SZENT ISTVÁN EGYETEM

Methodological development of *ex situ*, *in vitro* propagation of *Himantoglossum adriaticum* - adriatic lizard orchid

PhD thesis

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1. INTRODUCTION AND OBJECTIVES

The topic of my doctoral dissertation is the possibilities of *ex situ* propagation of wild orchid species in Hungary. The *Orchidaceae* family with its nearly 880 genus (CHASE et al. 2015) and 25-30.000 species is one of the largest among plants (CHASE et al. 2003; PILLON és CHASE. 2007). At the same time, it is probably one of the most species-rich, but one of the most vulnerable plant group as well (CLEMENTE 2009). The Orchidaceae family includes herbaceous, perennial species, and according to their life form, they can be epiphytic, lithophytic or terrestrial species. However, among the orchids rooted in soil or trees, it is also worth mentioning the crawling lifestyle, which is very common in the tropics, especially in the subfamily of *Vanillioidae*. In the temperate zone only terrestrial orchid species can be found in the wild, so in Hungary. Their seeds are minute, and in the absence of endosperms, these seeds are incapable of germination alone, which requires so-called „orchid mycorrhizas", symbiotic fungal partners. Their *ex situ* germination and cultivation is a great challenge even for professionals. Because of their extraordinary beauty, the everlasting ambition of the human race and their attraction to exotics, more and more attempts are being started to breed and cultivate them far from their habitats. In addition, most of them are very sensitive to the slightest change in their environment. That is why they have become such endangered species with such rapidity and magnitude. Over the past 30 years, the natural habitats of European terrestrial orchids have been decreasing with an increasing tendency (BARMAN and DEVADAS 2013), the population decline in more and more cases is irreversible and leads to the complete extinction of the population. The loss of natural habitats is not only due to human intervention, but also to the climate change, which causes significant, most often negative effects on the abiotic and biotic balance of habitats on orchid populations. Due to the high level of trade caused by the desire to collect and possess them, several species have become threatened with extinction. To prevent this, the CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna), issued in Washington in 1973, plays a huge role in protecting all species of orchids, thus their trade became regulated by their appropriate conservation category (Appendix I, Appendix II.) (MATHEW and BURTON 1994). Hungary's law of LIII. 1996 (Law on Nature Conservation) intended to protect them as well, according to which all species of orchids in Hungary are protected *ex lege*. The Convention on Biological Diversity, which was concluded in Rio de Janeiro in 1992, is proclaimed by Hungary with the law of LXXXI 1995. n the spirit of the Convention, Hungary has undertaken to preserve the diversity of Hungarian species and biological systems. Article 9th deals with the obligation of *ex situ* conservation. I also conducted

and prepared my researches and dissertation on the *ex situ* conservation of orchids in Hungary in the spirit of this Convention. In the course of my PhD research, the main objectives of my work were the following:

- Development and optimization of germination and culture conditions of *Himantoglossum adriaticum in vitro*: determination of the pH optimum on modified Fast medium for seed germination, determination of potassium (potassium chloride) and nitrogen (ammonium nitrate) optimum for seed germination, examine the effect of coconut water and silver nitrate on the seedlings *in vitro* and the determination of the physical parameters and optimal culture conditions of the *in vitro* plants.
- Cultivation of fungal partners of *Himantoglossum adriaticum* root on malt agar with benomyl, malt agar with bengal rose and PDA medium.
- Determination of pH optimum of the fungal species grown out from the root of *Himantoglossum adriaticum* on PDA medium, and compare the results with the pH optimum of *H. adriaticum*.
- Symbiotic germination experiment on *Himantoglossum adriaticum* seeds and the fungal species grown from root, on H1 oat medium.
- Determination of endo- and ectomycorrhiza fungal partners from the root of *Himantoglossum adriaticum* by molecular genetic methods.
- Transplantation of *in vitro Himantoglossum adriaticum* plants and observation of their survival success in a growth medium made by us and in the soil brought from its natural habitat.

2. MATERIALS AND METHODS

2.1.*In vitro* asymbiotic germination of *H. adriaticum*

2.1.1. Origin, storage and quantity of *H. adriaticum* seeds used for *in vitro* asymbiotic germination

The seeds of *Himantoglossum adriaticum* used for *in vitro* germination experiments were obtained from four Hungarian habitats of the species: from Nagytevel, from the Keszthelyi-hills, from Sümeg-Tapolca and from the Kőszegi-hills. The seeds were harvested between July and August 2016-2019, when the fruits were ripened and their capsules dried up. During seed collection, our basic plan was to collect 3-3 capsules from 10-10 individuals of the four habitats in every year, then to make a seed mixture from them before every sowing. Due to the number of different flowering specimens and different amount of ripened capsules in the populations, we changed the collection strategy each year. This was done because the priority in each case was to collect as many capsules and seeds from the habitats which does not affect the survival of the population. Based on our previous studies, we have determined during the analysis of variance that there is no significant difference between the germination averages of seeds from different habitats (GILIÁN 2015). Thus, it was known during the sowing that neither the different origin can be a problem while sowing, nor the proportion of seeds from the habitats in the seed mixture. The seeds were stored in a dark refrigerator at 4°C in 2 ml eppendorf tubes until use, without any treatment of drying them. Approximately 500-500 seeds were sown on the surface of the media in every flask during each experiment of asymbiotic germination.

2.1.2. Experiments of *in vitro* asymbiotic germination, modifications of the media

All the experiments were made in Szent István Univesity, in the laboratory of the Institute of Genetics, Microbiology and Biotechnology, under sterile conditions.

The basic media I used for the experiments was modified Fast medium (R. ESZÉKI and SZENDRÁK 1992), and I modified it every time according to my experiments (Table 1.).

I sterilized the media on 120°C for 40 minutes after boiling, then, after cooling, I poured the media into flasks under laminar flow. After solidification I put folpack ont he top of the flask. The medium was prepared 5-7 days before each sowing. The time between preparation of media and sowing was used as incubation time.

Table 1. The amount of ingredients I modified in the media during different experiments.

