Anatolian Journal of Botany
9(1): 51-54 (2025)
Research article *doi:10.30616/ajb.1570258*

Endemic *Dactylorhiza osmanica* subsp. *osmanica:* Ex vitro symbiotic germination approach

Bengisu ŞENTÜRK[1*], Erdi Can AYTAR[2], Yasemin ÖZDENER KÖMPE[3]
[1,3]*Ondokuz Mayıs University, Science Faculty, Department of Biology, Samsun, Türkiye*
[2]*Uşak University, Agriculture Faculty, Department of Horticulture, Uşak, Türkiye*
**bengisusenturk@gmail.com, [2]erdicanaytar@gmail.com, [3]yasemino@omu.edu.tr*

Received : 19.10.2024
Accepted : 27.01.2025
Online : 15.02.2025

Endemik *Dactylorhiza osmanica* subsp. *osmanica:* Ex vitro simbiyotik çimlenme için bir bakış açısı

Abstract: In this study, the potential effects of *Ceratobasidium* sp. fungus on the ex vitro symbiotic germination of *Dactylorhiza osmanica* (Klinge) P. F. Hunt & Summerh subsp. *osmanica* seeds were evaluated. Results, *Ceratobasidium* sp. caused that about 51.95% germination rate was obtained in seeds inoculated. Protocorm and primordium formation rates were determined to be 25.27% and 26.67%, respectively. These findings indicate that the fungus promotes the germination of seeds. *Ceratobasidium sp*. on ex vitro symbiotic seed germination of *D. osmanica* subsp. *osmanica* shows the potential effect of the fungus Longer term and more detailed studies should be conducted to reach adult plants and adapt to natural conditions. These findings are very promising for protecting of endemic and rare orchid species.

Key words: *Ceratobasidium*, conservation, endemic orchid, ex vitro symbiotic germination, mycorrhiza

Özet: Bu çalışmada, *Ceratobasidium* sp. fungusunun, *Dactylorhiza osmanica* (Klinge) P. F. Hunt & Summerh subsp. *osmanica* tohumlarının ex vitro simbiyotik çimlenmesi üzerindeki potansiyel etkileri değerlendirilmiştir. Sonuçlar, *Ceratobasidium* sp. ile aşılanan tohumlarda yaklaşık %51,95 çimlenme oranı elde edildiğini göstermiştir. Protokorm ve primordiyum oluşum oranları sırasıyla %25,27 ve %26,67 olarak belirlenmiştir. Bu bulgular, fungusun tohum çimlenmesini teşvik ettiğini göstermektedir. *Ceratobasidium* sp.'nin *D. osmanica* subsp. *osmanica* üzerindeki ex vitro simbiyotik tohum çimlenmesi üzerindeki etkisi, fungusun potansiyel etkisini ortaya koymaktadır. Yetişkin bitkilere ulaşmak ve doğal koşullara adaptasyonu sağlamak için daha uzun süreli ve detaylı çalışmalar yapılması gerekmektedir. Bu bulgular, endemik ve nadir orkide türlerinin korunması için oldukça umut vericidir.

Anahtar Kelimeler: *Ceratobasidium*, koruma, endemik orkide, ex vitro simbiyotik çimlenme, mikoriza

Citation: Şentürk B, Aytar EC, Özdener Kömpe Y (2025). Endemic *Dactylorhiza osmanica* subsp. *osmanica:* Ex vitro symbiotic germination approach. Anatolian Journal of Botany 9(1): 51-54.

