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Effect of different substrates on *in vitro* symbiotic seed germination for soilless production of *Anacamptis laxiflora* orchid

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Abstract – In recent years, orchid species have become endangered due to excessive collection and habitat destruction. As with most flowering plants, seed production is the primary strategy for reproduction in orchids. Orchids produce tiny seeds consisting of a seed coat and a rudimentary embryo. However, it lacks the endosperm, which is generally required as the primary energy source during germination. The only way to germinate orchid seeds is to get nutrients from an external source. In nature, this is achieved by mycorrhizal symbiosis. This study used *Ceratobasidium sp.* inoculation of *Anacamptis laxiflora* (Lam.) seeds combined with media with various organic substrates to determine their effectiveness on *in vitro* germination and seedling development. The highest germination rate (35.78%) was obtained in the medium with the addition of young hazelnut leaves. Then, soilless *ex vitro* symbiotic germination was performed on young hazelnut leaves, the most effective organic substrate. Seed germination was determined to be 19.01% in this medium while 14.87% seedlings with developed leaves and roots were formed. For the first time, success was achieved by producing *A. laxiflora* from seed in *ex vitro* conditions without soil and adapting it to nature.

Keywords: *Anacamptis laxiflora*, *Ceratobasidium*, Orchidaceae, reintroduction, symbiotic seed germination

Introduction

Orchids are one of the most diverse and widespread families of flowering plants, with approximately 28,500 species, and are classified among the most threatened plant groups worldwide (Indan et al. 2021, Štípková et al. 2021). Orchids are facing a rapid decline in their population worldwide; therefore, it is among the main species with respect to plant protection (Yi-Bo et al. 2003, Tikendra et al. 2021). Although a single orchid produces thousands of seeds during the breeding season, very few seeds survive as adult plants and the germination of seeds depends on the presence of compatible fungi in the soil and optimal microclimate and micro-edaphic conditions (Nicole et al. 2005, Jacquemyn et al, 2010, Cardoso et al. 2020, Phillips et al. 2020).

The density of fungi in the distribution area of the orchid affects the germination rate of seeds (Jacquemyn et al. 2012). Habitat destruction due to global climate change, urbanization, agricultural purposes, environmental pollution, and over-collection for food and medical purposes threaten the existence of orchids (Rasmussen 1995, Sezik 2002). Orchids with their very different life types, specific pollinators, and mycorrhizal associations are very interesting plants and the focus of interest for scientists from different research disciplines (Rasmussen 1995, Yi-Bo et al. 2003).

Terrestrial orchids (temperate) are mostly distributed in Eurasian-Mediterranean regions and most of them have tubers under the soil. Glucomannan, a starch-like polymer found in these tubers, is an important food additive (Sezik 2002, Hossain 2011). Especially in Greece, Iran, and Turkey, the use of orchids as a food additive is the most important factor threatening their extinction (Sezik 2002, Ghorbani et al. 2014). Against these threats, seedling production by *in vitro* and *ex vitro* symbiotic seed germination with compatible fungi seems to be a very favourable method for the propagation and reintroduction success of temperate orchids (Aewsakul et al. 2013, Mutlu and Kömpe 2021, Deniz et al. 2022). It has been determined that the adaptation success of healthy and strong seedlings to the natural environment is quite high (Deniz et al. 2022). It has also been shown that rich media and substrates for strong seedlings are effective in symbiotic reproduction (Aewsakul et al. 2013).