Experiment	**Ingredient(s)**	**New quantity**
Changing Peptone	Triptone (peptone from casein)	1g/1200ml
Changing Fe-EDTA	MS Mesoelements	6ml/1200ml
Heller-mikro	Heller-mikro	0,6ml/1200ml
Optimizing pH	↑NaOH, ↓HCl	pH 4,5 – 8,5 beállításához megfelelő
Optimizing K	KCl	40;120;200;280;360 mg/1200ml
Optimizing N	NH_4NO_3	40;120;200;280;360 mg/1200ml
Observing bactericidal, growth promoter, ethylene inhibitor	$AgNO_3$	4mg/1200ml
Observing germination- and growth promoter	Coconut water	10;20;30;40;50 ml/1200ml
Observing bactericidal, germination- and growth promoter, ethylene inhibitor effects together	$AgNO_3$ + Coconut water	4 mg/1200ml + 25ml/1200ml

During each experiment, I modified the followings in the medium: I replaced the peptone with tryptone each time. The reason of this is that peptone is usually a soy protein, which has already been partially degraded by the papain enzyme. In contrast, tryptone is usually casein (a milk protein), which is partially degraded by trypsin. Compared to tryptone, peptone has a higher carbohydrate content but a lower weight percentage rate of nitrogen. In order to increase the absorbable nitrogen content of the medium, I chose tryptone. Fe-EDTA was added to the media as "MS mesoelements" each time. MS Mesoelements is technically equivalent to Fe-EDTA, but is individually prepared in the laboratory of the Institute of Genetics, Microbiology and Biotechnology at Szent István University, and it is a liquid, so it can be added very accurately. The components of the Heller micro (special micronutrients) remained the same, but I received a new concentrate solution from Eszter R. Eszéki, the head of the Orchid Lab at ELTE Botanical Garden. so I reduced it to 6 ml /1200 ml.

In 2016 and 2017, in order to determine the optimum pH for the species, 1 molar hydrochloric acid was used to decrease the pH of the medium and sodium hydroxide to increase it. These were used to set the following five pH: pH 4.5; pH 5.5; pH 6.5; pH 7.5; pH 8.5.

In the case of the seeds collected in 2018, the potassium chloride content of the medium was changed to 40; 120; 200; 280 and 360 mg / 1200 ml to determine the optimal potassium requirement of the species. In parallel, in an other experiment, to determine the opimal ammonium nitrate requirement of the species, the ammonium nitrate content of the medium was changed to 40; 120; 200; 280 and 360 mg / 1200 ml. In both experiments, the medium was adjusted to the optimal pH already determined on the basis of the results of the previous years' experiments. In 2019, the coconut water used for the experiments with coconut water was a DM-brand organic coconut water obtained from green coconuts as a food product.

When sowing the seeds harvested in 2016, I made 12-12 flasks for each pH (60 flasks in total), and when sowing the seeds collected in 2017, I made 5-5 flasks, so 25 flasks in total. Unfortunately, the seeds harvested in August 2016 were not sown until January 30, 2017 due to the late arrival of the laboratory equipments. At this point, the seeds were stored for 5 months in the refrigerator at 4^{o}C without any treatment of drying. The seeds harvested in August 2017 were sown 4 days after harvesting. In the case of seeds harvested in 2018, either for the experiment to find the optimal amount of potassium chloride and ammonium nitrate, 3-3 flasks/amount of KCl or NH_4NO_3, so 15-15 flasks in total were sown.

2.1.3. *In vitro* seed sowing, storage of flasks until the first protocorms appear

Before sowing *H. adriaticum* seeds *in vitro*, the seeds were pre-treated with calcium hypochlorite, whereby 10 g of calcium hypochlorite was dissolved in 90 cm^3 distilled water, then filtered through a filter paper, and that filtrate was used for sterilizing the seeds. We poured the filtrate onto the seeds until the 1/3 of the test tubes, closed the top of the test tubes with plugs, and let the seeds stay in the filtrate for 10 minutes, while shaking them sometimes. After 8 minutes, we turned the test tubes upside down, so that the seeds go to the top of the filtrate, and when removing the plugs, the seeds stay sticked on the side of the test tubes.

The pretreated seeds were applied under laminar flow with sterile scalpels from the sides of the test tubes to the surface of the media, then spread with a drop of sterile distilled water on the media using a sterile glass rod to distribute the seeds uniformly.

After that, the flasks were covered with foil to prevent the infections and dehydration, then placed in a box at room temperature (24^{o}C) for one week, stored in darkness.

After the incubation period, the flasks were placed into 4°C temperature, darkness, for 3 months, then, until the germination started, the flasks were stored in a dark box at 22-24°C.

2.2.*In vitro* asymbiotic growth experiments of *H. adriaticum* plants

In June 2017, flasks containing protocorms from seeds sown earlier in 2014 were placed in rooms of different conditions (temperature and light sources) to determine the optimal temperature and light requirements for the *H. adriaticum* seedlings.

The first room was a fixed 21°C lightroom with POLYLUX 36W/F840, 4000K fluorescent lamps, with 16 hours light, 8 hours dark period of artificial lighting. The second room had a south-eastern window, with summer temperatures between 24-30°C, with natural light. The third room had a north-facing window, with summer temperatures between 22-26°C, with natural light. The fourth room had a south-eastern window, and was an air conditioned, fixed 24°C temperatured room with natural light.

To represent the results, three categories were distinguished based on the condition of the *in vitro* cultured plants: green, brown, and dead. The "green" category indicates that everything is okay with the conditions. "Brown" already indicates some negative change in circumstances. There are two options in this category for the future: either a new, green, healthy shoot will start or the plant dies. So this category indicates the state between the two others, which is worse than the green state, but can even be reversed. The "dead" category is obviously the result of the inadequate conditions for the *in vitro* plants.

In 2019, two new components were used in my experiments to help the development of the seedlings besides the already specified components of the optimal medium: silver nitrate and coconut water. In separate experiments, I observed the addition of 4 mg / 1200 ml silver nitrate; 10; 20; 30; 40 and 50 ml / 1200 ml coconut water; and 4 mg / 1200 ml silver nitrate combined with 25 ml / 1200 ml coconut water on the development of *H. adriaticum* seedlings. These flasks were stored in the lightroom of the ELTE Botanical Gardens Orchid Laboratory, in fixed temperature of 24°C, next to the window, in natural light, protected from the lightrooms fluorescent light.

In 2019, I made 10 flasks for silver nitrate medium, 10 for coconut water and 25 for silver nitrate + coconut water, then placed 1-1 seedling with approximately 0.3 to 1 cm shooting in each flask.

