Journal of Applied Botany and Food Quality 97, 150 - 157 (2024), DOI:10.5073/JABFQ.2024.097.018

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Annual germination cycle of salep orchid (*Anacamptis sancta* L.) and adaptation to outdoor conditions

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(Submitted: June 3, 2024; Accepted: September 29, 2024)

Summary

Orchids have an important place in plant biodiversity. Although many orchid species are endangered, millions of tubers are removed and destroyed every year. *Anacamptis sancta* is one of the most widespread and most collected species. Since orchid seeds do not have endosperms, their reproduction rate in nature is low. It can be germinated asymbiotically in the laboratory environment, but the critical stage in this process is the acclimatisation of the plants from the *in vitro* growing media to the outdoor conditions. Seedlings that cannot establish mycorrhizal relationships in the transferred growing media die. Studies on acclimatisation of salep orchids to the outdoor environment are quite limited. In this study, the germination cycle of *Anacamptis sancta* was determined by sowing seeds in monthly intervals into asymbiotic growing media, and adaptation studies were carried out by transferring the seedlings to different growing environments. Starting from May, seeds were sown on modified Knudson C (KC) medium between the 15th and 20th of each month. The seedlings, which reached the transplant size after approximately five months, were transplanted to three different growing media consisting of peat, peat/perlite (3/1) and soil. In this study, which was repeated every month, 300 seedlings were transplanted into each growing media in three replicates, and a total of 900 seedlings were transplanted into three growing media. As a result, germination percentages in all months were higher than the reported studies. Besides, for the first-time direct transfer of orchid transplantation from laboratory to field was carried out and statistically the most successful results in outdoor adaptation were obtained from the seedlings transferred to the peat in August.

Keywords: *Anacamptis sancta* L., Salep orchids, Germination, Acclimatisation, Outdoor Conditions, Transplantation

Introduction

The family with the highest number of endangered species is Orchidaceae. It is also among the most threatened of all flowering plants (Huang et al., 2018). Orchids are at the front line of extinction, with more species under threat globally than in any other plant family (Kull and Hutchings, 2006; Swarts and Dixon, 2009). The number of orchid species has continued to decline dramatically over the last decades (Seaton et al., 2010). These species are facing a serious threat with an uncertain future because of collection for commercial uses (Swarts and Dixon, 2009). Especially orchids in the Mediterranean are under threat from over-zealous collectors and particularly from land-use changes (Neiland, 1994). The existence and population density of many terrestrial orchids have decreased due to climate change, changing ecological conditions, careless land use practices, habitat loss, over-harvesting, and illegal trade (Diantina et al., 2020; Djordjević and Tsiftsis, 2022).

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Salep orchids are used in the pharmaceutical, food and cosmetic industries (Kasparek and Grimm 1999) and in traditional medicine (Khasim et al., 2020). Supply for these uses is generally provided by collecting from nature (Hinsley et al., 2018). Orchid species in Turkey are under threat due to excessive harvesting in the wild (Bozdemir et al., 2018) and unless alternative solutions are found, some salep species will become extinct (Tamer et al., 2006). Salep is obtained for the food industry from the tubers of the *Anacamptis* genus. Although it is illegal to harvest orchid tubers from the wild, *Anacamptis* spp. tubers are widely harvested and traded due to their economic value (Teoh, 2019). *Anacamptis* genus is distributed in the Caucasus, Central Europe and Eastern Mediterranean countries (Arcidiacono et al., 2021). *Anacamptis sancta* is the most commonly used species in salep production due to its widespread occurrence (Bozdemir et al., 2018).

On the other hand, the seeds of all orchid species are very small, have no endosperm and do not contain sufficient nutrient reserves for growth, so they need an external nutrient resource during the germination period (Bewley et al., 2012). Besides, germination rate is low due to germination suppressing hormones such as abscisic acid (ABA) (Jevšnik and Luthar, 2015). For this reason, in vitro asymbiotic germination of orchid seeds in different nutrient media is preferred. In vitro production from seeds is especially preferred in terms of preserving biodiversity and variation (Arcidiacono et al., 2021).

Since salep orchids have a single growing eye in their tubers, vegetative propagation by tuber division is not possible. In this regard, tissue culture methods have some advantages compared to traditional methods in the production of species that are endangered and difficult to germinate (Babaoğlu et al., 2001). Additionally, by tissue culture methods, it can produce seedlings faster and throughout the year without being affected by seasonal and weather conditions by ensuring germplasm preservation of plants, less space required, and protection against diseases and pests (Emiroğlu and Gürel, 2005). Germination of orchids under in vitro conditions increases the effectiveness of conservation and breeding programmes. Although the germination rate of seeds is very low in natural environmental conditions, germination rates in in vitro growing media are generally over 70% (Kunakhonnuruk et al., 2018).