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Anacamptis laxiflora is an orchid distributed in wetlands and at altitudes up to 1600 m above sea level in the Mediterranean countries of Western Asia, and Central and Southern Europe (Wood and Ramsay 2004). *A. laxiflora*, attracts attention due to its specific wetland habitats, which are becoming gradually reduced, making the species more vulnerable to extinction threat (Kömpe et al. 2020). These orchids with showy flowers and large tubers are one of the most collected species for the well-sought-after hot beverage, salep (Sezik 2002). One individual with loose flowers and the pollination of all of these flowers produces hundreds of thousands of seeds (Wood and Ramsay 2004). Some research has been conducted on the propagation of this species through *in vitro*, symbiotic and asymbiotic germination (Özkoç and Dalci 1993, Kömpe et al. 2020). The most important goal in both commercial and conservation studies is to produce healthy and strong seedlings that are easily adapted to the natural environment. It has been determined that healthy seedlings obtained with compatible fungus under *in vitro* and *ex vitro* conditions can easily adapt to nature (Kömpe et al. 2020, Deniz et al. 2022). The success of symbiotic germination is strongly dependent on the compatible fungus as well as the additives added to the nutrient medium. This is because the symbiotic fungus transports carbon and other nutrients from the nutrient medium to the embryo. The presence of suitable substrates in the germination medium positively affects germination and seedling rate (Aewsakul et al. 2013). In addition, *ex vitro* symbiotic germination seems to be a practical and effective method by which to obtain strong seedlings in ashort time and in simpler environments, as well as to ensure easier adaptation to nature (Aewsakul et al. 2013, Kömpe and Mutlu 2021, Deniz et al. 2022, Kömpe et al. 2022). In this context, the aim of this study is to determine the effect of different organic substrates added to a modified oat medium (Clements et al. 1986), which is used as an effective *in vitro* symbiotic culture medium, for the germination of *Anacamptis laxiflora* (Lam.) seeds and to demonstrate that the most effective substrate can be used as an *ex vitro* orchid propagation medium.

Materials and methods

Plant material

Seeds of *Anacamptis laxiflora*, which is commonly found in wet meadows in Samsun (Turkey), were collected in July 2017. The mature capsules were taken, opened in the laboratory, dried for a few days at room temperature, and then stored at 4 °C in brown glass bottles.

Fungal isolate

In this study, we used *Ceratobasidium* sp. (NCBI-accession number is MT605389) from the roots of *A. laxiflora* from previous research (Kömpe et al. 2020). The isolate is kept in the orchid-fungi collection of the Department of Biology at the University of Ondokuz Mayıs. A piece of the stock culture of this fungal isolate was taken and transferred to Petri dishes containing the fungus isolation medium (Clements et al. 1986) and the Petri dishes were wrapped with aluminium foil. They were incubated at 25 °C in the dark until the fungal hyphae completely covered the dishes. Fungus activation procedures were performed under sterile conditions and with sterile equipment throughout the entire study.

In vitro symbiotic seed germination

Modified oat medium (MOM) was used as a positive control in the symbiotic seed germination method (Clements et al. 1986). MOM contains $Ca(NO_3)_2 \times 2\,H_2O$ (0.2 g), KH_2PO_4 (0.2 g), KCl (0.1 g), $MgSO_4 \times 7\,H_2O$ (0.1 g), yeast extract (0.1 g), agar (10 g), ground oat (3.5 g) and sucrose (2 g) per litre and the pH is 5.8. Inorganic substances used for this medium were obtained from Merck (USA), and yeast extract and agar were obtained from Sigma.

Instead of ground oats, the same amounts (3.5 g L^{-1}) of ground old hazelnut leaves, young hazelnut leaves, straw, old oak leaves and young oak leaves, with sucrose (2 g L^{-1}) or without it, were added in the MOM. As young leaves, we used spring leaves and dried them in an oven at 40 °C. For old leaves we used dry brown leaves collected directly from the trees in autumn which were then dehumidified at 40 °C. These substrates were ground separately and added to each medium.

The medium prepared as above and adjusted to pH 5.8 was sterilized in an autoclave at 121 °C for 15 minutes and then poured evenly into Petri dishes in sterile laminar flow. After the MOM in the Petri dishes solidified, they were used for germination tests. In addition, MOM without sucrose and fungi were prepared as a negative control. All substrates added to MOM in this study and their modifications are given in Tab. 1.

Tab. 1. Characteristics of substrates tested for germination of *Anacamptis laxiflora* seeds.