2.3.Determination of mycorrhizal fungal partners from the root of *H. adriaticum*

For the determination of mycorrhizal fungal partners of *H. adriaticum*, root samples were derived from individuals from the habitat of the Kőszegi-mountains. The root samples were collected in April 2019 and placed the next day on the pre-made medium under sterile conditions in the laboratory of the Institute of Plant Protection of Szent István University.

2.3.1. Cultivation of mycorrhizal fungi on different media

Mycorrhiza fungal cultivation experiments were performed in the laboratory of the Institute of Plant Protection of Szent István University. Three types of media were used to grow fungi. The first was malt agar with benomyl (BM) because benomyl is selectively toxic to most of the members of Ascomycetes, while members of Basidiomycetes are largely resistant. So it was hoped that basidiomycetes, which are known to be orchid mycorrhizal fungi, will grow on these media (BOLLEN and FUCHS 1970).

The second type of medium was malt agar with bengal rose (BRM) because bengal rose slows down the growth of fungi. So we hoped that the growing fungi might be easier to isolate, observe and inoculate (SMITH and DAWSON 1944).

For these two media, the basic medium was malt agar for fungi cultivation developed by THOM and CHURCH (1926).

The third type of medium was the standard version of potato-dextrose-agar (PDA) medium which is the most commonly used for fungal cultivation (DHINGRA and SINCLAIR 1986), since fungi grow best and fastest on it. Chloramphenicol was added to the medium due to its antibacterial action to prevent the growth of bacteria.

When the roots were collected in April 2019, they were thoroughly washed in tap water and transported to the laboratory. The root samples were thoroughly rinsed again in tap water after arrival and cut into 1-2 cm pieces. Two third of the samples were placed in 1% sodium hypochlorite (NaOCl) for 5 minutes. Then, these samples were washed three times for 10 minutes with sterile distilled water and placed on Petri dishes containing BM, BRM and PDA media. The remaining 1/3 of the samples were pre-treated differently: after washing them with tap water, the root pieces were placed in 70% ethanol for 30 seconds, then in 4% sodium hypochlorite for 90 seconds. The samples were then washed three times for 5 minutes in sterile distilled water and placed on Petri dishes with PDA medium.

After placing the root segments on the media, fungi were allowed to grow and then inoculated continuously into fresh PDA media until pure cultures were obtained. Photographs of the pure cultures were made and samples were analyzed by morphological features using an Olympus microscope with phase-contrast illumination at 400x and 600x magnification.

2.3.2. Determining the optimum pH for the cultivated fungi

To determine the pH optimum of the different cultivated fungal species grown on BM, BRM and PDA media, pH range of pH 4.5; pH 5.5; pH 6.5; pH 7.5 and pH 8.5 were prepared in 5.9 cm diametered Petri dishes. 5 mm diametered samples of each fungi were placed onto

the middle of these media, and the growth rate of the fungi on different pH was monitored in a 14-day experiment.

Three replicates were made from each fungal species on the 5 different pH, so we had 15 Petri dishes per fungal species. During the inoculation we were working under laminar flow with sterile tools in the laboratory of the Institute of Plant Protection of Szent István University.

The Petri dishes were scanned once a day for 14 days. Every day, the mean growth area was measured in mm^2 using ImageJ program. From the data obtained, R program was used to plot the different growth rates of each fungal species along the pH gradient. Then we compared the obtained pH optimum of fungi with the optimal soil and medium pH of *Himantoglossum adriaticum*.

2.3.3. Isolation of the given cultured fungal species in Petri dish with molecular genetic testing

The cultivated fungus which appeared to be the most mycorrhizal-like under the microscopic observation was given to the DNA Laboratory of BIOMI Biotechnology Services Ltd. for isolation. The other cultured specimens were not given, because, when observed under the microscope, it was unequivocally stated that non-basidiomycete fungi were grown on the media. These were species that could be identified under the microscope on family or genus level, because those were fungal species commonly found in the soil. Specifying these species accurately by molecular genetic testing would have been very costly and unfortunately we did not have the budget for that. However, they were included in the *in vitro* experiment, and if they were found to give positive result, they could subsequently be determined at species level.

The DNA was isolated using the ABI Prepman Ultra Kit (INTERNET-1). The evaluation was done with BioNumerics version 7.6.3 (made by Applied Maths NV. Available from http://www.applied-maths.com).

2.3.4. Determination of *H. adriaticum* fungal partners by molecular genetic analysis of a root segment

To isolate the fungal partners, one root segment of about 10 cm length was selected from the population of the Kőszegi-mountains. Next to the mother plant, the soil was gently scraped until one of the emerging roots was carefully cut off. Subsequently, the plant was placed back into the soil carefully, taking care to minimize the damage. The cut root is thoroughly washed by water and cleaned of surface contamination. After that, the cleaned root segment was delivered to Gödöllő the same day by placing it in a centrifuge tube. The isolation was made by the workers of DNA Laboratory of BIOMI Biotechnology Services Ltd., using NucleoSpin

Microbial DNA Isolation Kit. Then, sequencing was made using LoopSeq ™ Mycobiome 18S ITS Kit (Loop Genomics). The kit was used by the User Manual (INTERNET-2) and the Quick Guide (INTERNET-3). This Kit is capable of sequencing the entire 18S-ITS1-ITS2 region.

The DNA was isolated in several ways by using liquid nitrogen.

Illumina sequencing was performed on a MiSeq machine with a V3 (600) kit with 2*300bp paired-end reads. Based on the Loop Genomics Mycobiome Kit, the sequencing results were evaluated from Unite and Silva international databases.

2.4.Experiments of symbiotic germination of *H. adriaticum* seeds

For the symbiotic germination and cultivation of seedlings, in September 2019, I made "H1 oat medium", a fine-ground oatmeal medium based on CLEMENTS et al. (1986), modified by RASMUSSEN et al (1990).

I made the finely ground oats from oatmeal, which I thoroughly ground and then sieved through a small meshed kitchen sieve. The culture medium was prepared and stored in the same way as the modified Fast medium for asymbiotic germination.

After 1 week of incubation period, approximately 200-200 *Himantoglossum adriaticum* seeds were sown onto the H1 oat medium the same way as during asymbiotic sowing, but the seeds were spread on only one half of the oat medium. After 4 days of incubation period, a fungal sample of 5 mm in diameter, cultivated from *H. adriaticum* root, was placed on the other side of the H1 oat medium, at the farthest point from the seeds. For each fungal species I made 1 experimental symbiotic sowing.

