room atmospheric pressure was used as control.

Preparation of the medium: Sigma-Phytamax P-6668 medium was used in germination (Table 1). Since *H. robertianum* mostly grows in calcareous soils in its natural environment (Davis, 1984; Rasmussen, 1995; Rossi, 2002), the pH of the medium was adjusted to 7 using 0.1 N HCl. 7 g/L agar was added for medium hardness. After pH adjustment, the medium was autoclaved at 121°C for 20 minutes. It has cooled to 65-70°C, then one liter of medium was poured in 60 petri dish in flow cabinet.

Table 1. The composition of the germination medium.

Germination medium components Sıgma-Phytamax P-6668)	Amount/Medium (mg l-1)
Ammonium nitrate	825.0
Boric acid	3.10
Calcium chloride anhydrous	166.0
Cobalt chloride hexahydrate	0.0125
Cupric sulfate pentahydrate	0.0125
Disodium EDTA dihydrate	37.240
Ferrous sulfate heptahydrate	27.850
Magnesium sulfate anhydrous	90.350
Manganese sulfate	8.450
Potassium iodide	0.4150
Potassium nitrate	950.0
Potassium phosphate monobasic	85.0
Sodium molybdate dihydrate	0.1250
Zinc sulfate heptahydrate	5.30
Charcoal	2000.0
MES (free acid)	1000.0
Myo-Inositol	100.0
Nicotinic acid (free acid)	1.0
Peptone type I	2000.0
Pyridoxine hydrochloride	1.0
Sucrose	20000.0
Thiamine hydrochloride	10.0
Dosage	27.3 g of powder are used to prepare 1 L of medium.

Table 2. Developmental stage of *In vitro* asymbiotically cultured *H. robertianum* according to Yamazaki & Miyoshi (2006).

Phase	Description
0	No germination phase: No growth of embryo occurs
1	Pre-germination phase: Embryo swells to fill the seed coat
2	Germination phase: Embryo emerges from the seed coat
3	Protocorm phase: Embryo is completely discharged from the seed coat
4	Rhizoid phase: Rhizoids are formed on the protocorm surface
5	Shoot phase: Shoot is differentiated from the protocorm

Sowing seeds and maintaince of cultures: Seeds were sown in sterile plastic Petri dishes with a diameter of 90 mm. Petri dishes were wrapped with a double layer of transparent stretch film. Seeds of many orchids germinated in high percentages when incubated at constant temperatures (Baskin & Baskin, 2014), Petri dishes were placed in a cultivation chamber set at 20°C (± 0.1°C) constant temperature. The surface of the cultivation chamber was covered with aluminum foil to provide a dark environment. Each of the treatments were performed in nine replicates. The average number of full seeds in Petri dishes varies between 280 and 300.

Seed count and statistical analysis: The number of seeds in the grids were counted periodically. Germination counts were carried out in the same grid area by determining 3 cm square areas in each Petri dish. During the 210-day incubation period, the counts were made every 15 days for the first 90 days, and then at 30-day periods. The seeds were sown on October 2019 and observed periodically and were counted by stereo microscope under 3.35- to 180x-fold magnification. Germination phase of the seeds were evaluated according to Yamazaki & Miyoshi, (2006) (Table 2, Fig 1).

The germination percentages were calculated using the following formula for each medium (Acemi *et al*., 2019).

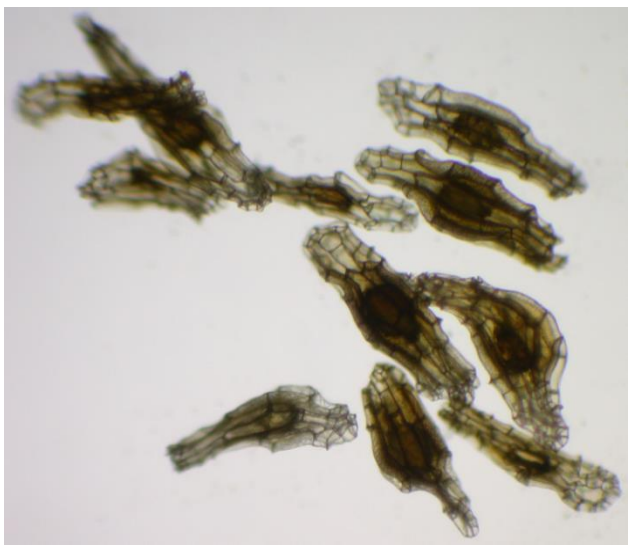
$$\text{Germination percentage (\%)} = \Sigma \text{ Seed number (Stage 2}-4) \times 100 / \Sigma \text{ Seed number (Stage 0}-4).$$