Substrates	Abbreviations
Modified oat medium + fungus	MOM
Modified oat medium + fungus (no sucrose)	MOM (-S)
Modified oat medium (no fungus)	MOM (-F)
Modified oat medium (no sucrose and fungus)	MOM (-S and -F)
Old hazelnut leaf medium + sucrose+ fungus	OHL
Old hazelnut leaf medium + fungus (no sucrose)	OHL (-S)
Old oak leaf medium + sucrose + fungus	OOL
Old oak leaf medium + fungus (no sucrose)	OOL (-S)
Straw medium + sucrose + fungus	S
Straw medium + fungus (no sucrose)	S (-S)
Young hazelnut leaf medium + sucrose+ fungus	YHL
Young hazelnut leaf medium + fungus (no sucrose)	YHL (-S)
Young hazelnut leaf + fungus – *ex vitro*	YHL-EV
Young hazelnut leaf (no fungi) – *ex vitro*	YHL-EV (-F)
Young oak leaf medium +sucrose+ fungus	YOL
Young oak leaf medium + fungus (no sucrose)	YOL (-S)

The seeds were disinfected by immersion in 1% (v/v) solution of sodium hypochlorite (NaOCl) plus 0.1% (v/v) of Tween 20, followed by six rinses with distilled, autoclaved water. Seeds (approximately 100–150) were sown with a sterilized inoculation loop in Petri dishes (diameter 9 mm) containing the organic substrates tested in the study and incubated at 25 ± 2 °C in the dark for a week. Then, an agar block (approx. 0.5–7 mm^2) containing fungal hyphae was taken from a Petri plate containing the fungal isolate and put next to the seeds. Each application was repeated three times. All treatments were cultivated in a tissue culture chamber under 16:8 hours (light/dark) conditions at 25 ± 2 °C for 8 weeks.

Ex vitro symbiotic seed germination

After determining that the most effective substrate for total germination and seedling growth in *in vitro* conditions was young hazelnut leaves, this substrate was also used in *ex vitro* germination medium. Young hazelnut leaves were collected from a hazelnut orchard in Samsun/Turkey during spring (April-May), dried at 40 °C in an oven, ground, and then sterilized in an autoclave at 121 °C for 20 minutes before being placed into pots. Approximately 200–250 seeds were placed between sheets of water-resistant nylon mesh (45 µm pore size) and fixed between the dia frame (Rasmussen and Whigham 1993). Six seed packets were buried in each pot, and the pots were filled with equal amounts of young hazelnut leaves. In three pots, eight 1 cm^2 agar blocks containing fungal hyphae were placed around the seed packets, while the other pots were kept as a control group without any fungal inoculation. The pots were incubated under 16:8 hours (light/dark) conditions at 25 ± 2 °C in a climate chamber. Pots were irrigated once a week with a sterilized solution containing MOM's minerals and sucrose (1/2 strength).

Germination parameters

Three germination parameters were examined during 28 days to find out the potential effect of different substrates on seed germination of *A. laxiflora*. The observations were recorded daily for seeds.

Germination percentage was calculated according to Elezz et al. (2019), while germination index and mean germination time according to Marvin and Gonzales (2015):

$$\text{Germination percentage} = \frac{\text{seeds germinated}}{\text{total seeds tested}} \times 100$$

$$\text{Germination index} = \sum_{i=1}^{k} = \frac{\text{No. of germinated seed}}{\text{the count day}}$$

where: i = 1 is day one, k is the last day of observation.

$$\text{Mean germination time} = \frac{\sum_{i=1}^{k} ni * ti}{\sum_{i=1}^{k} ni}$$

where: ti is the time from day one to the last day of observation, ni is the observed number of germinated seeds every day, and k is the last day.

During seed germination, development was also monitored and described according to the following stages: S0 – no germination, S1 – swollen embryo with rupture of the seed coat, S2 – rupture of testa, globular embryo, rhizoids present (germination), S3 – protocorm formation, S4 – formation of first leaf and S5 – elongation of the first leaf and rooted (Zettler and Hofer 1998, Johnson et al. 2007). Stage 3 was considered the beginning of germination.

