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CONSERVING THE CRITICALLY ENDANGERED *Anacamptis Coriophora* L. IN TÜRKİYE THROUGH *EX VITRO* SEED GERMINATION

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Abstract: *Anacamptis coriophora* (Orchidaceae) is a highly endangered orchid in Türkiye due to its excessive collection and the continuing deterioration of its habitat. In this study, the cultivation conditions of *A. coriophora* were determined. A sterile soil mixture was filled into jars and the fungal isolate (previously isolated from *A. coriophora* roots), Ceratobasidiaceae MG762693 was inoculated in separate glass jars, producing fungal compost when hyphae were developed. This fungal compost was then filled into pots where *A. coriophora* seed packs (0.001 g) were placed and subsequently moistened with sterile liquid nutrient medium. After 45 days of germination, fifty seedlings of approximately equal size were transferred directly to a natural environment and after 6 months of development the measuring of the tubers was done. The phenological process was then monitored until flowering. After 45 days, germination and developmental stages rates were determined from the seed packs in the pots inoculated with the Ceratobasidiaceae MG762693 fungal isolate and 64.3% germination and 11.75% leaf-rooted seedlings (stage 4) occurred. Plants flowered in June the following year, and the seeds ripened in July. The largest tuber in adult individuals was about 3 times the weight of first-year tubers. Each individual formed 2 or 3 tubers, thus increasing the number of tubers approximately 2.5 times in 2 years. In this study, *ex vitro* symbiotic seedlings were planted in the natural environment and a small population was formed in a 2-year period. The results revealed that orchids can be grown on a large scale with this method, both economically and for conservation and reintroduction.

Keywords: Orchidaceae, Orchid cultivation, Seed germination, Symbiotic

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1. Introduction

The Orchidaceae family ranks among the world's largest, comprising more than 27,000 species (Zhang et al., 2018). Orchids are able to grow in diverse climates and ecosystems, spanning from sea level to temperate and tropical mountains (Oktalira et al., 2019; Steinfort et al., 2010). Orchids have both terrestrial and epiphytic growth habits and produce a large number of tiny seeds (Pujasatria et al., 2020). Orchids hold a prominent place in the list of endangered plant species in certain countries like Türkiye (León-Yánez et al., 2011; Qin et al., 2017). Nevertheless, despite many orchid species being familiar in biodiverse tropical countries, the number of threatened orchid species is expected be higher in national and international registries during the few coming years (Joppa et al., 2011a, b).

Tubers of Euroasian orchids are over-harvested, especially in Türkiye, Greece, Iran, and some Middle Eastern countries for the salep beverage and ice cream additive production. The harvesting of these orchids is so extreme that all orchid species are now protected by law, but unfortunately, the illegal collection continues extensively (Ghorbani et al., 2014; Kreziou et al., 2016; Sezik, 2002). In the last 30 years, orchids and the related ecosystem have become more vulnerable to extinction, especially due to the extreme pressure brought on by human activities. The fragmentation and destruction of habitats, fires, and a decrease in pollinators have caused serious losses in orchid populations and diversity (Sosa and Platas, 1998; Hopper, 2000; Coats and Dixon, 2007). Therefore, orchids face a risky future due to habitat loss caused by human activities and climate change. Both *in vitro* asymbiotic and symbiotic methods have been used to successfully achieve seed germination and seedling development for Euroasian temperate orchids (Eşitgen et al., 2005; Çığ et al., 2018; Fatahi et al., 2022). However, these methods are expensive and time-consuming for the germination and seedling development processes. The difficulty of adapting seedlings to the natural environment complicates the applicability of these methods. All tuberous orchids, especially *Anacamptis* species, are collected from nature to meet this demand. A sustainable production model can be an effective solution to both industrial demands and especially, the conservation and reintroduction efforts of endemic and severely threatened orchid species. *Ex vitro* symbiotic

seedling production of some tropical and temperate orchids and then the formation of mature plants by easily adapting these seedlings to the natural environment (Quay 1995; Aewsakul et al., 2013; Kömpe and Mutlu 2021; Kömpe et al., 2022; Deniz et al., 2022) is very promising for their mass production. The genus *Anacamptis* (Orchidaceae) consists of about 34–35 species and hybrids in Europe, west-northwest Asia, and the Mediterranean. All species of this genus have underground tubers (Govaerts et al., 2017). Otherwise, even the most common orchids will face the threat of extinction shortly, and unfortunately, orchids in Türkiye will only be kept in cemeteries (Löki et al., 2015). Although protocols for mass production were suggested in studies on the *in vitro* asymbiotic germination and seedling development of this species, it could not be transferred from the laboratory to the field (Bektaş et al., 2013; Gümüş and Ellialtıoğlu 2012).