Arc-sin transformation was applied to the % values obtained by germination. Data were subjected to analysis of variance (ANOVA) and means were compared by Duncan's multiple range test at $p<0.05$ using the SPSS ver. 22 (IBM SPSS ver 22). The effects of ultrasonic and vacuum treatments durations on the germination rates were analysed with the Duncan multiple comparison test.

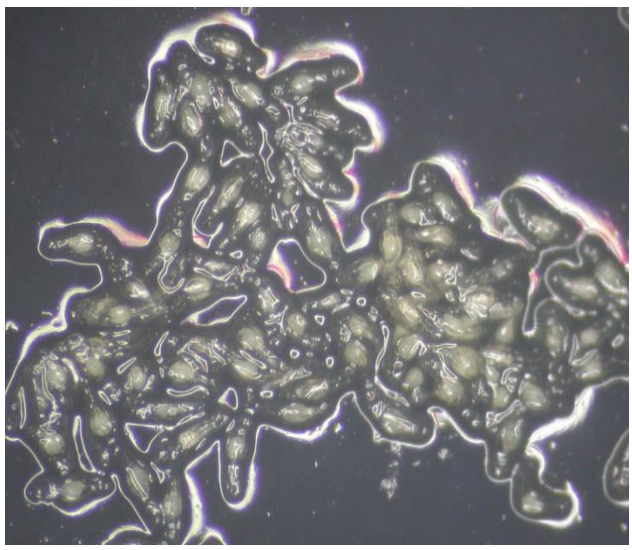
Results and Discussion

Ultrasonic treatments: *H. robertianum* is an orchid that is difficult to germinate and takes a long time to germinate (Clevo *et al*., 2017). This germination difficulty is mostly due to the impermeable structure of the seed coat (Pierce & Belotti, 2011). If the dose and duration of the chemicals applied to make the seed coat permeable are not chosen appropriately, the seeds may be damaged. Another way to make the seed coat permeable is ultrasounic applications. In particular, shortening the germination period will facilitate the production of this species and ensure its protection.

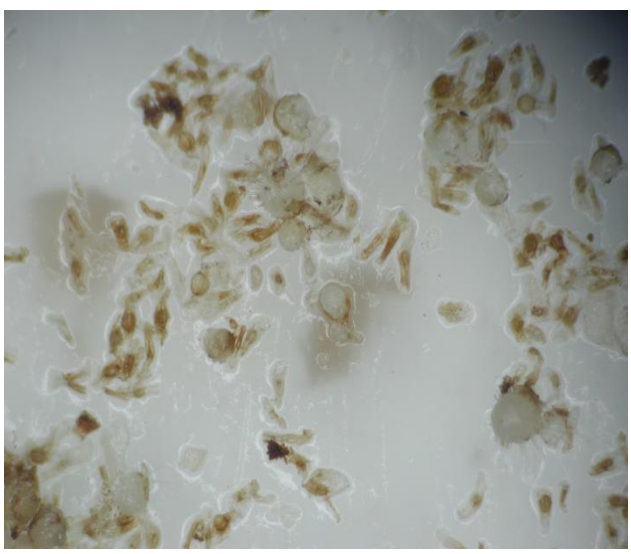
With the method used in our study, the germination rate of *H. robertianum* seeds was increased and the germination period was shortened. The analysis of variance on the data collected in the study revealed that the ultrasonic treatment was an effective method for the germination of *H. robertianum* seeds (Table 3). As shown in (Table 4) the lowest germination rate among the applied treatments occurred in the control group. The germination rate of the control group was 1.9%, and the germination rate was 20.5% in the seeds subjected to ultrasonic treatment for 3 minutes (Fig. 2). However, by increasing duration of ultrasonic treatment ended up with decreasing germination percentage, still statistically better than controls.