Data analysis

Data were subjected to analysis of variance (ANOVA) and mean values were compared by Duncan's multiple range test (DMTR), $\alpha = 0.05$) using IBM SPSS Statistics 24.

Results

Anacamptis laxiflora seeds germination

In order to evaluate the germination parameters, the germination status of the seeds in all culture media was checked daily from seed sowing. Germination started within 4 weeks of seed sowing and germination parameters were evaluated for the next 4 weeks (Tab. 2).

The effects of sucrose in combination with oat and other substrates on symbiotic germination and seedling development of *A. laxiflora* were firstly tested in *in vitro* conditions. MOM basic medium and its modifications, namely sucrose-free (MOM (-S)), fungus-free (MOM (-F)), and both sucrose- and fungus-free (MOM (-S and-F)), were used as control groups. No germination was observed in fungus-free substrates, including MOM (-F) and MOM (-S and-F), and consequently, data related to germination index (GI), germination rate (GR), and mean germination time (MGT) could not be obtained (Tab. 2). The highest GI values (Tab. 2) was achieved in YHL and OHL substrates (Tab. 1) and were significantly higher than that in MOM (Tab. 2). Other sucrose-containing substrates gave similar results to MOM. In the groups without sucrose, the lowest GI (Tab. 2) was observed in MOM (-S), OOL (-S), YOL (-S), and YHL (-S) substrates (Tab. 1).

The YHL substrate has been found to have the highest germination rate (GR) among all tested substrates *in vitro* (Tab. 2). It exhibited a germination rate three times as high as YHL (-S). Among other sucrose-containing media, including MOM, OHL had a significantly higher GR value. Among the sucrose-free substrates, MOM (-S), YHL (-S), and YOL (-S) were found to have lower GR rates. In contrast, OHL (-S) had the highest GR rate among the sucrose-free group, which was twice that of MOM (-S) (Tab. 2).

Regarding the mean germination time (MGT), sucrose-containing substrates *in vitro* exhibited the highest and lowest values in OOL and OHL media, respectively. However, there were no significant differences from MOM. Among the sucrose-free substrates, MOM (-S) had the highest MGT, and the lowest MGT (Tab. 2) was observed in YOL (-S) substrate, which was significantly different from the value observed in the same medium with sucrose.

Tab. 2. The effects of different organic substrates (see Tab. 1. for abbreviation explanation), with or without sucrose (-S) on symbiotic seed germination of *Anacamptis laxiflora*. The results are expressed as mean ± standard deviation, n = 3. One-way ANOVA and the post-hoc Duncan multiple range test was used to analyse differences between means. Statistical significance was set at $P < 0.05$. Means followed with the same letters denote that there was no statistically significant difference between groups.

Substrates	Germination index (GI)	Mean germination time (MGT)	Germination rate (GR) (%)
MOM	0.6 ± 0.4d	18.3 ± 4.7ab	15.60 ± 6.87cd
MOM (-S)	0.9 ± 0.1d	20.1 ± 2.7ab	14.42 ± 3.04d
MOM (-F)	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0e
MOM (-S and -F)	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0e
OHL	3.4 ± 1.5a	13.7 ± 3.6bc	31.16 ± 9.73ab
OHL (-S)	2.7 ± 1.4ab	13.6 ± 8.3bc	30.85 ± 5.67ab
OOL	1.3 ± 0.4bcd	22.1 ± 4.3a	24.95 ± 5.90bc
OOL (-S)	0.7 ± 0.4d	18.5 ± 1.1ab	15.87 ± 5.41cd
S	1.3 ± 0.9bcd	19.4 ± 4.1ab	24.13 ± 0.99bc
S (-S)	0.9 ± 0.2cd	19.4 ± 1.6ab	24.87 ± 6.12bc
YHL	2.6 ± 1.9abc	18.7 ± 2.3ab	35.78 ± 10.43a
YHL (-S)	0.7 ± 0.1d	18.4 ± 3.8ab	11.65 ± 1.11d
YOL	0.7 ± 0.1d	17.7 ± 1.9ab	14.40 ± 2.35d
YOL (-S)	0.5 ± 0.4d	7.9 ± 2.3c	9.54 ± 3.00d
YHL-EV	3.4 ± 1.21a	12.3 ± 0.5ab	19.01 ± 4.15cd
YHL-EV (-F)	0.0 ± 0.0d	0.0 ± 0.0d	0.0 ± 0.0e