1. Introduction

The Orchidaceae family consists of 736 genera and about 28.000 species, and about 500 new species are defined in these two families every year (Chase et al., 2015). This family presents a wide variety of epiphytic and terrestrial specimens. It finds its habitat in all parts of the world and is famous for its morphological species diversity. Representing monocotyledons, these species impressively represent diversity with an unlimited number of variations (de Vasconcelos et al., 2023). Türkiye has a rich diversity of flora due to its geographical location and different climatic conditions, which is reflected in the diversity of orchid species (Karakaya et al., 2019) Orchidaceae species richness is quite high in Türkiye. According to current data, there are 33 taxa belonging to the genus *Dactylorhiza* Neck. in our country, 14 of which are endemic (Fırat et al., 2015). In the current climate change scenario, orchid species are threatened due to their unique habitats, complex seed germination mechanisms, and specialized pollination processes. In addition, in our country, orchid tubers are collected in an uncontrolled manner depending on economic factors such as food and medical drug production. Therefore, orchids urgently need to be protected and resettled. Seeds of all orchids form a symbiotic relationship with certain types of fungi to germinate naturally (Wani et al., 2020; Deniz et al., 2022) In nature, the seeds of almost all orchids need a certain fungal partner to germinate due to the lack of endosperm. These fungi are usually orchid mycorrhizal fungi that promote the formation of structures called pelotons in stem cells or protocorms. These fungi play an important role in in situ reproduction and conservation processes by promoting seed germination (Selosse et al., 2017). The symbiotic method may be more suitable for producing tuberous orchids for both conservation and commercial purposes to obtain fast and high-yielding results. Studies have shown that symbiotic seedlings adapt more easily to the natural environment and grow faster. Therefore, the use of symbiotic methods in orchid conservation efforts can provide a significant advantage in terms of producing orchids more effectively and adapting to natural habitats better (Quay et al., 1995; Aewsakul et al., 2013; Özdener Kömpe et al., 2022; Deniz et al., 2022).

Many orchid species, such as *D. osmanica* (Klinge) P. F. Hunt & Summerh. *var. osmanica*, require conservation efforts due to their rarity and dwindling populations. This species is classified as "Endangered" on the IUCN Red List of Threatened Species as a result of habitat loss and fragmentation caused by human activities such as agriculture and urbanization (Zhou et al., 2021). However,

conservation efforts for *Dactylorhiza osmanica* subsp. *osmanica* are unfortunately scarce in Türkiye (Sazak and Özdener 2006). For the conservation of *Dactylorhiza osmanica* subsp. *osmanica* and similar orchid species, the importance of ex vitro studies is emerging. The aim of this study is to determine the germination and development of *Dactylorhiza osmanica* subsp. *osmanica* seeds under ex vitro conditions with *Ceratobasidium* sp. to investigate the symbiotic development obtained by AG A (Accession Number: OR036462) vaccination and to provide important data for this endemic species. This study can be considered a fundamental step to develop strategies for conserving *Dactylorhiza osmanica* subsp. *osmanica*.

2. Materials and Method

2.1. Material

Dactylorhiza osmanica subsp. *osmanica* seeds were collected from East Black Sea Region in Trabzon, Türkiye (2300 m). The flowering period is between May and July, the altitude at which it grows is 1000-2400 m, its distribution area is riversides and meadows with high ground water. The mature capsules were taken, opened in the lab, dried for a few days at room temperature, and then stored at 4 °C in brown glass bottles.

2.2. Method

2.2.1. Fungal isolate

In this research, *Ceratobasidium* sp. AG A (accession number: OR036462) derived from the orchid-fungi collection at the University of Ondokuz Mayıs, Department of Biology. A segment of the fungal stock culture was aseptically transferred to a petri dish containing a meticulously prepared fungus isolation medium. Following an activation period, diligent observation was carried out to track the progressive colonization of the petri dish surface by the fungal hyphae. Upon achieving complete coverage, this cultivated fungus was utilized as the pivotal element for executing germination assessments.

2.2.2. Ex vitro symbiotic seed germination

For ex vitro seed germination, a soil mixture of ratio 2:1 (soil, perlite, respectively) was prepared. Afterwards, this mixture was sterilized in an autoclave (121 °C, 1.5Atm) and added to the pots. Previously activated fungi were inoculated into these pots. 6 seed packets were placed in each pot. About 1 mg of seeds (approx. 100 seeds) was added into the seed packets. The finished pots were placed in a climate chamber under 12:12 hours (light/dark) conditions at 23 ± 2 °C. Seed packs were placed in the control group, but the fungal isolate was not inoculated. All experimental groups were irrigated twice a week with ground oats and modified oat medium without agar. Developmental stages were evaluated according to the following stages, modified by (Clements et al., 1986): 0: Ungerminated seed, 1: Protocorm, 2: Leaf primordium, 3: First leaf, 4: Developed leaves and/or roots

2.2.3. Statistical analysis

Data were analyzed by the ANOVA method, which is analysis of variance, and the means were analyzed by Duncan's comparative test. Statistical analyzes were performed using the SPSS program. The visualizations of the statistical analyzes were made using the R program.

3. Results

3.1 Ex vitro symbiotic seed germination

Ceratobasidium sp. approximately 51.955% germination occurred in seeds inoculated with AG A (Table 1, Figure 1). However, seedling development did not occur. Protocorm and primordium formation rates differ (25.27% and 26.67%, respectively). This indicates that the fungus promotes germination of seeds. Germinated seeds are shown in Figure 2. No germination occurred in any of the fungal-free control groups.