During the *in vitro* symbiotic germination, the interaction between the seeds and the fungi was observed under an Olympus microscope with phase contrast illumination at 400x and 600x magnification, and the microscopic images were taken through the microscope's Olympus lens.

2.5.Planting experiments of *in vitro* grown *H. adriaticum* plants

For the planting experiments into the specially formulated growth medium made by us, we used 2-year-old *in vitro* plants, germinated in 2017. For the planting experiments into the soil from the natural habitat of *H. adriaticum,* we used 4-year-old *in vitro* plants, germinated in 2015.

In July 2019, I planted a total of 8 specimens. Six of these were planted into specially formulated growth medium made by us, containing oak leaf litter, quartz sand, limestone chipping and pine mulch in a 2:1:1:1 ratio. I placed burnt clay balls in the bottom of the 9 cm

diameter, 9.5 cm high planting pot for proper drainage. On this, I placed one unit of oak leaf litter, one unite of quartz sand, one unit of limestone chippings, another unit of oak leaf litter and finally one unit of mulch. I planted 6 pieces of *in vitro* grown plants individually into this medium.

After this I treated 3-3 transplanted specimens in different ways: in case of 3 plants, I watered them by diluting 2.5 ml of Greenman Floraria liquid in 1 liter water. Greenman Floraria is a concentrated multimicrobial preparation offered by Greenman Ltd. for my experiment. Greenman Floraria has a total germ amount of at least 10 x 10^6 pieces/cm^3. The other 3 plants were irrigated with the liquid, agar-free version of the modified Fast medium I used for asymbiotic germination. These 6 individuals were kept next to each other, at room temperature between 24-28°C during the day, and 18-20°C at night on natural light.

The remaining 2 plants were planted into the soil from the Kőszegi-mountains habitat of the plant, into a 27 cm diameter pot, and was placed in the Botanical Garden of Szent István University. The plants were irrigated with rainwater in the dry days of summer, otherwise they got that amount of moisture and light as the nature gave.

The experimental transplanted plants were observed and photographed daily until December.

In December, before the onset of frost, a pyramid shape from reed was made around the 2/3 of the pot, which somehow protected the plants from the winter frosts. The one-third space left allowed the light and moisture to reach the plants, thus slightly imitating the open wintering period in the natural habitat.

2.6.Methods of data processing

The data obtained during my work were recorded in a Microsoft Excel sheet, and the basic operations were calculated with this program. Statistical analyzes and diagrams were made in R statistical software (R CORE TEAM 2017). For fungi grown in Petri dishes, a linear trend line was plotted on the graphs and the square of the correlation coefficient was calculated (R^2). If the value of the correlation coefficient (r) is close to -1 and + 1, it means that the two variables (in this case, the pH and the amount of growth) are strongly related (correlated).to each other. Then the result was tested at 0.05 significance level by t-test. For this, we used the t-distribution table, where if the correlation coefficient is higher than the t-value for the number of samples (in our case n = 14 samples (days) $\nu = n-2$, so $\nu = 12$), the correlation is significant (HOGG és TANIS 1998). The p-value was then determined by analysis of variance (ANOVA), which shows that if $p < 0.05$, there is, and if $p > 0.05$, there is no significant difference between the

averages of fungal growth at different pH. The amount and growth rate of *H. adriaticum* protocorms and seedlings within the flasks were based on visual observations.

3. RESULTS

3.1.Results of *in vitro* asymbiotic germination of *H. adriaticum*

3.1.1. Germination results along the pH gradient

Since the seeds could not be sown until 5 months after harvesting them in August 2016, when the seeds were sown on January 30, 2017, 63% of the flasks became infected. Thus, instead of 12-12 flasks of each pH, 5-5 flasks, which stayed sterile, were used for our experiment. As a result, seeds of *Himantoglossum adriaticum* can not be stored for a long period in a refrigerator at 4°C without any drying treatment. The viability of untreated seeds decreased radically after 5 months of harvesting, and the infection rate increased significantly.

From these 5-5 flasks left, the first protocorm appeared 6 months after sowing both at pH 6.5, 7.5 and 8.5. 7 months after sowing, protocorms formation began in all flasks. The best germination rate was found at pH 7.5. From the 12th month after sowing, protocorm formation was reduced, and several protocorms put on natural light were died. This is noticeable in flasks of pH 8.5 and pH 4.5, but at pH 7.5 was also observed. At 20 months after sowing, the total germination rate was 0.2% and this rate was 0.76% only in the flasks at pH 7.5.

In August 2017, all 15-15 flasks remained sterile. In 2017, the first protocorm appeared at 7 months after sowing, at pH 6.5. Interestingly, while from the seeds harvested in 2016, protocorms appeared at pH 4.5 as well. In contrast, from seeds harvested in 2017, no protocorms were formed at pH 4.5. 20 months after sowing, most of the protocorms were formed again at pH 7.5 (28 protocorms), but the germination was fairly good at pH 6.5 as well (23 protocorms).

12 months after sowing, during the holiday period in August at Szent István University, an unaware electrical maintenance happened, therefore in the air-conditioned room where the flasks were kept, the air-conditioner turned off and did not restart automatically, so almost all of our protocorms and seedling were died. This decline is visible between the 12th-14th months.

Therefore, these flasks were placed into a dark, 4°C refrigerator for 1 month. 16 months after sowing new protocorms developed at pH 6.5 and 7.5.

20 months after sowing, the total germination rate is 0% in flasks of pH 4.5, 0.12% in flasks of pH 5.5, and 0.92% in flasks of pH 6.5 , 1.12% in flasks of pH 7.5, and 0% in flasks of pH 8.5. The average germination rate in the flasks of the two best pH (pH 6.5 and pH 7.5), the germination rate was 1%. However, if from each pH, only those flasks are counted, in which the most protocorms were formed by the 20th month after sowing, the germination rate would increase to 0.6% in flasks at pH 5.5, 3.2% in pH 6.5 and 4% in pH 7.5. Since the initial

germination rate was the best at pH 6.5, but the best result was given by the pH 7.5 flasks at the end, the optimal medium was adjusted to pH 7 between for the further experiments.