Stage 0. No germination phase. No growth of embryo occurs

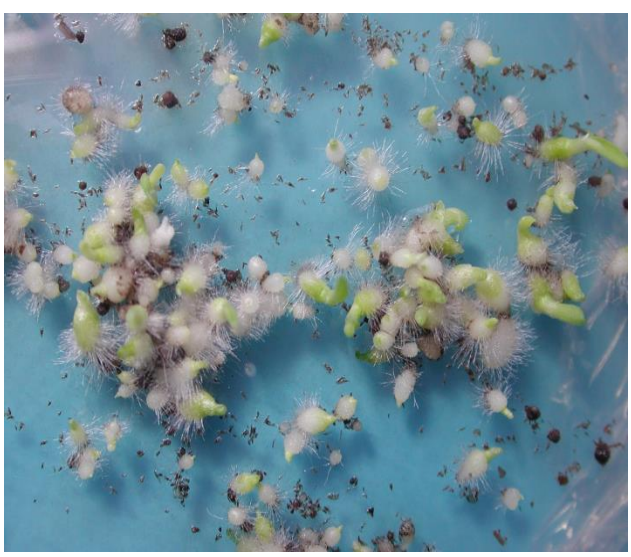


Stage 1. Pregermination phase. Embryo swells to fill the seed coast



Stage 2. Germination phase. Embryo emerges from the seed coat

Stage 3. Protocorm phase. Embryo is completely discharged from the seed coat



Stage 4. Rhizoid phase. Rhizoids are formed on the protocorm surface

Stage 5. Shoot phase. Shoot is differentiated from the protocorm

Fig. 1. Developmental stages of *In vitro* asymbiotically cultured *H. robertianum* according to Yamazaki & Miyoshi, (2006).