Since under *in vitro* conditions, YHL had the highest GR and GI, it was used as the substrate for the *ex vitro* experiment. GI obtained in *ex vitro* conditions was slightly better than in *in vitro* conditions. However, GR was found to be approximately half of that in the *in vitro* and MGT was also slightly lower.

The developmental process of *Anacamptis laxiflora*

From the data collected for the germination of *A. laxiflora* seeds in the different treatments, the developmental stages were assigned to a qualitative scale, according to a modification from Zettler and Hofer (1998), and Johnson et al. (2007).

The effect of substrates added instead of oat in the modified medium on seedling development in the presence and absence of sucrose is shown in Tab. 3. Seedling growth was evaluated 8 weeks after seed sowing. According to the results, leafy seedlings (stage 5) were formed at different percentages in all *in vitro* substrate modifications as well as in *ex vitro* condition (FHL-EV). No growth was observed in

Tab. 3. Effects of substrates and mycorrhizal inoculation on the developmental stages of seeds of *Anacamptis laxiflora* at 8 weeks after seed sowing. Development of the seedlings was divided into stages: S0 – no germination, S1 – swollen embryo with rupture of the seed coat, S2 – rupture of testa, globular embryo, rhizoids present (germination), S3 – protocorm formation, S4 – formation of first leaf; S5 – rooted and leafy plantlet. The effects of substrates and their sucrose-free modifications on developmental stages were analyzed using one-way ANOVA. Results were compared using the standard deviation of the means (n = 3) and the post-hoc Duncan multiple range test. Statistical significance was set at $P < 0.05$. There is no statistically significant difference between groups with the same letters.

Substrates	Development stages %					
	S0	S1	S2	S3	S4	S5
MOM	77.64 ± 5.2abcd	6.77 ± 2.1ef	4.15 ± 1.2abc	3.14 ± 1.7bcde	4.15 ± 2.2abcd	4.15 ± 2.2cde
MOM (-S)	72.30 ± 3.8bcdef	13.28 ± 4.8bcd	3.34 ± 1.5bcd	2.91 ± 1.1bcde	1.66 ± 0.8de	6.50 ± 2.0bcd
MOM (-F)	82.10 ± 3.5ab	17.90 ± 2.42b	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e
MOM (-S and -F)	72.41 ± 5.9bcdef	27.59 ± 5.9a	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e
OHL	65.57 ± 9.3ef	3.27 ± 1.3f	5.21 ± 1.8ab	7.37 ± 4.9ab	3.84 ± 0.5abcd	14.73 ± 3.5a
OHL (-S)	63.27 ± 7.5fg	5.88 ± 2.3ef	4.52 ± 2.2abc	6.13 ± 3.8abcd	5.05 ± 2.4ab	15.16 ± 3.3a
OOL	67.76 ± 3.6def	7.30 ± 2.3def	3.42 ± 0.5bcd	7.28 ± 1.8ab	4.93 ± 2.1abc	9.32 ± 2.4b
OOL (-S)	78.52 ± 2.0abcd	5.62 ± 3.3ef	3.27 ± 1.2bcd	3.31 ± 1.3bcde	3.67 ± 1.8abcd	5.62 ± 1.9bcd
S	70.09 ± 2.8cdef	5.78 ± 2.4ef	3.14 ± 1.3bcd	6.99 ± 1.4abc	6.13 ± 2.6a	7.87 ± 2.6bc
S (-S)	67.72 ± 6.5def	7.41 ± 2.8def	4.83 ± 0.8ab	7.47 ± 4.6ab	3.34 ± 0.5bcd	9.23 ± 1.6b
YHL	55.47 ± 12.0g	8.75 ± 2.1def	6.81 ± 3.5a	9.04 ± 5.1a	4.88 ± 1.0abc	15.05 ± 4.3a
YHL (-S)	82.04 ± 3.5ab	6.31 ± 2.5ef	1.55 ± 0.3cde	2.21 ± 0.3cde	3.84 ± 0.5abcd	4.05 ± 0.9cde
YOL	74.50 ± 5.3abcde	11.11 ± 5.2cde	3.20 ± 1.9bcd	4.14 ± 1.8bcde	2.31 ± 0.8cde	4.74 ± 0.7cd
YOL (-S)	79.26 ± 3.abc	11.20 ± 3.8cde	1.72 ± 0.2cde	2.05 ± 0.7de	2.88 ± 1.1bcd	2.88 ± 1.1de
YHL-EV	78.07 ± 3.2abcd	2.93 ± 1.3f	0.98 ± 0.44de	1.60 ± 1.18de	1.56 ± 0.52de	14.87 ± 3.88a
YHL-EV (-F)	83.71 ± 4.8a	16.29 ± 4.8bc	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e	0.0 ± 0.0e