3.1.2. Germination results among KCl gradient on MFA medium on optimized pH 7

The first protocorm appeared 6 months after sowing, in April, on MFA media containing 200 mg / 1200 ml potassium chloride. 13 months after sowing, the best germination results were obtained in flasks containing 280 mg / 1200 ml potassium chloride with 76 protocorms (15.2%), which is slightly higher than the original FA medium recipe contains. Then, the second best result was given in the flasks containing 120 mg / 1200 ml potassium chloride, with 42 protocorms (8.4%), interestingly, this is slightly less amount of KCl, what the original prescription contains. Then, in the flasks containing 40 mg / 1200 ml KCl, 38 protocorms (7.6%), in 200 mg / 1200 ml KCl, 26 protocorms (5.2%) were formed. No protocorms was developed at 360 mg / 1200 ml KCl.

The experiment gave very interesting results, since the best results were obtained with a little more amount of potassium chloride than the original medium contains, and the second best was given by the slightly less potassium chloride amount. By the end of the experiment, the original KCl amount of the Fast medium gave the second worst result.

3.1.3. Germination results among NH_4NO_3 gradient on MFA medium on optimized pH 7

The first protocorm appeared 8 months after sowing, in June, on MFA media containing 120 mg and 280 mg / 1200 ml ammonium nitrate. 13 months after sowing, the best germination results were obtained in flasks containing 40 mg / 1200 ml ammonium nitrate, with 22 protocorms (4.4%), which is significantly less than what the original FA medium formula contains. Then, the second best result was given in the flasks containing 120 mg / 1200 ml ammonium nitrate, with 16 protocorms (3.2%).

In the flasks containing 200 mg / 1200 ml NH_4NO_3, 6 protocorms (1.2%) and in the flasks containing 280 and 360 mg ammonium nitrate, only 3 (0.6%) and 2 (0.4%) protocorms were formed over 13 months. The results clearly show that increasing the amount of ammonium nitrate has a negative effect on the development of *H. adriaticum* protocorms.

3.2.*In vitro* asymbiotic growth experiments of *H. adriaticum* plants

3.2.1. Effect of coconut water on the development of seedlings

One month after putting the seedlings on modified Fast medium contained coconut water, the growth stimulating effect of coconut water was visible. It is noticeable that increasing the amount of coconut water also increases the growth stimulating effect. The amount of 10 ml /

1200 ml coconut water initiates the development of the green leaf, but with increasing the amount, this process becomes more intense; 40 ml / 1200 ml amount starts the root development, and with 50 ml / 1200 ml the root development increases.

After 2.5 months, the growth was approximately the same in media contained 10 and 20 ml / 1200 ml coconut water. It was noticeable that the growth of the plants in the medium contained 30 ml and 40 ml / 1200 ml of coconut water is more intense, however, in case of 40 ml of coconut water the root development enhanced further. In the medium contained 50 ml / 1200 ml of coconut water, the growth seemed to slow down after the initial rapid growth and root development within 1 month, moreover, in one flask the seedling has not developed further.

3.2.2. Effect of silver nitrate on the development of seedlings

One month after putting the seedlings onto MFA media containing 4 mg / 1200 ml silver nitrate, no significant change was observed in the condition of the seedlings. After 2.5 months, the seedlings did not show much change compared to the first month. In one of the flasks, a seedling started to grow and continued to develop, but it appeared rather weak, thin and brittle. Based on these results, silver nitrate alone had no growth promoting effect on *H. adriaticum* seedlings.

3.2.3. Effect of coconut water and silver nitrate together on the development of seedlings

One month after putting the seedlings onto MFA media containing 25 ml / 1200 ml coconut water and 4 mg / 1200 ml silver nitrate together, this combination had an evident growth stimulating effect. From the three experiments, this gave the most spectacular growth stimulator effects on the *H. adriaticum* seedlings.

In two and a half months, the seedlings continued to grow, and I found very strong, viable plants with dark green leaves in several flasks.

3.2.4. Development of *in vitro* grown plants under different conditions (temperature and light sources)

In the lightroom, at 21°C, with the alternation of 16 hours of light and 8 hours of dark periods, the browning of the *in vitro* grown plants started already in the first month. The browning of the leaves was continuous in this room in the 16 weeks long experiment. The decay of the plants began at week 6, and became more intense from week 10. At the end of the experimental period, the death rate was 60%. 20% of the plants remained green and 20% of them became browned.

In the second room with southern exposure, the temperature fluctuated between 24-30°C, the number of green plants halved in the first two weeks and from week 4, the brown plants

started to die. By week 14, no green plants remained. At the end of the experimental period, the death rate was 60% and the remaining 40% of the plants were browned.

In the third, northern exposured room, with a temperature fluctuated between 22-26°C, no decay was observed at all during the 16-week experimental period. From week 4, the plants started to be browned, and this became more intense until the end of week 16. At the end of the experimental period, 60% of the flasks remained green and 40% of them were browned.

In the fourth, air conditioned, fix 24°C temperatured room with natural light, results during 12 weeks are remarkable, as an unannounced electrical maintainance happened in August, because of what the air conditioner did not work for up to 1 week. At the point the problem was noticed, it was too late, 70% of the *in vitro* grown plants had already died. By the 12th week, only 20% of the plants became browned, 80% remained green, so we assume that this tendency would have persisted until the end of week 16.

3.3.Experiments and determination of mycorrhizal partners of *H. adriaticum*

3.3.1. Cultivation of mycorrhizal fungi on different media

In case of the surface disinfection of the root samples, fungi grew on all three types of media (PDA, BM, BRM) with the pre-treatment of 1% sodium hypochlorite solution for 5 minutes. The other pretreatment, first placing the root segments into 70% ethanol for 30 seconds, then into 4% sodium hypochlorite solution for 90 seconds, proved to be too strong, since no fungi were grown on any of the media from any of the treated root segments.

Pure cultures of fungi grown on PDA, BM and BRM media were inoculated onto PDA media. This resulted 5 different types of fungi from the root segments. These have been named A, B, C, D, and E fungi until precise identification.

3.3.2. Determining the optimum pH for the cultivated fungi

During the 14-day growth experiment, we found that the cultivated fungal species had a very wide pH optimum. Fungal species „A” prefers pH 4.5. The optimal pH for fungal species „B” was pH 6.5, but there was not really much difference in the growth rate of the species at various pHs, so this species may be found anywhere on a wide range of soil pH.

Fungal species "C" gave the best growth results at pH 5.5 and the worst at pH 4.5.