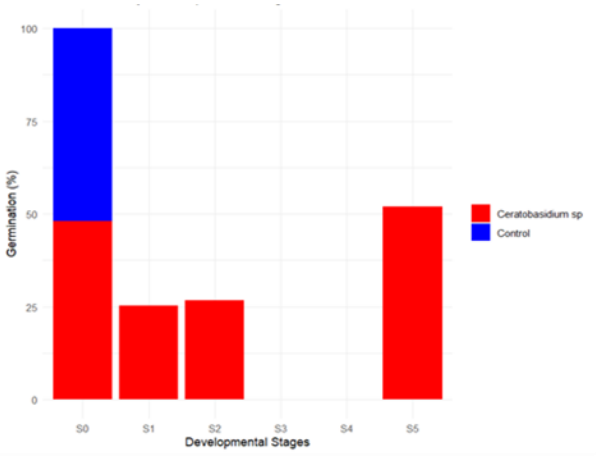

Figure 1. Column chart of *Dactylorhiza osmanica* subsp. *osmanica* development stages with R program. S0: Ungerminated seed, S1: Protocorm, S2:Leaf primordium, S3: First leaf, S4: Developed leaves and/or roots

Figure 2. *Dactylorhiza osmanica* subsp. *osmanica* protocorm and primodium stages

4. Discussions

The initial findings of our study indicate a positive impact on various stages of orchid seed germination, including the germination of *Dactylorhiza osmanica* subsp. *osmanica* seeds, protocorm development (the first germination phase), and leaf primordium formation. This is particularly remarkable considering that terrestrial orchid seeds typically possess hydrophobic and lignified seed pods, which act as a waterproof barrier, impeding water and nutrient absorption (Barsberg et al 2018; Gao et al., 2022). As a result, these seeds heavily rely on a mutually beneficial relationship with specific fungi, which provide them with essential nutrients (Zeng et al., 2012; Deniz et al., 2022). The process of symbiotic germination in orchids is

Table 1. Germination and development of *Dactylorhiza osmanica* subsp. *osmanica* seeds with *Ceratobasidiaceae*

	Developmental Stages (%)					Germination
	S0	S1	S2	S3	S4	%
Control	100±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
***Ceratobasidium* sp**	48.04± 1.52	25.27±1.46	26.67±2.98	0.00±0.00	0.00±0.00	51.95±1.52

S0: Ungerminated seed, S1: Protocorm, S2:Leaf primordium, S3: First leaf, S4: Developed leaves and/or roots

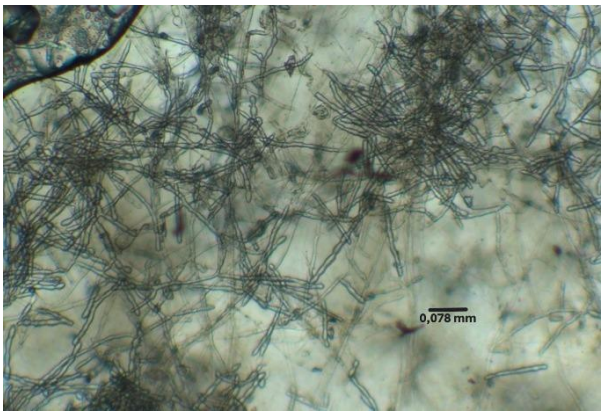

Figure 3. Isolated *Ceratobasidium* sp. derived from the orchid-fungi collection at the University of Ondokuz Mayıs, Department of Biology.

known to be exceedingly intricate and challenging. While there have been some in vitro investigations conducted on orchid species belonging to the *Dactylorhiza* genus (Rasmussen et al., 1990, Warghat et al., 2014), studies conducted ex vitro are relatively scarce (Aggarwal and Zettler 2010). Consequently, our study represents a significant contribution as the first ex vitro exploration specifically focused on *Dactylorhiza osmanica* subsp. *osmanica*. The outcomes of this study have shed light on the ex vitro germination potential *D. osmanica* subsp. *osmanica* seeds, facilitated by the mycorrhizal fungus *Ceratabaisidum* sp. However, it is worth noting that this particular fungus did not contribute to the subsequent growth and development of the seedlings. Nonetheless, the data generated from this research not only serve to aid in the conservation and propagation efforts of this species but also serve as an impetus for further investigations in this field.