In light of this information, the aim of this research are to make mass production of *A. coriophora* seeds from Türkiye and to determine the morphological characteristics of the tubers formed.

2. Materials and Methods

2.1. Plant Materials

In this research, *Anacamptis coriophora* seeds were collected from the capsules of adult plants formed by natural pollination around Bolu–Abant lake (Black Sea Region, Türkiye). The seeds were collected from mature capsules, kept at room temperature for a few days to dry, and stored at 4 °C until used in germination tests.

2.2. Fungal Inocula

Ceratobasidiaceae MG762693, a fungal isolate that was isolated from the roots of *A. coriophora* in a previous study (Mutlu and Kömpe 2020) was tested in this study to investigate seed germination and seedling development. The fungal isolate was inoculated into PDA (Potato Dextrose Agar) medium, incubated in the dark at 25 °C for 5 days and activated.

2.3. *Ex Vitro* Symbiotic Seed Germination

For *ex vitro* symbiotic seed germination, after collecting the soil from *A.coriophora* habitat, the soil mixture was prepared (2:1 soil: perlite), filled in glass jars, and sterilized in an autoclave (121 °C, 1.5 Atm). The fungal isolate was inoculated first into the jars containing a sterile soil mixture and incubated at 25 °C in the dark for 15 days. When fungal hyphae developed, this compost was filled into sterile pots (20×31×15 cm), and 6 seed packs were buried in each pot and six repetitions were made for germination tests.

The soil mixtures in the pots were moistened with a modified oat medium once a week. After two months of incubation, the germination and development stages in the seed packs were evaluated.

For the control group, the soil mixture was put in the pots without fungus-inoculated compost. Six seed packs were embedded in each pot.

Development was assessed according to the following stages by modifying Clements et al. (1986):

0: Ungerminated seed
1: Protocorm
2: Leaf primordium
3: First leaf
4: Developed leaves and/or roots

2.4. The Seedling Plantation

After 50 seedlings (reaching stages 1-2 and stages 3-4) were planted in a natural field at a distance of 50-60 m from the main habitat of *A.coriophora* adult plants, the phenological process was followed and evaluated from the seedling stage to the flowering adult stage. From the seedlings transferred to nature, the formation rate, sizes and weights of the tubers were measured. All of the tubers were buried in the ground again after their measurements were taken and the flowering process was followed. The number of tubers in adult plants at the flowering stage and tuber sizes were determined.

2.5. Statistical Analysis

The rates of seeds at germination and seedling development stages were compared against the control group (without fungal inoculation). The rate of germination and seedling growth were evaluated by using one-way ANOVA. Statistical significance was set at $P<0.05$. Differences among the means were compared with the Duncan test. All the statistical analyses were performed using SPSS software 25.0 (SPSS Inc., Chicago, USA).

3. Results and Discussion

Ceratobasidiaceae (Access Number MG762693), which is an earlier isolate from *A. coriophora* roots, promoted germination and seedling development. 64.3 % of the seeds germinated and developed. 14.06 % and 11.75 % of the seeds reached stages S3 and S4, respectively. (Table 1, Figure 1A).

Table 1. Germination and development of *Anacamptis coriophora* seeds with Ceratobasidiaceae (±: Standard deviation)

	Developmental Stages (%)					Germination (%)
	S0	S1	S2	S3	S4	
Control	100.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ceratobasidiaceae MG762693	35.92 ± 10,72	14.23 ± 5.6	24.01 ± 7.74	14.06 ± 4.65	11.75 ± 3.03	64.30 ± 10.71

Figure 1. A: Seedlings that develop 45 days after seeds sowing , B: Seedlings transferred to nature, C: Flowering plants after 2 years of growth, D: The tubers of the mature individual of *A. coriophora*. (The arrow shows the previous (old) tuber).

The results of this research conducted on the relationship between orchid seed germination and mycorrhizal fungi showed varying effectiveness of the different isolates on orchid seed germination. In some orchids, different fungi are effective at the germination and development stages, while in some species, a certain type of fungus supports both germination and development (Wang et al., 2011; Zhang et al., 2020; Kömpe et al., 2021). In addition, this study supported the previous research result explaining that the compatible fungus for *A. coriophora* is *Ceratobasidium* (Kömpe and Mutlu 2021).