The optimal pH of fungal species „D” was pH 6.5, but there are very small differences between the growth rate of the species on each pH, so this species may also be a widespread soil fungus.

Fungal species „E" prefers pH 5.5 and had the slowest growth at pH 8.5. The growth rate of this species is clearly reduced as the pH of the media was increased.

The R^2 values for each fungal species were calculated. The null hypothesis is that there is no significant difference between the means. According to the mean of the target variable, the treatments in all samples are all the same. Each of the R^2 values indicates that the linear trend line assigned to the growth points at a given pH significantly correlates. As a result of the t-test, the correlation between pH and growth rate is significant for each pH of each fungal species.

Based on the results of the analysis of variance it can be stated that the significance level is> 0.05, thus the null hypothesis is maintained, the growth rates of the cultivated fungi species at different pH do not differ significantly from each other; they are not really depend on the pH of the soil.

3.3.3. Result of fungal species cultivated from *H. adriaticum* roots given for isolation, and the visual detection of the other cultivated species under microscope

According to the 506 nucleotide-length ITS1-2 sequence, the species: *Ilyonectria robusta* was isolated from the cultivated fungal sample „A" by BIOMI Biotechnology Service Ltd. This species is a member of the *Ascomycota* phylum of the kingdom Fungi, which is recognized as a common soil fungus, asymptomatic root endophyte and opportunistic plant root pathogen (CHAVERRI et al. 2011). No literature was found about this species as having mycorrhizal relationships with any orchid species.

Based on the visual microscopic examinations, species „B" was determined as *Penicillium sp*., species „D" and „E" as *Fusarium sp*. Species „C" could not be identified by morphological features at family/genus level, but it was apparently certain that the species was *Ascomycota*.

3.3.4. Fungal partners determined from the *H. adriaticum* root segment given for isolation

During the root sample sequencing, in most of the cases (206), the DNA sequence of the *H. adriaticum* species itself came out, which is not surprising, but proves that the root of the species was processed. The DNA sequences of other orchid species (*Dactylorhiza incarnata*, *D. fushsii*, *Himantoglossum hircinum*, *Cephalanthera austiniae*, *Orchis quadripunctata*, *Dendrobium catenatum*) came out in relatively lot cases. These results are probably due to the similarities in the DNA sequences within the *Orchidaceae* family. Other taxa appearing with much lower abundance are also extremely uncertain.

Our relevant potential mycorrhizal partners belong to the Phylum: Basidiomycota., Therefore, Basidiomycota species that have been identified from the root sample, are being discussed below.

Among the Basidiomycetes, *Rhizoctonia solani*, *Tulasnella* sp. and *Cryptococcus neoformans* species were identified from the submitted root sample.

Species from *Rhizoctonia* and *Tulasnella* genus are well known mycorrhizal partners of the *Orchideaceae* family.

In my previous research, *Tulasnellaceae* family has already been isolated from *H. adriaticum* protocorms (GILIAN 2015). So by identifying *Tulasnella* sp. in the *H. adriaticum* root as well, it is certainly clear that some of the species of this family are mycorrhizal partners of *H. adriaticum*.

According to the literature, *Rhizoctonia solani* is a mycorrhizal partner of orchid species found in Hungary as well. According to research by DOWNIE (1959), *R. solani* is a symbiont or partly symbiont mcycorrhizal partner of *Orchis purpurella*, *Dactylorhiza viridis*, *Goodyera repens* and *Gymnadenia conopsea*. Based on these, even if the abundance is quite low (0,003) it can not be excluded that it is also a symbiont partner of *H. adriaticum*.

Cryptococcus neoformans is an obligate aerobic yeast that can cause disease in apparently healthy and immunodeficient animals and plants. In case of orchids, *Cryptococcus* species have already been isolated from the roots of *Gymnadenia conopsea* (STARK et al. 2009) in Germany and *Epipactis albensis* (MALINOVÁ 2009) in the Czech Republic.

3.4. Results of the experiments of symbiotic germination of *H. adriaticum* seeds

Except fungi species "B", the other species did not, or just reached the midline of the media within 1 week, where they could encounter *H. adriaticum* seeds.

Within 2 weeks, all five fungal species crossed the midline of the medium and reached H. adriaticum seeds. No interaction had been observed between the seeds and the fungi after this time.

One month after the inoculation of the fungi next to the seeds, the fungal species completely overspreaded on the surface of the media, but no change was observed on the seeds.

Two months after the inoculation, the fungi continued to grow and the fungal species still did not interact with the seeds.

Three months after the inoculation, still none of the fungal species started any interaction with the orchid seeds. In case of the fungal species „E”, the hyphae had completely overgrown the medium and it had grown above the seeds so much, that they became not visible under microscope.

Four months after the inoculation, we can state that none of the five cultivated fungi have initiated the germination of *H. adriaticum* seeds on H1 oat media, so these species did not become symbiotic fungal partners under our experimental conditions.

3.5. Results of the planting experiments of *in vitro* grown *H. adriaticum* plants

The leaves of the 4-year-old individuals with large tubers planted into soil brought from the natural habitat, turned brown in the early September and disappeared in November.

At the time of finishing the dissertation, the plants were still overwintering. Hopefully, by the spring of 2020, we will see if there will be new shoots on the plants. Taking into consideration the life cycle of *H. adriaticum*, they will certainly not bloom. The question and the important result will be whether they will survive the winter, and if they will, then how many leafs will be observed on them in the spring and summer.

In the *in vitro* planting into the specially formulated growth medium we prepared, *H. adriaticum* plants could be kept for about 1 month during the experimental period, then they started to turn browish followed by death of the plant. Three plants of *H. adriaticum* irrigated with Greenman Floraria could be kept for 6 weeks after which they died. The plants were watered with liquid MFA, could be kept for 10 weeks. In fact, I used 1.5-year-old individuals for this experiment, so it might be the problem that they may not have been strong enough to overcome the external stress associated with transplanting. But in my personal opinion this kind of transplanting into the specially formulated growth medium would require more specific conditions (proper lighting, temperature and humidity) which I unfortunately could not provide for them at the University.