The genus *Anacamptis* is one of the Mediterranean orchids with facile seed germination (Betty et al., 2014; Dulić et al., 2020). *A. sancta* is a species with relatively easy germination (Pritchard, 1989; Bozdemir et al., 2018). Some species of the *Anacamptis* genus are reported to be tolerant of high temperatures and arid conditions (Djordjević and Tsiftsis, 2022). This feature provides an advantage for orchid cultivation. In natural habitats, the seeds of salep orchids disperse after they mature in the spring, and the seeds that remain in the soil for a year germinate in the spring of the following year. During this long waiting period, germination obstacles caused by the seed are eliminated by the influence of natural factors. In an asymbiotic growing media, this germination period can be shortened.

This study aimed to determine whether germination changes over time and the effect of the natural biological cycle on germination in the tissue culture environment. In addition, it is aimed to directly adapt the seedlings to outdoor conditions when they reach the seedling stage, without using an intermediate acclimatisation environment such as a greenhouse. The aim of this study was to ensure the asymbiotic regeneration cycle of *A. sancta* L. seeds on in vitro media and the adaptation of seedlings to outdoor conditions.

Materials and methods

Materials

The developmental stages of the *A. sancta* L. plant were followed, and fully matured seeds were used in the study. Seeds were collected from Menemen Agricultural Research Institute's Collection Garden, İzmir/ Türkiye. The seeds were removed, cleaned and mixed from the capsules dried at room temperature and stored in Eppendorf tubes and ziplock polyethylene bags at 4 °C. Germination studies were carried out in a digitally controlled germination cabinet (Lovibond TC 140 G-Liebherr, Dortmund/Germany). Seed counts were made with a stereo microscope (Irmeco, IM SZ550-B-ST5-H, Geesthacht/ Germany). A sterile biosafety cabinet (class II) was used for seed planting, and an autoclave was used for disinfection of the germination media (Tomy SX- 700e, Tokyo/Japan). Elga DV 35 (ELGA LabWater/UK) model pure water device was used to obtain pure water, and Hanna H1991002 model pH meter was used to regulate the ambient pH. Disinfection of the seeds was done with hydrogen peroxide (H_2O_2) (Sigma-Aldrich, Nord., 34.5-36.5%). Seeds were sown in sterile plastic Petri dishes with a diameter of 90 mm and wrapped in a double layer of transparent stretch film. Knudson C Modified Orchid Medium (KC) (Sigma) was modified by adding coconut water to the medium. Fungicide was added to the mixture to prevent fungal infections, and agar (Sigma-Aldrich 05039) was used to harden the medium. Digital devices were used to measure temperature and relative humidity. The temperature and humidity values of the outdoor conditions where the seedlings were transferred in the study are given in Tab. 1. Images of germinated plants were photographed by a Nikon E990 camera (Fig. 2 and 4).

Methods

Preparation of the growing media for asymbiotic germination and seedling production under laboratory conditions

In preparation of tissue culture medium, 21.6 g of Knudson C (KC) medium was dissolved in pure water. 7 g/l agar and 100 ml of fresh coconut water were added to this medium and the entire solution was made up to 1000 ml with pure water. To prevent fungal infections, 0.1 ml Maxim XL 035 FS (Active ingredient - 25 g/l Fludioxonil 10 g/l+Metalaxyl-M) fungicide was added to the mixture. the pH of the medium was adjusted to 7.0 using 0.1 N KOH and 0.1 N HCl (Rasmussen, 1995). The solution was sterilized for 20 minutes at an autoclave temperature of 121 °C to prevent degradation of sugary compounds in the medium (Lee and Yeung, 2018). After sterilization, when the ambient temperature dropped to 65-70 °C, the solution was poured into Petri dishes in the sterile cabin and left for 2 hours to solidify. Since orchids do not have endosperm, the amount of sugar present in the embryos is generally insufficient for germination (Ingold, 2012). 100 ml/l coconut water (HCS) was added to the KC medium as an organic compound before autoclaving to promote protocorm formation and grow tissues and seedlings (Kang et al., 2020).

Sterilization of seeds, planting and culture care of seedlings

Nearly 200 seed capsules were used in the seed study which were collected at the same time and stored in Eppendorf tubes at +4 °C. Cold stratification is needed for orchid seeds to germinate (Baskin and Baskin, 2014). Surface sterilization applied to mature seeds also eliminates dormancy (Pierce and Belotti, 2011). For this purpose, *A. sancta* seeds were treated with in 10% hydrogen peroxide (H_2O_2) in 2 ml Eppendorf tubes for 60 minutes for superficial sterilization (Bektaş, 2014; Lee and Yeung, 2018).

In the study, seeds were planted in the culture medium in Petri dishes under in vitro conditions between the 15th and 20th of each month, starting from May 2021. Seeds were spread homogeneously on the medium. In each planting period, 5 Petri dishes were planted with seeds. Each Petri dish contained approximately 800-1000 full seeds. The glass covers of the germination chambers were covered to prevent light and dark environment conditions were provided (Yamazaki and Miyoshi, 2006; Teoh, 2019). It has been determined that the most suitable temperature for orchid seed germination and seedling development is 20-25 °C (Arditti, 1980) and there is no significant difference in germination rates between 15 and 20 °C temperatures (Rasmussen, 1995). Since the seeds of many orchid species germinate at high rates at constant temperatures (Baskin and Baskin, 2014), the Petri dishes were kept at constant temperature conditions of 20 °C in the study. The experimental plan of the study is given in Tab. 2.