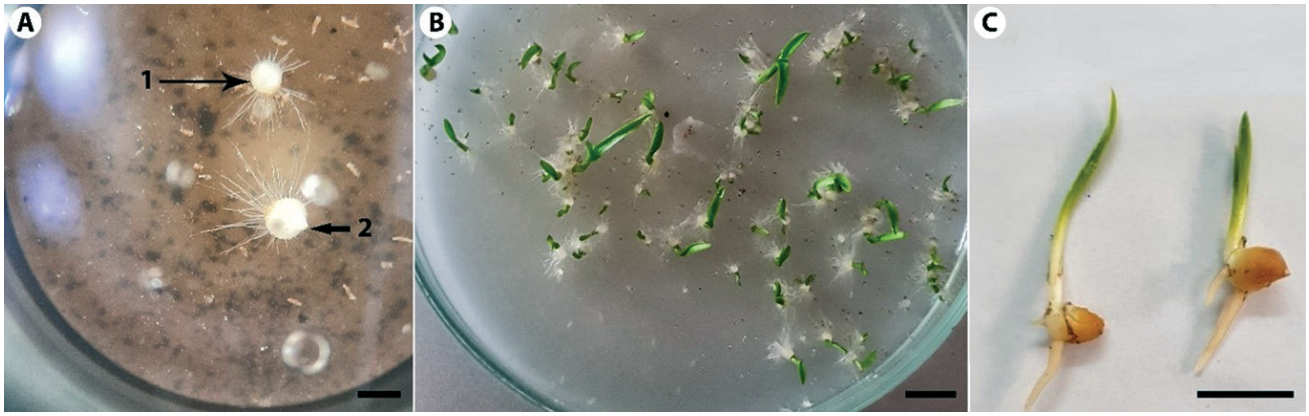

Fig. 1. Protocorm and progression of seedling development of *Anacamptis laxiflora* cultured on YHL medium. A) Stage 2 - protocorms and stage 3 - leaf primordium (arrows 1 and 2 show protocorm and leaf primordium, respectively), B) Stage 4 - leafy seedlings, C) Stage 5 – rooted and leafy seedlings. Scale bars: A - 1 mm, B and C - 1 cm.

the control groups without fungal inoculation (MOM (-F), MOM (-S and -F)).

The highest percentage of protocorm formation (S3) was observed in the YHL substrate (Fig. 1A) and it was approximately three times higher than in MOM. Results on other substrates showed no difference from those obtained with MOM. The protocorm formation in substrates that did not contain sucrose were similar to that obtained for substrates with sucrose except for YHL (-S) where significantly lower protocorm formation was observed (Tab. 3).