The germination results observed in *Dactylorhiza osmanica* subsp. *osmanica* are consistent with the findings reported in previous studies on asymbiotic germination on *Dactylorhiza osmanica* subsp. *osmanica* (Sazak and Özdener, 2006) and other *Dactylorhiza* taxa (Hayakawa et al., 1999; Kömpe and Mutlu, 2007; Çığ and Yılmaz, 2017; Çığ et al., 2018; Fatahi et al., 2023).

By elucidating the germination dynamics and symbiotic associations in *Dactylorhiza osmanica* subsp. *osmanica*, our study expands the understanding of orchid conservation biology and the importance of fungal interactions in orchid seed germination. As seen in the control group, no germination was observed in pots without *Ceratobasidium* sp. In seeds inoculated with *Ceratobasidium* sp., a germination rate of approximately 51.95% was achieved. The protocorm formation rate was 25.27%, and the primordium formation rate was 26.67%. These findings suggest that *Ceratobasidium* sp. promotes seed germination. Future research endeavors could explore alternative mycorrhizal fungi that might exhibit a more comprehensive symbiotic relationship, encompassing both germination and subspequent seedling development. Additionally, investigates the factors that hinder the growth of *Dactylorhiza osmanica* subsp. *osmanica* seedlings in the presence of *Ceratabaisidum* sp. could provide valuable insights into the physiological and ecological aspects of orchid-fungal interactions.

In summary, our study highlights the ex vitro germination potential of *Dactylorhiza osmanica* subsp. *osmanica* seeds with the aid of the mycorrhizal fungus *Ceratabaisidum* sp. This research not only contributes to the conservation and propagation efforts of this orchid species but also paves the way for further exploration into the intricacies of orchid symbiotic germination.

Our findings provide evidence of the potential impact of *Ceratobasidium* sp. fungus on the ex vitro symbiotic germination of *Dactylorhiza osmanica* subsp. *osmanica* seeds. However, the lack of support for seedling development by this fungus raises a significant area of further investigation. These findings serve as a catalyst for future research endeavors in fields such as orchid conservation and restoration, where greater efforts are needed.

The discovery of the positive effect of *Ceratobasidium* sp. fungus on orchid seed germination represents a valuable contribution to the understanding of symbiotic interactions in orchids. While the current study focused on *Dactylorhiza osmanica* subsp. *osmanica*, it opens up avenues for exploring similar relationships in other orchid species. Investigating alternative fungal species and their potential role in facilitating both germination and subspequent seedling growth would enhance our understanding of the complexities of orchid-fungal symbiosis.

Overall, this study underscores the importance of further investigations into the ex vitro symbiotic germination of *Dactylorhiza osmanica* subsp. *osmanica*, specifically concerning the factors inhibiting seedling development in the presence of *Ceratobasidium* sp. These insights will not only advance our knowledge of orchid biology but also inform conservation and restoration strategies for orchid species. Continued research in this area will contribute to the effective management and preservation of these remarkable plants and their delicate ecological relationships.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

The authors contributed equally.

References

Aggarwal S, Zettler LW (2010). Reintroduction of an endangered terrestrial orchid, *Dactylorhiza hatagirea* (D. Don) Soo, assisted by symbiotic seed germination: First report from the Indian subcontinent. Nature and Science 8(10): 139-145.

Aewsakul N, Maneesorn D, Serivichyaswat P, Taluengjit A, Nontachaiyapoom S (2013). Ex vitro symbiotic seed germination of Spathoglottis plicata Blume on common orchid cultivation subsptrates. Scientia Horticulturae 160: 238-242.

Barsberg ST, Lee YI, Rasmussen HN (2018). Development of C-lignin with G/S-lignin and lipids in orchid seed coats–an unexpected diversity exposed by ATR-FT-IR spectroscopy. Seed Science Research 28(1): 41-51.

Clements MA, Muir H, Cribb PJ (1986). A preliminary report on the symbiotic germination of European terrestrial orchids. Kew Bulletin 41: 437-445. https://doi.org/10.2307/4102957

Chase MW, Cameron KM, Freudenstein JV, Pridgeon AM, Salazar G, Van den Berg C, Schuiteman A (2015). An updated classification of *Orchidaceae*. Botanical Journal of the Linnean Society 177(2): 151-174.

Çığ A, Yılmaz H (2017). In vitro symbiotic culture studies of some orchid species. Journal of Agricultural Sciences 23(4): 453-463.