3.6. NEW SCIENTIFIC RESULTS

I. For germinating the seeds of *Himantoglossum adriaticum*, the optimal pH of modified Fast media is between pH 6.5 and 7.5. At these pHs the seeds germinated the fastest (6 months) and with the highest rate (3.2-4%). This pH is nearly the same as the soil pH measured taken from its habitat. Twenty months after sowing, in the best germinating flasks, the germination rate was 0% at pH 4.5, 0.6% at pH 5.5, 3.2% at pH 6.5, 4% at pH 7.5 and 0% at pH 8.5. The pH of the media significantly influences the germination rate of *H. adriaticum* seeds and the number of protocorms formed.

II. For germinating the seeds of *H. adriaticum*, the optimum KCl concentration of modified Fast media (with optimized pH) was 280 mg / 1200 ml. The concentration of KCl in the medium also influenced the germination rate of *H. adriaticum* seeds and the number of protocorms formed.

III. For germinating the seeds of *Himantoglossum adriaticum*, the optimum NH_4NO_3 concentration of modified Fast media (with optimized pH) was 40 mg / 1200 ml. Increasing the concentration of NH_4NO_3 in the media is strongly inhibiting the germination of *H. adriaticum* seeds.

IV. For *Himantoglossum adriaticum* protocorms, the optimum amount of coconut water added to the media (with optimized pH, KCl and NH_4NO_3) is 30-40 ml / 1200 ml. At this time, the growth and development of the seedlings is the fastest. Addition of 4 mg $AgNO_3$ and 25 ml coconut water to the media enhances the growth and development of the seedlings further, making them very strong, viable plants with dark green leaves

V. DNA sequencing of the root of *Himantoglossum adriaticum* gave 14 *Ascomycota* and 3 *Basydiomycota* taxa, of which besides the genus *Tulasnella*, which is already indicated from protocorm as a possible mycorrhizal partner of the species, the form genus *Rhizoctonia* and *Cryptococcus neoformans* can be the most possible symbiont mycorrhizal partners of *H. adriaticum*. The latter two have been previously described as a symbiont of other orchid species according to literature

4. CONCLUSION AND RECOMMENDATIONS

Based on our results, we found that *ex situ, in vitro* germination of *H. adriaticum* seeds is more effective on pH 7, than on the base pH 5.5 of the Fast media. The optimal pH 7 is almost the same as it was measured on its natural habitats according to BÓDIS et al. (2011), and according to our field measurements during seed collections as well. This is a promising result, that is why it is necessary to determine the pH optimum for seed germination of other domestic orchid species, with measuring the soil pH of their natural habitat at the same time. We have already started our experiments on *Himantoglossum jankae, Orchis militaris* and *Orchis purpurea*, and in the future we aim to determine the pH optimum of as many domestic orchid species as possible.

For germination of *H. adriaticum* seeds, the optimal concentration of KCl of the original Fast media should be increased to 280 mg / 1200 ml. The second best germination results were obtained with slightly less potassium chloride (120 mg / 1200 ml) than the original. However, in spite of the rapid development of the protocorms in the beginning, the medium containing potassium chloride according to the original formula (200 mg / 1200 ml) gave the second worst germination results by the end of the experiment.

The germination of *H. adriaticum* seeds is inversely proportional to the ammonium nitrate concentration of the medium. The lower the amount of ammonium nitrate in the media, the higher the germination rate is and the more protocorms are being formed. This is consistent with the literature review of section 2.5.3, according to the results based on VAN WAES (1984), VAN WAES and DEBERGH (1986) and EIBERG (1970).

Coconut water has shown to have a very spectacular growth stimulating effect on protocorms and seedlings. According to our experiments, the optimal amount of coconut water is between 30 and 40 ml / 1200 ml of modified Fast media. This effect is even more enhanced when 4 mg / 1200 ml silver nitrate is added to the medium. This is probably due to the presence of cytokinins and other phytoregulator compounds of coconut water and the ethylene inhibitor effect of silver nitrate.

Because of the success of the combined growth stimulating effect of coconut water and silver nitrate on *H. adriaticum* seedlings, it would be worthwhile to investigate this effect on the germination and seedling growth of several temperate orchid species.

Further studies are currently under investigaton: we observe the common effect of 25 ml / 1200 ml coconut water and 4 mg / 1200 ml silver nitrate on the germination rate and time of *H. adriaticum* seeds, and on the quantity and condition of the protocorms which will be formed.

In an other ongoing experiment, we are checking, how does the length of the chilling period influences the germination success of *H. adriaticum* seeds, as keeping them for 3 months at 4°C (as it is the process according to the literature) is a long time, therefore we would like to consider if it is essential or not for the seeds. In the future, it would be worth to examine the amount of other components of the modified Fast media both in case of germinating *H. adriaticum* and several domestic orchid species as well.

In our experiments, the biggest problem for us was keeping *in vitro* seedlings and plants, because neither a constant temperatured lightroom with artificial light, nor a southeastern exposured room, with natural light and temperature of 24-30°C was available. Keeping them in a northern exposured room on natural light and temperature of 22-26°C is also moderately appropriate, as the condition of the individuals has gradually deteriorated over the weeks (turned more and more brown). It is very difficult to find a place where the temperature and light conditions are adequate, and there is place for large quantities of *in vitro*-grown plants (flasks) for a long time without having access there for other people. Therefore, we are still conducting our experiments in cooperation with the ELTE Botanical Garden, where we keep our flasks with great care. This problem, however, would be good remedy, and a room set at a fixed 24°C would provide the best conditions for our experiments should be found or developed to ensure continuous, accurate experimentation locally at the University.

In our experiments to determine the mycorrhizal partners of *H. adriaticum* by isolating them from a root segment by molecular genetic testing did not provide reliable results, as taxa with quite high abundance (eg, orchids and plants not found in the area) could be excluded, while mycorrhizae species mentioned in literature as well had very low DNA sequence abundance. This is why none of the detected taxa can be said certainly to be present in the root of *H. adriaticum*.

Unfortunately, media suitable for the cultivation of various fungi did not produce the expected results. None of fungi cultivated from the root segments of *H. adriaticum* could initiate the germination of *H. adriaticum* seeds symbiotically.

Among the basidiomycetes isolated from the root of *H. adriaticum* by molecular genetic testing, *Rhizoctonia solani*, which has already found to be a symbiont of other terrestrial orchid species by DOWNIE (1959), *Tulasnella* sp., which was isolated as a mycorrhizal partner of terrestrial orchid species according to SUFAATI et al. (2012), ILLYÉS et al. (2012) and GILIAN (2015), and *Cryptococcus* genus, previously isolated by STARK et al. (2009) from the root of *Epipactis albensis* and by MALINOVÁ (2009) from *Gymnadenia conopsea* are the most

possible mycorrhizal partners of *H. adriaticum*, but their presence is still doubtful because of the low abundance.