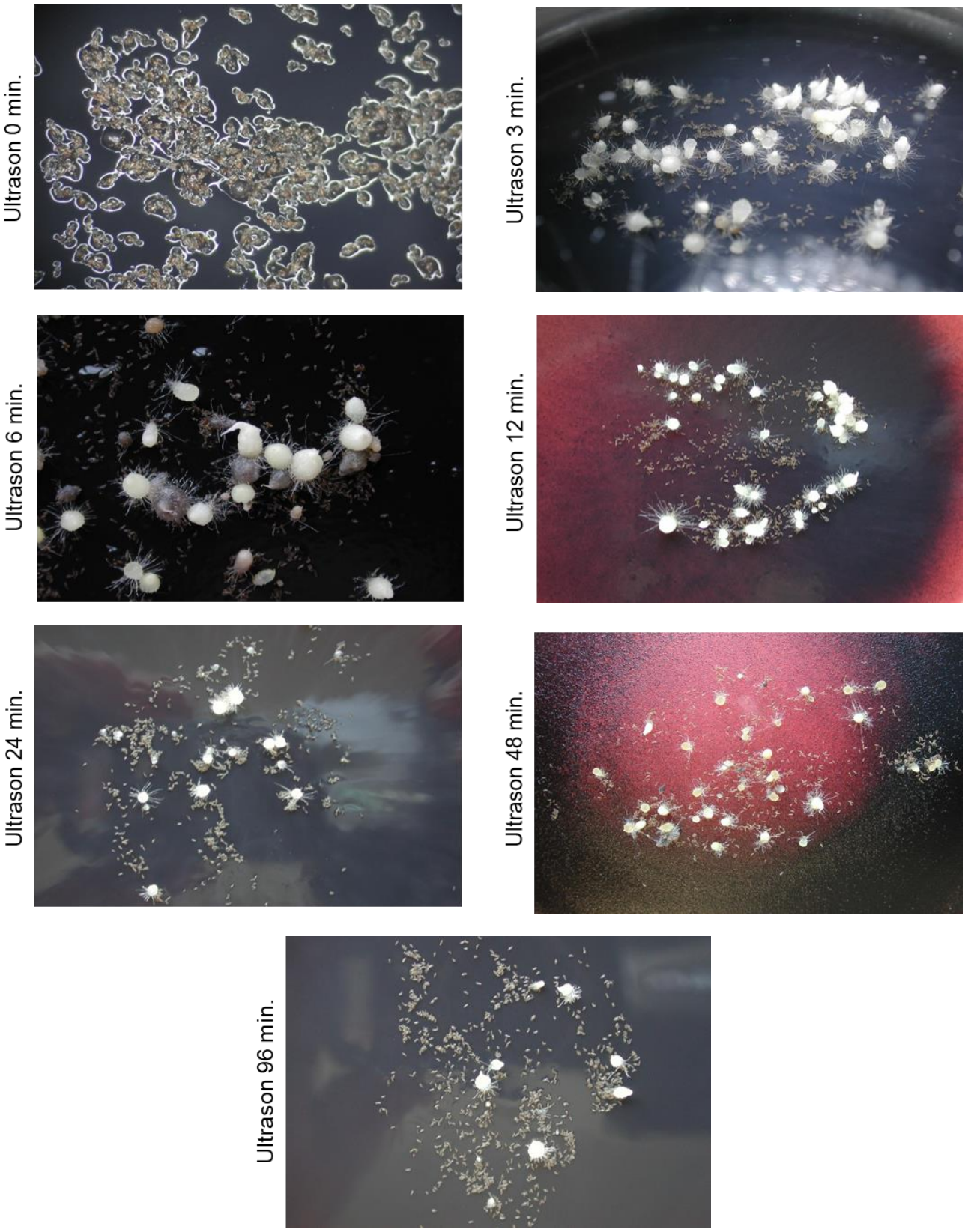


Fig. 2. Wiev of ultrasonic treatments on 210 days after seed sowing.

Table 3. Efficiency of ultrasonic treatments for *H. robertianum* germination.

	Sum of squares	df	Mean square	F	Sig.
Between groups	0.130	6	0.022	13.732	0.000
Within groups	0.060	38	0.002		
Total	0.190	44			

Table 4. Duncan tests of ultrasonic treatments for *H. robertianum* germination.

Ultrasonic treatment	Petri dish (N)	Average germination (±se)
0 min (Control)	5	0.0186 ± 0.003a
3 min.	6	0.2070 ± 0.027 c
6 min.	9	0.1113 ± 0.020 b
12 min.	6	0.0595 ± 0.005a
24 mn.	6	0.0683 ± 0.013 ab
48 min.	5	0.0646 ± 0.010 ab
96 min.	8	0.0605 ± 0.006 a

(Means and standard errors within a column followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$)

Our findings are consistent with those reported by previous studies. Ultrasonic treatment creates small cracks in orchid seeds, which are enlarged with water intake. As a result, seed testa becomes more permeable, and this increases germination (Neiland, 1994; Rasmussen, 1995; Ribera & Vicient, 2017). There are different studies on the increase of germination in seeds applied ultrasound. For example, ultrasonic treatment of *Calanthe discolor* seeds may break the coating and increase seed germination (Miyoshi & Mii, 1988; Kull & Arditti, 2002). Lauzer *et al*., (1994) also used sonication to improve germination of *Cypripedium acaule*. The seed germination rate was significantly increased by sonication treatment. While control seeds germinated less than 10%, whereas 60% germination occurred in seeds that were sonicated for 4-16 minutes (Miyoshi & Mii, 1988). In *Calanthe tricarinata*, mature seeds pretreated with ultrasound were effective in improving the germination (Lee *et al*., 2007). After ultrasonication pretreatment, a strong correlation was found between triphenyl tetrazolium chloride (TTC) embryo staining (embryo coloring) and seed germination (Zhang *et al*., 2015). Pretreatment of mature seeds with ultrasound for 15 to 60 minutes improved germination. Seed pretreatments may increase orchid seed germination by affecting the physical characteristics of the testa (Lee *et al*., 2005; Lee *et al*., 2007). In this study, germination was found to decrease as the ultrasonic treatment time increased (Fig. 3).

There are studies arguing that long-term ultrasonic treatment of seeds may damage the embryo. Miyoshi & Mii, (1988) found that the degree of damage to the embryo increased when the *Calanthe discolor* seeds were subjected to ultrasonic treatment for more than 8 minutes. However, the 7-minute treatment decreased germination when compared to the 3.5-minute (Dutra *et al*., 2008). In addition, treatment time significantly affected seed germination and protocorm formation of *Calanthe* hybrids. While protocorm formation rise up to 7 minutes, increasing ultrasonic time significantly reduced protocorm formation (Shin *et al*., 2011). The effects of ultrasound treatments on seedling growth and stimulate seed germination have been investigated in many seed species and positive results have been obtained (Miyoshi & Mii, 1988; Aladjadjiyan, 2002; Yaldagard *et al*., 2008; Shekari *et al*., 2015; Sharififar *et al*., 2015; Ding *et al*., 2018; Ghaghelestany *et al*., 2020; Kibar, 2020; Kalita *et al*., 2021; Erken, 2021). These earlier studies are correlating with our findings.