Fifty healthy and approximately equal-sized seedlings of the S4 stage were then planted directly in a natural area at a small distance from adult plants of *A. coriophora* in autumn (October - 2019) (Figure 1B) and the field was protected against potential animal destruction. The seedling's life cycle was followed until May 2020 without any interference. All the seedlings were removed in the spring, and the tuber formation was evaluated. It was determined that tubers of different sizes developed in 35 seedlings. After cleaning the soil residues from the surfaces of the tubers, their dimensions and weights were determined. The tubers were buried again in the soil, and the phenological process was followed up to the flowering stage. In the first year (T1, Table.2), 35 seedlings produced only one tuber. During the summer, the tubers remained dormant in the soil, and in October 2020 the first leaves and roots developed. From October to June 2021, the vegetative phase continued, and flowering occurred for the first time in June 2021 (Figure 1C). All tuber-forming seedlings (35 seedlings) flowered. The flowering process continued for about 1 month, and as a result of natural pollination, seeds were formed. All individuals were removed, and tuber numbers and sizes were determined (Figure 1D).

Tubers that were formed in 35 of the 50 developed seedlings (T_0) were retransferred to nature to control its growth and development during the first and second year of growth. T_0 presented relatively low measurements; the weight was about 0.35g, the width was about 0.72cm, and the length was about 0.88cm. After one year, 35 adult orchid plant's tubers (T_1) developed and it was determined that presented relatively higher measurements than T0; where the width attended 2.34cm and the weight was the highest (7.15g).

After two years, it was determined that 31 adult plants (from the 35 plants that formed first tuber during the first year) formed second tubers (T_2) with also high measurements and only 22 adult plants (from 31 plants that formed two tubers) formed the third tubers (T_3). These small tubers (T_3) presented the lowest

measurements in terms of weigh, width, and length.

Accordingly, for the first time in two years, a small population was able to form under natural conditions. The acclimatization process from using *in vitro* asymptotic or symbiotic production methods, (Zhang et al., 2015; Fatahi et al., 2022) has not been applied before the seedlings are planted in nature. In this study, we have shown that new individuals can be obtained with a compatible fungus isolated from *A. coriophora* roots for the reintroduction of orchids. Only a few studies have been conducted on the *ex vitro* and *in situ* germination and reintroduction of both tropical and temperate orchids and their mass production for economic purposes (ornamental and medicinal) (Quay et al., 1995; Aewsakul et al., 2013; Khasim et al., 2020; Kömpe and Mutlu 2021). In a similar study, the reintroduction of *ex vitro* seedlings of A. *sancta* with a compatible fungus was successful (Deniz et al., 2022). One of the most effective ways of protecting orchids is by ensuring that they can be produced in sufficient quantities to meet industrial demands. Because sustainable production will meet the demand, there will be no need to harvest naturally occurring orchids. In this context, the cultivation provided will bring about protection, because the application of this method for endemic, rare, and threatened species also possible to create a successful and self-sufficient population in the wild. Therefore, filling the gap between orchid conservation theory through practice with cultivation and reintroduction will provide significant benefits both for industrial production and for the reintroduction of threatened species into the wild (Wu et al., 2014; Deniz et al., 2022).

Table 2. Measurements of *Anacamptis coriophora* tubers.

Tubers	Tuber Width (cm)	Tuber Length (cm)	Tuber Weight (g)
T0 (N=35)	0.72 ± 0.28	0.88 ± 0.31	0.35 ± 0.27
T1 (N=35)	2.34 ± 0.64	2.70 ± 0.61	7.15 ± 4.06
T2 (N=31)	1.49 ± 0.44	1.76 ± 0.52	2.57 ± 1.86
T3 (N=22)	1.04 ± 0.20	1.30 ± 0.43	0.93 ± 0.59

T0= tubers formed 6 months after seedlings plantation, T1= first year's biggest tubers of the adult plants, T2= second year's medium sized tuber of the adult plants, T3= second year's third smallest tubers. All values are reported as mean ± SD, N= number of adult orchid plants that formed tubers.

4. Conclusion

This report is the first study on the cultivation of *A. coriophora*, which is important as a food supplement and medicinal orchid. It is also a pioneering method of protection and reintroduction for temperate orchids, which are threatened with extinction. The seedlings easily adapted to nature, and flowering occurred in the second year. 2 and 3 tubers were formed from each *A. coriophora* plant individual, and the number of individuals increased by about 2.5 times in a year. With this method, it will be possible to protect endemic, rare, and threatened orchids in small populations, and mass production of medicinal and economic orchids will be made easily and at a low cost.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	I.H	Y. ÖK
C	50	50
D	50	50
S	50	50
L	70	30
W	60	40
CR	20	80
SR	80	20
PM	50	50
FA	40	60

C=Concept, D= design, S= supervision, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or

humans.

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