Based on our results, we have come to the conclusion that in the future it may be worthwhile to try other media for cultivation of basidiomycetes from roots, and trying to find already cultivated *Basidiomycota* fungi from those, which we get as a result from the isolation.

Experiment of transplanting *in vitro H. adriaticum* plants into the specially formulated growth medium we prepared has been unsuccessful either watering them with Greenman Floraria or liquid modified Fast medium. In my opinion, this experiment would also require a special room where we could provide adequate conditions for acclimatization of the plants before transplanting. As for the future of the species transplanted into the soil from the natural habitat, we can not provide data until the dissertation is finished, and we must wait for the results of this experiment at least until spring 2020.

In the future, we aim to replicate experiments on the determination of optimal pH, KCl and NH_4NO_3 concentration for *Himantoglossum adriaticum* so that the results can be evaluated using appropriate statistical methods as well. Furthermore, we would like to perform similar experiments on as many orchid species as possible in Hungary, to determine their optimal media pH and their *in vitro* germination success. First, we plan to carry out these experiments with an other domestic species of the *Himantoglossum* genus: *Himantoglossum jankae*, since, with that result we could compare the *in vitro* germination properties and pH requirements of the two *Himantoglossum* species found in Hungary, which will give us a more complex picture of the family.

In addition, we would like to show the methods of *in vitro* germination of orchid species to more and more enthusiastic young researchers, work with them and develop strictly protected and protected species *ex situ* transplanting and replanting process to their natural habitats.

5. SUMMARY

Until now, 67 orchid species have been described in Hungary, although some of them have not been found since more than 10-50 years. Thus, these species can be considered as forbidden or extinct in Hungary. Due to the effects of climate change and human activities in the environment, the populations of endangered species are rapidly decreasing worldwide, therefore it is necessary to develop and elaborate the possibilities of their protection as soon as possible.

The target species of my dissertation, *Himantoglossum adriaticum*, is a highly protected, Natura2000, Adriatic-Mediterranean, temperate, terrestrial orchid species. In Hungary, there are smaller or larger, relatively stable populations known in only five areas namely in the Keszthelyi-Mountains, the Sümeg-Tapolcai Banks, the Bakony-Mountains, the Kőszegi-Mountains and in the Sopron-Mountains. In my previous research, the seeds of this species germinated at the basic pH (pH 5,5) of modified Fast medium (1,3% germination rate), and from *in situ* germinated protocorms, the *Tulasnellaceae* and the *Telephoraceae* families were isolated as possible symbiotic fungal partners by molecular genetic testing.

The aims of my doctoral dissertation were to determine the possibility of an *in vitro asymbiotic* germination and the germination success of *Himantoglossum adriaticum* along the pH gradient, to determine the optimal potassium-chloride and ammonium-nitrate content of the modified Fast medium for *H. adriaticum* seeds, and to determine the effects of coconut water and silver nitrate on the growth of *H. adriaticum* seedlings and seeds. Furthermore, my aim was to isolate and cultivate the fungal partners of *H. adriaticum* from a root sample, and to try the symbiotic germination of *H. adriaticum* seeds with these cultivated fungi. Lastly to monitor the viability of *H. adriaticum* seedlings and plants grown in the flasks, and the viability of the plants after planting them out either into an individually prepared mixture of planting medium or into the soil of the natural habitat of the species.

Our experiments have shown that the optimum pH of modified Fast medium for germination of *Himantoglossum adriaticum* seeds is between 6,5 and 7,5. The germination of seeds are the fastest and highest between these two pHs (6 months, 4%).

Our results showed that the optimal KCl amount of modified Fast medium for *Himantoglossum adriaticum* seeds was 280 mg/1200 ml and the optimal NH_4NO_3 amount was 40 mg/1200 ml on pH 7. Our experiments proved that 4 mg/1200 ml silver nitrate and 25 ml/1200 ml of coconut water added together to the optimal modified Fast medium of *Himantoglossum adriaticum*, stimulated the growth of the seedlings spectacularly. It became

clear that maintaining the best conditions for the plants in the flasks is one of the most difficult task, and we proved, that even a very small change from the optimum conditions can be enough for the entire flask-grown population to begin to decline rapidly.

According to our experiments, we could not cultivate basidiomycetes neither on PDA nor on malt agar with benomil and malt agar with bengal rose, so from this point of view this cultivation method does not seem appropriate.

Molecular genetic testing have given taxonomically very unreliable results, as several species DNA sequencing matches have appeared that definitely can not be present in the area, and those as well, which, according to the literature, may be found in *H. adriaticum* roots. Such as the three basidiomycete species, *Tulasnella sp*., *Rhizoctonia solani* and *Cryptococcus neoformans,* which have appeared as a result of sequencing. Compared to our previous isolation results from the protocorms, it can be stated that the *Tulasnella* genus was present in the root of the *Himantoglossum adriaticum* too, and despite the low abundance of the sample we may consider it as a symbiotic partner. *Rhizoctonia solani* and *Cryptococcus neoformans*, also appeared with very low abundance, but they are also likely to be present according to literature, and they can possibly be in a symbiotic association with *H. adriaticum*.

In vitro symbiotic germination, due to failure of cultivating basidiomycetes on the agar we used; was unsuccessful. None of the cultivated ascomycetes induced germination of *H. adriaticum* seeds on H1 oat medium symbiotically. This indicates that these species probably do not have mycorrhizal relationship with *H. adriaticum*, but it is also a possiblility, that this symbiotic relationship could not be established with the medium we used.

H. adriaticum plants grown in flasks and planted individually in the prepared planting medium survived for 6 weeks with Greenman Floraria concentrated multimicrobial preparation and 10 weeks when they were watered with Fast liquid medium. Hopefully we will be able to provide information about the viability of the species planted in the soil of their natural habitat in spring 2020.

We are currently conducting further experiments as well. In the future we would like to include more orchid species found in Hungary in our research and finally write a comprehensive study on the *in vitro* propagation possibilities of Hungarian orchids. In addition, we would like to conduct further experiments on the transplantation of *H. adriaticum*.

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