Germination counts were made periodically using millimetric paper according to Arcidiacono et al. (2021). In detail, counts were made by determining three 1 cm^2 areas on each Petri dish. Germination and development stages of the seeds were evaluated according to Yamazaki and Miyoshi (2006). As stated by Önal (1999), the seeds germinated in the dark at 20 °C were transferred to the incubator illuminated for 24 hours in order to perform photosynthesis in the 5th developmental period. As noted by Jevšnik and Luthar (2015), yellow light bulbs were placed in addition to the white light fluorescent lamp of the incubator, and an average of 2.200 lux was illuminated for 24 hours.

The pot experiments of the study were carried out in laboratory and uncontrolled outdoor conditions for two years. The census of plants that adapted to the external environment was made in the second

Tab. 1: The climate data of the study area for the period January-July 2022

Months	Temperature (°C)			Humidity (%)			
	Average temperature	Lowest temperature	Highest temperature	Average humidity	Lowest humidity	Highest humidity	Average humidity at minus temperatures
January	1.3	-5.1	25.3	71.7	58.2	92.4	80.7
February	7.6	-2.4	30.7	64.6	18.8	92.9	73.3
March	6.2	-3.9	32.3	60.0	15.0	93.6	75.9
April	17.2	2.9	32.4	47.9	15.0	91.6	-
May	21.0	7.8	39.1	43.8	15.4	91.7	-
June	24.5	16.8	39.1	47.5	15.2	89.1	-
July	26.5	16.5	46.6	37.1	15.4	77.4	-

vegetation period in April. If salep orchids can form tubers while adapting to outdoor conditions, they can emerge above the ground again during the following year's vegetation period. In this regard, the ability of the seedlings to emerge from the soil again the following year is taken into account as adaptation success.

Tab. 2: Sowing and potting dates of *A. sancta* seeds

Seed sown calender	Petri dish (N)	Seedling development period (months)	Planting time of seedlings	Number of seedlings planted (3 growing media × 3 replications × 100 seedlings = 900 seedlings)
17 May 2021	5	5	20 October 2021	900
16 June 2021	5	5	25 November 2021	900
10 July 2021	5	5	20 December 2021	900
15 August 2021	5	5	21 January 2022	900
17 September 2021	5	5	18 February 2022	900
15 October 2021	5	5	23 March 2022	900
23 November 2021	5	5	26 April 2022	900
20 December 2021	5	5	18 May 2022	900
14 January 2022	5	5	28 June 2022	900
23 February 2022	5	5	19 July 2022	900
16 May 2022	5	5	26 August 2022	900
19 April 2022	5	5	21 September 2022	900

Studies on acclimatisation to outdoor conditions

Seedlings grown under laboratory conditions were planted in different growing medias for 12 months to determine their adaptation to uncontrolled outdoor conditions. For adaptation studies the environments to which the seedlings will be transferred, were prepared to meet the conditions specified by Lee and Yeung (2018). Sterile peat (Swarts and Dixon, 2017), garden soil form Bursa plain and Perlite (Lee and Yeung, 2018), which provides good aeration, were used as organic matter sources in the mixtures. The experiments were carried out in pure peat (Portgrond P) (M-1), natural soil (M-2) and 1/3 volumetric peat/perlite mixture (M-3). The pH of the mixtures was measured, and no lime was added. The substrate used (Fig. 1) and their features are as follows:

M-1 (Peat): Portgrond P-Highly decomposed black sphagnum moss peat mixture, pH: 5.9, NPK; 14:10:18 ratios, 1.5 kg/m^3, EC: 0.40 dS/m (25%), Organic matter: 85-90%, Density: 180 kg/m^3, Production: Geeste-Germany

M-2 (Natural soil): Sandy, permeable and loose-structured, low in organic matter, pH: 8.8

M-3 (Perlite/peat mixture-(3/1-v/v): Super coarse agricultural perlite mixture, pH: 6.1

When the seedlings in the Petri dishes reached 2-3 cm in height, they were transferred to pots with a 17 cm mouth diameter and a capacity of 3 liters between the 20th and 25th of each month, starting from October. During planting, agar residue on the roots that could cause infection was removed (Park et al., 2018). 100 seedlings were planted in each medium of 3 replicates. After planting, spray irrigation was applied and the pots were randomly placed outdoors. To provide a semi-shade environment, the pots were covered with burlap that had a 50% shading effect (Manuel, 1998; Djordjević and Tsiftsis, 2022). They were covered with a polyethylene cover during extreme cold months (minimum measured temperature -13 °C) in January, February and March. The experiment field was checked every day and sprinkler irrigation was carried out manually with tap water as needed. (Karsten and Wodrich, 2007). The temperature and relative humidity of the experiment field were recorded with a climate data recorder (Hobo U10) that measured every 30 minutes. In the study, no nutritional supplements were given to the pots.