Ultrasonic treatment increases also the germination speed of the seeds. In our study, the seeds that were treated with ultrasound for 3 minutes were found to start germination in 45 days protocorm was formed, while in the control group, germination and protocorm formation started in 60 days. The protocorm formation in the control group reached the same rate as the seeds treated with ultrasound for 3 minutes in 120 days. A similar difference regarding germination was demonstrated by Yararbaş (2008) for *O. italica* seeds treated with ultrasound, and germination time was found to decrease 6 times compared to the control group. *O. italica* seeds treated with ultrasound had a germination rate of 81% in 15 days, while the control seeds reached a germination rate of 89% in 90 days. These results suggest that the developed methodology may be a useful method for the propagation of endangered orchid species and for commercial use in orchid cultivation.

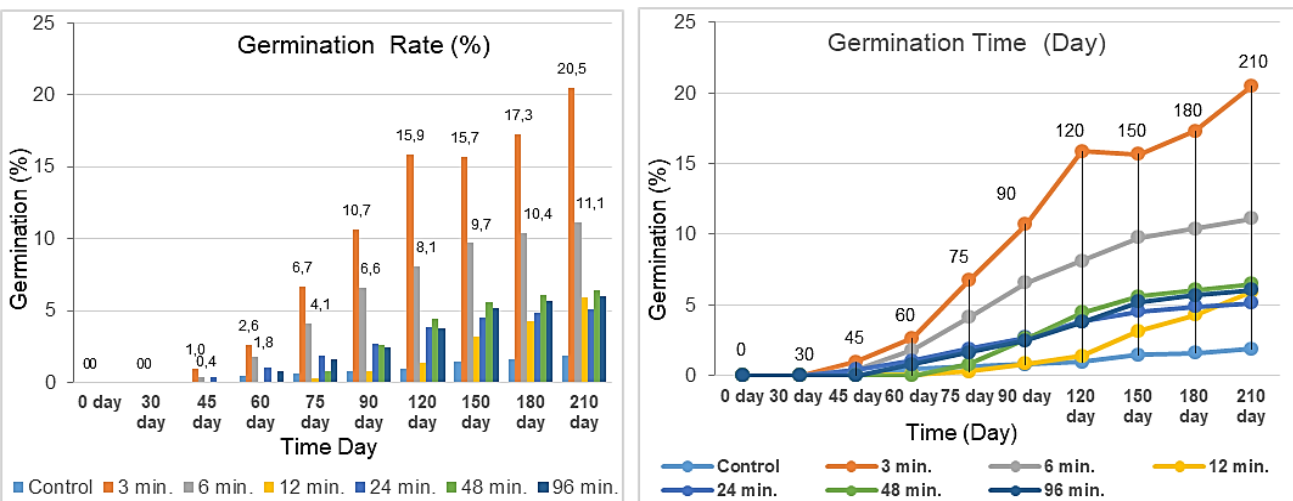


Fig. 3. Germination rates (left) and speeds (right) by the ultrasonic treatment time.