Çığ AD, Durak E, İşler S (2018). In vitro symbiotic germination potentials of some *Anacamptis, Dactylorhiza, Orchis* and *Ophrys* terrestrial orchid species. Applied Ecology and Environmental Research 16(4):5141-5155.

de Vasconcelos FM, Andrade EHA, Teixeira LOA, Figueiredo PLB, Maia JGS (2023). Volatile constituents from *Catasetum* (*Orchidaceae*) species with occurrence in the Brazilian Amazon. Plants 12(4): 703.

Deniz İG, Kömpe YÖ, Harzli I, Aytar EC, Mutlu VA, Uysal Dİ (2022). From seed to flowering tuberous orchid using ex vitro symbiotic seed germination: A breakthrough study with Anacamptis sancta. Rhizosphere 24: 100597.

Fatahi M, Anghelescu NE, Vafaee Y, Khoddamzadeh A (2023). Micropropagation of *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski through asymbiotic seed germination and somatic embryogenesis: A promising tool for conservation of rare terrestrial orchids. South African Journal of Botany 159: 492-506. https://doi.org/10.1016/j.sajb.2023.06.036

Fırat M, Karavelioğulları FA, Aziret A (2015). Geophytes of East Anatolia (Turkey). Manas Journal of Agriculture Veterinary and Life Sciences 5(1): 38-53.

Gao Y, Peng S, Hang Y, Xie G, Ji N, Zhang M (2022). Mycorrhizal fungus *Coprinellus disseminatus* influences seed germination of the terrestrial orchid *Cremastra appendiculata* (D. Don) Makino. Scientia Horticulturae 293: 110724.

Hayakawa S, Uetake Y, Ogoshi A (1999). Identification of symbiotic *Rhizoctonias* from naturally occurring protocorms and roots of *Dactylorhiza aristata* (*Orchidaceae*). Journal-Faculty Of Agriculture Hokkaido Unıversity 69: 129-141.

Karakaya S, Polat A, Aksakal Ö, Sümbüllü YZ, İncekara Ü (2019). Plants used in traditional medicine and other uses in South of Erzurum (Turkey): An ethnobotanical study. Ethnobotany Research and Applications 18: 1-18.

Kömpe YO, Mutlu VA (2017). Mycorrhizal diversity in some species of *Dactylorhiza* genus (*Orchidaceae*). Biological Diversity and Conservation 10(1): 55-64.

Özdener Kömpe Y, Akin Mutlu V, Özkoç İ, Demiray S, Serhat B (2022). Fungal diversity and ex vitro symbiotic germination of *Serapias vomeracea* (*Orchidaceae*). Acta Botanica Croatica 81(1): 108-116.

Quay L, McComb JA, Dixon KW (1995). Methods for ex vitro germination of Australian terrestrial orchids. HortScience 30(7): 1445-1446.

Rasmussen H, Andersen TF, Johansen B (1990). Temperature sensitivity of in vitro germination and seedling development of *Dactylorhiza majalis* (*Orchidaceae*) with and without a mycorrhizal fungus. Plant, Cell & Environment 13(2): 171-177.

Sazak A, Özdener Y (2006). Symbiotic and asymbiotic germination of endangered *Spiranthes spiralis* (L.) Chevall. and *Dactylorhiza osmanica* (Kl.) Soó var. *osmanica* (endemic). Pakistan journal of Biological sciences 9(12): 2222-2228.

Selosse MA, Minasiewicz J, Boullard B (2017). An annotated translation of Noël Bernard's 1899 article 'On the germination of *Neottia nidus-avis'*. Mycorrhiza 27: 611-618.

Wani I A, Kumar V, Verma S, Tasleem Jan A, Rather IA (2020). *Dactylorhiza hatagirea* (D. Don) Soo: A critically endangered perennial orchid from the North-West Himalayas. Plants 9(12): 1644.

Warghat AR, Bajpai PK, Srivastava RB, Chaurasia OP, Chauhan RS, Sood H (2014). In vitro protocorm development and mass multiplication of an endangered orchid, *Dactylorhiza hatagirea*. Turkish Journal of Botany 38(4): 737-746.

Zeng S, Wu K, da Silva JAT, Zhang J, Chen Z, Xia N, Duan J (2012). Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. Scientia Horticulturae 138: 198-209.

Zhou Z, Shi R, Zhang Y, Xing X, Jin X (2021). Orchid conservation in China from 2000 to 2020: Achievements and perspectives. Plant Diversity 43(5): 343-349.