Statistical evaluation of results

The development of the seeds germinated in the asymbiotic growing media in Petri dishes was monitored every month and germination counts were made periodically. Germination percentages were determined, and it was tested statistically whether there was a difference in germination percentages depending on the monthly sowing period. Comparison of germination tests and survival rates of seedlings transferred to the outdoor environment was determined by one-way analysis of variance and Duncan and Friedman tests with the SPSS statistical program (IBM SPSS vers. 22).

Results and discussion

Asymbiotic germination studies

Anacamptis sancta seeds germinate easily and at a high rate. 5 days after sowing, the seed coat began to crack, and the first rhizoids began to form after 12 days. It was determined that almost all of the seeds containing embryos germinated (Fig. 2). The germination percentage of *A. sancta* seeds was found to be 69.4% and the empty seed rate was 30.6%. Germination was higher and more uniform in filled seeds than in other species (Fig. 3). Germination of *A. sancta* seeds begins 24 hours after water intake and is completed in approximately two weeks.

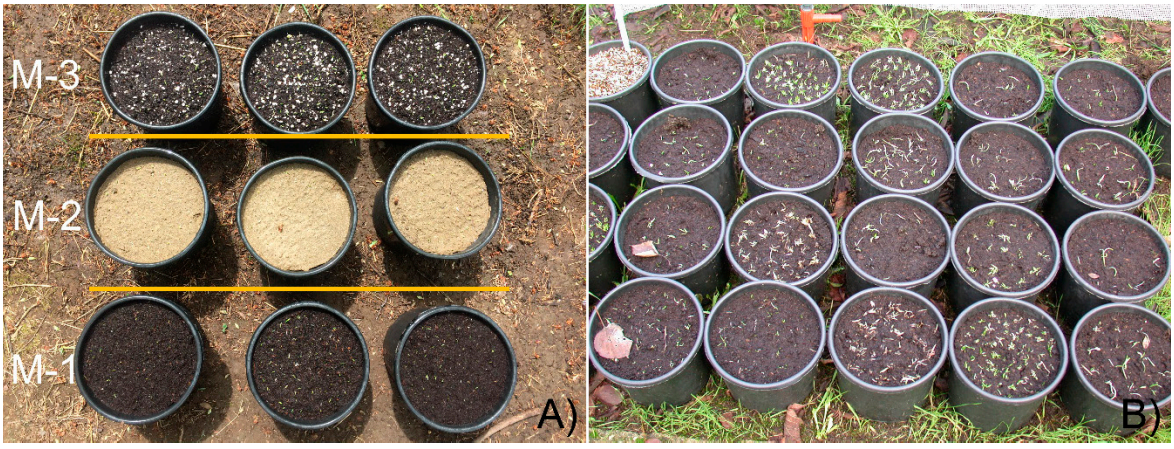

Fig. 1: A) Transplanted seedlings to three different substrates (top to bottom M-3, M-2, M-1) and B) General view of transplanted seedlings

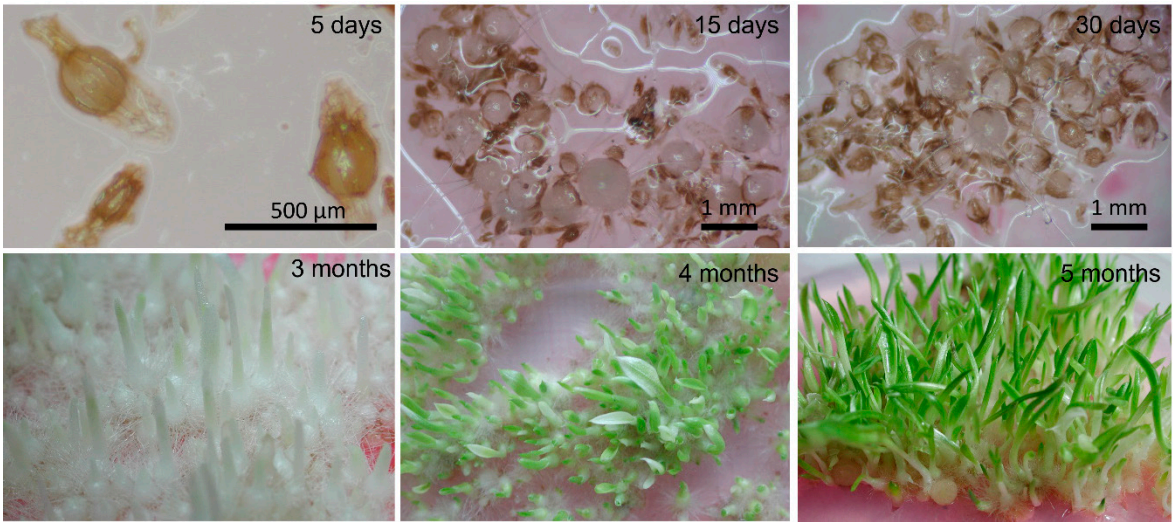

Fig. 2: Photographic images after germination on 5, 15, 30 days and 3, 4, and 5 months.