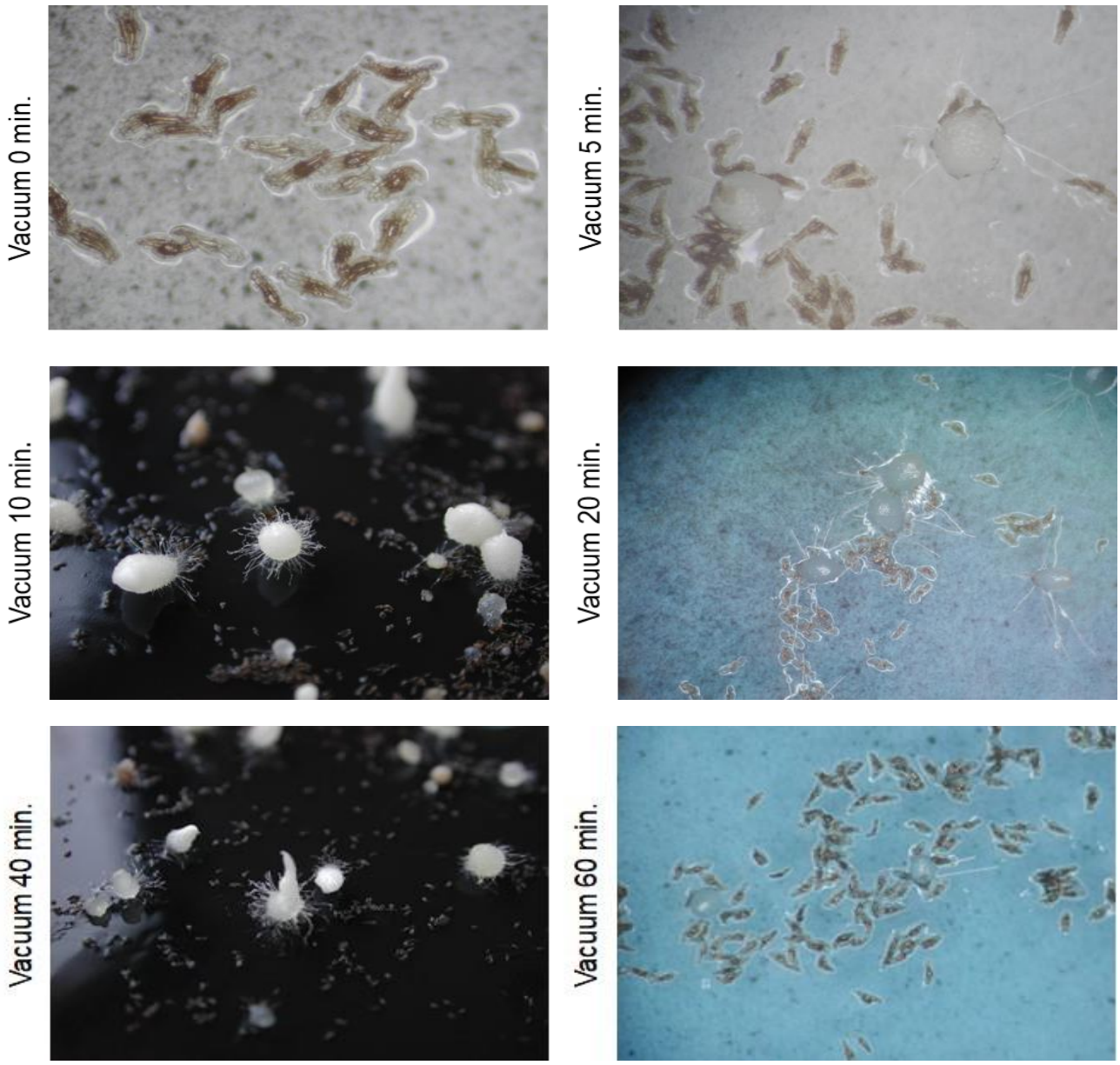


Fig. 4. Wiev of vacuum treatments on 210 days after seed sowing.