Protocorms can reach a size that can be transferred to the subculture medium one month after seed planting. This feature makes *A. sancta* one of the fastest germinating terrestrial orchid species (RASMUSSEN 1995; OIKONOMIDIS and THANOS, 2021).

Germination can vary depending on the season (JOHNSON, 2011). In the statistical analysis, it was determined that the germination percentages of *A. sancta* seeds showed a normal distribution according to months. Since seed planting was carried out regularly every month, the non-parametric Friedman test was used for repeated measurements. According to the results, it was determined that there was no significant difference in germination percentages according to months with a probability of 99.9%.

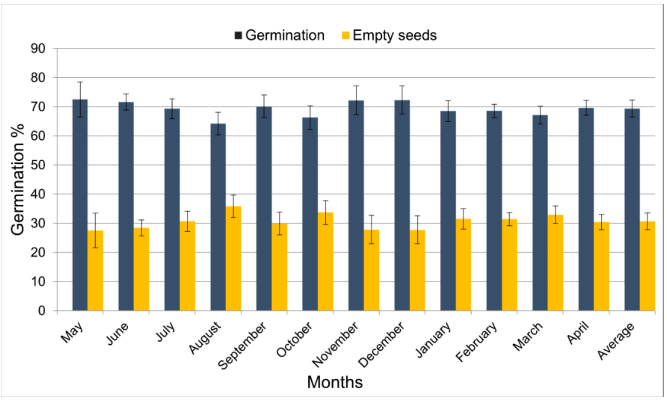

Fig. 3: Germination and empty seed rates in *A. sancta* (Statistically the difference is not significant) ($p \leq 0.5$))

In the studies conducted by BOZDEMIR et al. (2018) and PRITCHARD's (1989) studies, 74% and 84% germination were obtained for *A. sancta*, respectively. In the study conducted by OIKONOMIDIS and THANOS (2021) on *A. sancta*, the highest germination was obtained with 85.5%. In parallel with our study, ÖNAL (1999) reported in his study on *A. sancta* that all seeds (100%) germinated in Knudson C medium. The Petri dishes, which remained in the dark environment during the germination phase, were transferred to the lighted incubator approximately 2 months after planting, when promeristem began to appear. Terrestrial orchids should be kept in a dark environment during the first germination period, and the seedlings should be transferred to a light environment when they have formed good roots (JAKOBSONE, 2009). Light intensity can be understood from the colour of the leaves. Healthy plants in the in vitro growing media are bright green in colour. Excessive illumination can turn the leaves yellowish-green or red and may destruct chlorophyll and reduce chlorophyll content. Whitened leaves are an indication that the plant is severely blighted (JEVŠNIK VE LUTHAR, 2015; PARK et al., 2018). In the study, when the light intensity was increased to ensure rapid development of the seedlings, yellowing and whitening of the leaves began to occur

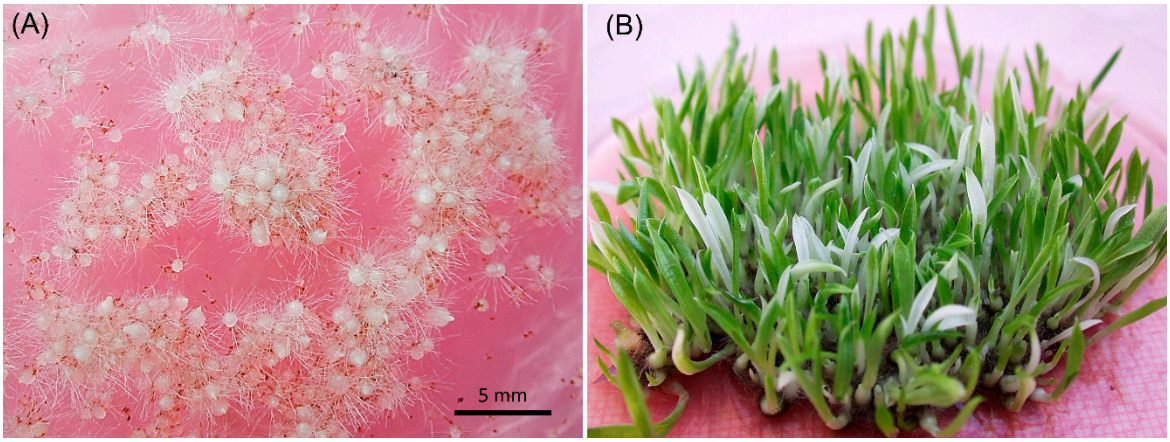

Fig. 4: Photographic images of homogeneous germination in *A. sancta* (A) and whitening of leaves caused by excessive light (B)

(Fig. 4). It is thought to be due to nutrient deficiency in the Petri dish, as excess light increases the rate of photosynthesis.

Outdoor environment acclimatisation studies

A. sancta seedlings grown in vitro were transferred to different prepared media and adapted to outdoor conditions. The first seedlings were transferred to the outdoor environment in September 2021, and the last group in September 2022. In transferring to the outside environment, the formation of a new tuber by the seedlings and the emergence of seedlings from the formed tubers were taken as success criteria. The seedlings were counted in March 2023. According to the statistical analysis results given in Tab. 4, it was determined that the date of transfer of the seedlings to the environment and the substrate used created a difference between the seedling survival rates. The interaction between planting date and environment was found to be ineffective (Tab. 3).

Tab. 3: Variance analysis of effects between applied procedures

Source	Interactions between processes Type III sum of squares	df	Mean square	F	*P*
Corrected model	691.229[a]	35	19.749	1.787	0.019
Intercept	283.573	1	283.573	25.659	0.000
Date of planting	**316.288**	**11**	**28.753**	**2.602**	**0.008**
Substrate	55.779	2	27.889	2.524	0.087
Date of planting * substrate	319.162	22	14.507	1.313	0.194
Error	795.726	72	11.052		
Total	1770.528	108			
Corrected Total	1486.955	107			

Seedling transplanting times and their development

Hardening is a prerequisite for plants grown in vitro to withstand adverse environmental conditions and adapt to ex vitro conditions. This exercise can be done with different methods (Teixeira da Silva et al., 2017). The transplanting stage is a major bottleneck in micropropagation of orchids (Klaocheed et al., 2021) and the survival of many terrestrial orchids may depend on seasonal rhythms (Pierce and Belotti, 2011). Mycorrhizal applications increase germination and seedling development (Teixeira da Silva et al., 2017). In their study, Harzli and Kömpe (2014) transferred seedlings from in vitro to ex vitro using suitable mycorrhizae and it was determined that the isolate used increased germination and seedling development. In a similar study, the reintroduction of ex vitro seedlings of *A. sancta* with a compatible fungus was successful (Deniz et al., 2022). However, mycorrhiza was not used in this study because no suitable strain was found. In the study, the rates of seedlings reforming tuber during transplantation periods and re-emerging during the vegetation period were found to be statistically significant. According to the transplanting periods, the re-emerging rates were 4.7% in August and 3% in June. No success was achieved with the transplants made in October and November, which is the time when orchids emerge from the soil in their natural environment. It is interesting to note that the highest survival rate was in seedlings transplanted in August (Tab. 4).

Similar to the findings in our study, Önal (1999) reported in his study that the optimum transfer time to soil for *Orchis laxiflora, A. sancta* and *Serapias vomeracea*, which are species produced by embryo culture, is August, and that the rate of developing cultures is low in transfers made in the spring (March - April). The highest survival rates were 81.2% and 74.2% in seedlings transferred to soil and in vitro subculture in August, respectively. The survival rate of tubers transferred to soil between November and April was lower (0.7%-14.6%). Arcidiacono et al. (2021) noted that in the *Anacamptis longicornu* subsp. *linkiana* species, approximately 90% of the seedlings survived in the study of transferring them to the outdoor environment under room temperature, natural daylight and a protective cover consisting of plastic film, and only 30% of the seedlings survived two months after the protective cover was removed. Antonetti et al. (2021) in their outdoor transfer studies on *Himantoglossum adriaticum,* H. Baumann and *H. robertianum* (Loisel.) P. Delforge species, the survival rate of the seedlings transferred in June was 100% at the end of 9 weeks, and the above-ground parts of all plants maintained their vitality until the end of the vegetative phase. In a period of approximately three months, from late October to early February, 19% of the plants were successfully acclimated to the outdoor environment.

Tab. 4: Seedling planting date and emerging rates Duncan test (Alpha = 0.05)

Date of planting	Seedlings (N)	Homogeneous groups
October	900	0.0000 *
November	900	0.0000 *
December	900	0.0000 *
March	900	0.0000 *
July	900	0.6377 *
January	900	1.2754 *
April	900	1.2754 *
May	900	2.0116 *
September	900	2.1787 *
February	900	2.5774 *
June	900	**3.6177 ** **
August	900	**5.8709 *** **
Sig.		0.155

The same asterisks are not significantly different using SPSS at $p \leq 0.05$

When the climate data is examined, the average temperature is measured as 1.3 °C in the lowest in January and 26.5 °C in the highest in July. The lowest temperature was -5.1 °C in January and the highest temperature was 46.6 °C in July. The lowest temperatures that *A. sancta* can tolerate in its natural distribution areas are at similar levels with this study. While the average humidity was 71.7% in January, it decreased towards July and became 37.1% (Tab. 1 and 2).

Tuber formation in the substrate used

The highest seedling emergence occurred in the peat environment. In the Duncan test and evaluation performed in the next vegetation season after planting, it was found that the highest seedling emergence from three different environments was in the peat environment (4.7%) and the lowest in the natural soil environment (0.3%). The Duncan test revealed the difference between the environments in seedling emergence (Tab. 5) (Fig. 5).