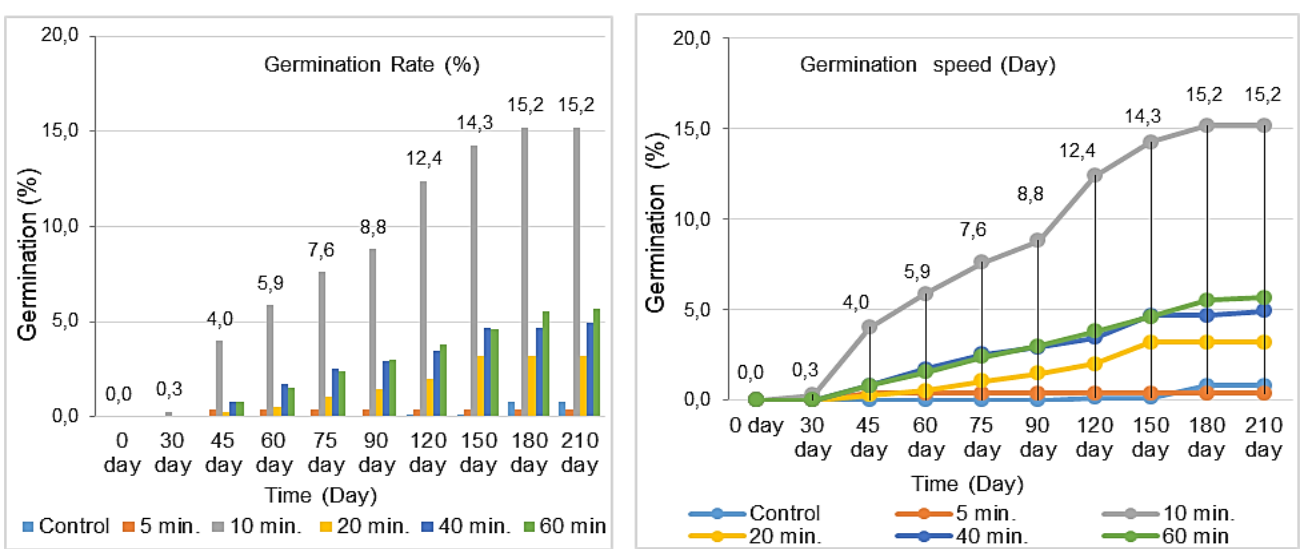


Fig. 5. Germination rate (left) and speed (right) by vacuum treatment time.

Vacuum treatments

The analysis of variance revealed that the vacuum treated seeds were germinated earlier and germination rates were found higher than the control group (Table 5).

It has been shown that applying vacuum to *H. robertianum* seeds is statistically effective on germination rate and rates. The highest germination rate was found in seeds with 10-minute vacuum treatments (Table 6). By the end of the 210-day incubation period, the germination rate of the control group was 0.8% while in the seeds treated with vacuum for 10 minutes was 15.2%. The seeds treated with 380 mm/Hg vacuum for 10 minutes started germinating in 30 days while the first germination occurred in 120 days after sowing in the control group (Fig. 4).

There are few studies that focus on vacuum treatment of orchid seeds and the effects on germination. Custódio *et al*., (2016) found that tetrazolium intake of the *Dactylorhiza fuchsii* seeds increased substantially when treated with 380 mm/Hg vacuum for 1 minute. The optimal germination was found in the seeds treated with vacuum for 2.5 and 5 minutes (85% of viability), whereas it decreased when seeds were treated for more than 10 minutes. In the study, no difference was found between 380 mm/Hg and 0 mm/Hg vacuum treatments. Vacuum infiltration has been shown to improve germination of orchid seeds. Also, applying a vacuum degassing treatment (Daws *et al*., 2006) has been shown to improve the germination in oily *Pinus* seeds. Treatment of *Pterostylis banksii* seeds for 30 minutes in 100% vacuum showed a significant 15% increase in viability compared to control treatment (Diantina *et al*., 2020). According to Erken, (2021) vacuum application affects germination positively in *Verbascum yurtkuranianum* seeds. The results obtained from the study appear to be in compatible with the results of previous studies obtained from vacuum-treated seeds.

Table 5. Efficiency of vacuum treatments for *H. robertianum* germination.

	Sum of Squares	df	Mean Square	F	Sig.
Between groups	0.045	5	0.009	3.860	0.026
Within groups	0.028	12	0.002		
Total	0.072	17			

Table 6. Duncan tests of vacuum treatments for *H. robertianum* germination.

Vacuum treatment	Petri dish (N)	Average germination (± se)
0 min (Control)	3	0.0080 ± 0.002 a
5 min	3	0.0040 ± 0.002 a
10 min.	3	0.1531 ± 0.065 b
20 min.	3	0.0320 ± 0.014 a
40 min.	3	0.0494 ± 0.012 a
60 mn.	3	0.0567 ± 0.009 a

(Means and standard errors within a column followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$)

Conclusion

In the present study, ultrasonic and vacuum treatments of *H. robertianum* seeds were found to inducing seed germination compared to the control group. Ultrasonic treatment was found to be better than vacuum treatment with regard to the penetration of water into seeds and breaking of dormancy. The highest germination in ultrasonic treatment was 20.5% and 15.2% in vacuum treatment. The seeds treated with ultrasound for 3 minutes germinated 10.8 times more than the control seeds, while the vacuum-treated seeds germinated 19 times more than the control group. If appropriate treatment time and dosage are not used to overcome dormancy with chemical substances, seeds will likely be damaged. Therefore, it was found that 3-minute ultrasonic treatment of seeds of *H. robertianum*, known to be an endangered species, was safe and could be used successfully for their germination.

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