The selection of suitable potting mixtures for acclimatisation is another condition for improving plantlet survival (Teixeira da Silva

Tab. 5: Seedling emergence rates in the substrate used (Alpha = 0.05)

Medium	Pot (N)	Subset 1	Subset 2
Soil	36	0.6377	
Peat + Perlite	**36**	**1.8872**	**1.8872**
Peat	**36**		**2.3363**
Sig.		0.115	0.568

et al., 2017). It is known that the aeration capacity of the medium, capillary water movement, and water and nutrient retention capacity of the medium are effective in growing orchids under artificial conditions (QIAN et al., 2013). A well-drained and aerated substrate is required for the successful cultivation of terrestrial orchids. The medium used must create the right atmosphere for optimum root development and provide nutrients to the plant (KARSTEN and WODRICH, 2007). Considering the growing media requirements of orchids, the appropriate growing media for orchids transferred to soil must have maximum water holding capacity, porosity and drainage (KISHOR et al., 2006). Substrate combinations can be created with different substances to provide the ideal environment for orchids. These substrates should always be well-drained and contain organic matter for mycorrhizal fungi (SWARTS and DIXON, 2017). Organic matter keeps the growing media moist and provides nutrients to the plants. Ideally, water should be absorbed quickly and excess water should be drained away (KARSTEN and WODRICH, 2007).

Calcium affects both the physical and chemical properties of the soil. It is known that most European terrestrial orchids, including the genus *Anacamptis*, grow in calcium-rich soils. These soils are suitable for most orchid species that prefer warm, dry habitats, especially *Orchis anacamptis*. At the same time, the activity of nitrogen-fixing bacteria is higher in calcareous soils (MÉRILLON and KODJA, 2019). In soils with high carbon and nitrogen content, protocorms of some orchid species could not develop a symbiotic relationship with mycorrhizal fungi (DJORDJEVIĆ and TSIFTSIS, 2022). Generally, terrestrial orchids prefer a heterogeneous mixture of organic and inorganic substances, the addition of soil, perlite and other substances that improve drainage and reduce the fertility of the soil (PIERCE and BELOTTI, 2011).

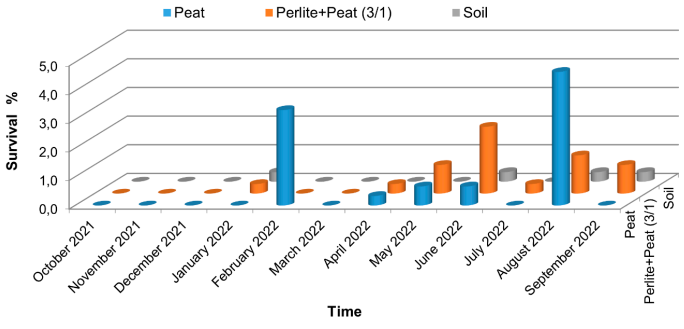

Fig. 5: Seedling planting periods and survival percentages

Many studies have been carried out on the adaptation of orchids to different substrate and the effect of substrate has been revealed. For example, PANTA et al. (2019) found that most orchid seedlings survived at rates of 70-85% in a mixture of 2:1(v/v) coconut fiber and sphagnum moss and 3:2:1 coconut fiber, decayed leaves and soil. In the study conducted on *Chloraea gavilu* Lindl terrestrial orchid, 95% of the seedlings survived after 40 days in a 1:1 mixture of peat and perlite (ROMERO et al., 2017). In the study conducted on *Dactylorhiza hatagirea* (D. Doon) Soo, all the seedlings preserved their viability in the mixture consisting of coir peat + vermiculite + perlite (1:1:1). A mixture of coir peat and vermiculite, which provides adequate moisture, aeration, and micronutrients for good growth of plants, resulted in good shoot and root growth (WARGHAT et al., 2014). HARTMANN et al. (2007) also stated that a mixture of perlite, coconut fiber and vermiculite provided faster shoot/root growth. A 1:1 mixture of moss and well-decomposed forest soil with the addition of charcoal and brick chips gave good results for terrestrial orchids (*Malaxis khasiana* (Hook. f. Kuntze) (DEB and IMCHEN, 2010). Three months after planting, 19% of the orchid seedlings were acclimated to the environmental conditions in a 7:2:1 peat, sand, and perlite environment by volume (PANTA et al., 2019). The highest survival rate of *Gastrochilus matsuran (*Makino) Schlechter grown in vitro, six weeks after planting, was observed in sphagnum moss with 79.7% (KANG et al., 2020). Seedlings were grown in mid-January and mid-February 2019. The medium containing 70:20:10 (v/v) peat, sand and perlite was found to be successful in acclimating species of the *Anacamptis* and *Serapias genera* to outdoor conditions (ANTONETTI et al., 2021). Seedlings of *Pecteilis radiata* (Thunb.) Raf. grown in vitro in sphagnum moss were 82% acclimated to outdoor conditions (KIM et al., 2019).

When *Dactylorhiza hatagirea* seedlings with 2-3 shoots grown in vitro were transferred to outdoor conditions, the highest survival rate was obtained from the mixture consisting of coir peat + vermiculite + perlite (1:1:1) by volume (WARGHAT et al., 2014). *Cattleya xanthina* seedlings survived 64% and 68% in two different substrates consisting of sphagnum peat and sphagnum+pine bark (50:50 v/v), respectively (JURAS et al., 2019). In the greenhouse environment, 98.8% of *Bletia purpurea* seedlings survived after 15 weeks of acclimatisation (DUTRA et al., 2008). During the acclimatisation phase after seed germination, the survival rate of *H. adriaticum* and *H. robertianum* seedlings was around 20% (ANTONETTI et al., 2021).

Soil reaction is one of the most important factors affecting the distribution of terrestrial orchids and controls the direct or mycorrhizal uptake of minerals. In general, most European terrestrial orchids grow in soils ranging from slightly acidic to slightly alkaline, with only a small portion of orchids growing in low pH values. The low number of orchids in strongly acidic soils with pH < 4.5 can be attributed to the fact that these soils contain high concentrations of harmful H^+ and Al^{3+} ions. The increase in orchid species with increasing soil pH can be attributed to the fact that increased pH positively affects the availability of nutrients. However, in highly alkaline soils and extremely acidic soils, mycorrhiza cannot survive and may cause a decrease in the number of orchids (DJORDJEVIĆ and TSIFTSIS, 2022).

The pH of the substrate used in the study was determined as 5.9 in peat, pH 6.1 in peat + perlite (3/1) mixture, and pH 8.8 in soil. Although *A. sancta* is found in slightly alkaline soils in its natural habitat, it is seen that peat and peat+perlite mixtures create an acidic environment.

In the adaptation of *A. sancta* seedlings to outdoor conditions, it was determined that the seedlings maintained their viability at a rate of 55% and 26%, respectively, after two months in peat and soil where antimicrobial chemicals (0.1% chlorocresol and 0.1% chloroxylenol) were applied. One month after the seedlings were uncovered, 87% continued their development, but no tuber formation occurred in the seedlings transferred to the soil (BEKTAŞ, 2014).

Another factor that is effective in the adaptation of orchids to the outdoor environment is outdoor environment or greenhouse conditions. Since it is not possible to control factors such as temperature and humidity in outdoor conditions, the survival rates of seedlings are lower than in controlled conditions. Acclimatisation is an important stage in adapting to outdoor conditions. Gradual acclimatisation of seedlings from tissue culture medium to outdoor conditions increases survival rates. To obtain nutrients and grow healthy, plants need well-developed roots and a suitable growing media that supports root development during the acclimatisation process (ASTARINI et al., 2015). Most seedlings produced in tissue culture substrate do not survive when transferred to a greenhouse or field environment. The reason for this is that these environments have lower relative humidity, higher and more intense light intensity compared to in vitro conditions, and these conditions create stress for plants (KLAOCHEED et al., 2021). Many different studies have been carried out on this subject. It is stated that the covers on the seedlings can be removed after 2-3 weeks and they can be accustomed to outdoor conditions. (KARSTEN and WODRICH, 2007). It has been observed that snails like to eat orchid leaves, especially in the spring, when acclimating them to the outdoor environment. This was also noted by KARSTEN and WODRICH (2007),

who found that they could destroy all the seedlings in a pot within a few hours.

Conclusions

All salep orchids in Turkey emerge from the soil in October-November, and while they are making their vegetative development in the winter period, if the tuber has formed, it remains latent in the soil in the summer period. Since some species have the capacity to form more than one tuber, they appear to multiply from one plant and form lumps over time. Seasonally changing development cycles make it necessary to take their physiological conditions into account in the production of these species. In this regard, germination and transfer of seeds to the outdoor environment were carried out periodically every month to determine the most suitable germination and transfer time to outdoor conditions. *A. sancta* seeds were germinated every month in the asymbiotic growing media, and after approximately 5 months, the seedlings reached the size to be transferred to the external environment. Although the highest germination percentage was obtained in plantings made in May, there was no statistically significant difference between germination rates according to time. Germination percentages were calculated based on the total number of empty and full seeds, and the average germination was calculated as 69.4 %. It was determined that *A. sancta* had a higher empty seed rate than other species.

There was a statistically significant difference between the transplantation time of the seedlings and the proportion of seedlings that formed a new tuber and started vegetative growth again. According to the transfer periods of the seedlings to the outdoor environment, the highest tuber formation and re-emergence rates occurred in the peat environment with a rate of 4.7% in August and 3% in June. Of the three different growing media, the highest survival rate was obtained from peat with 4.7%, and the lowest from soil with 0.3%. No success was achieved from the diversions made in October and November, which is the time when salep comes out of the soil in the natural environment.

As a result, although the seed sowing time did not make a difference in germination of *A. sancta*, it was determined that there was a statistical difference in the transfer studies to the external environment. In the propagation studies, seedling development can be achieved at any time of the year, but the vegetation period should be considered for tuber formation. The adaptation studies of the seedlings removed from the tissue culture to external conditions should definitely be carried out in a temperature and humidity-controlled environment, which will increase the success. Otherwise, the seedling survival rates decrease significantly.

Author contributions

SP conceived and designed the project. SP and KE carried out the germination and transplantation experiments. SP drafted the manuscript. KE reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This project was supported by Bursa Technical University BAP Coordination Office under project number 211N030.

Conflict of interest

No potential conflict of interest was reported by the authors.

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