The highest percentage of first leaf formation stage (S4) was observed in the straw (S) substrate with sucrose, while the OHL (-S) had the highest value among the sucrose-free modifications. The lowest percentage was in MOM (-S) under *in vitro* conditions.

The highest percentage of seedlings with roots and leaves (S5) was observed in the YHL substrate (Fig. 1C), which was approximately 3.5 times higher than that of MOM. OHL and OOL media had also significantly higher values than MOM. The lowest percentage in sucrose-containing substrates was observed in the YOL, following MOM. In sucrose-free substrates, the best percentage was observed in OHL (-S) which was similar to the value observed for OHL and more than twice that of MOM (-S). The lowest percentage was in the YOL (-S) medium.

Regarding *ex vitro* conditions, although the germination rate in YHL medium was lower than that in the *in vitro* medium containing the same substrate, there was an interestingly high percentage of seedlings in the S5 stage (Fig. 2C), with a value similar to that for YHL *in vitro*. Moreover, the seedlings successfully survived transfer to nature (Fig. 2D).

Discussion

Orchids are in danger of extinction because of habitat destruction, over-harvesting, and the lack of a suitable fungus for seed germination. *In situ* and *ex situ* conservation methods are recommended for the protection of orchids. However, *ex situ* protection is also insufficient due to the rapid destruction and disappearance of habitats and the vulnerability of the specific habitats they are found in (Batty et al. 2006, Lemay et al. 2015, Štípková et al. 2021). Seed banks are also included in conservation programs, as it is known that orchid seeds can maintain their viability for many years (Kömpe et al. 2020). However, as this longevity is limited seed banks may not be a long-term solution. In recent years, it has been accepted that the most effective

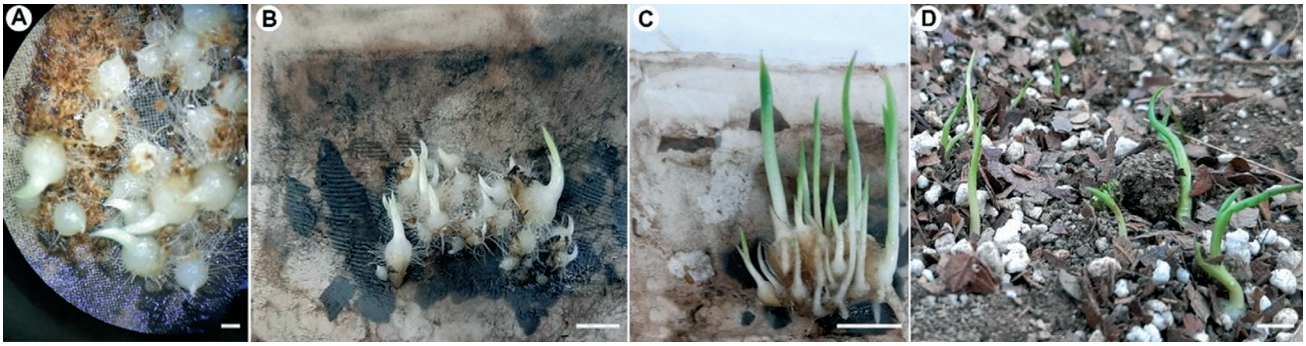

Fig. 2. *Anacamptis laxiflora.* A) Protocorms that developed 28 days after the seed packs were embedded in the fresh hazelnut leaf, B) the seedlings five weeks later, C) the seedlings with root that developed eight weeks later, D) transplanted seedlings in nature. Scale bar: A – 1 mm; B, C and D – 1 cm.

protection method is to transfer asymbiotic and/or symbiotic seedlings to the natural environment to establish a population (Smith et al. 2010, Duncan and Moloney 2018, Soares et al. 2020, Kömpe and Mutlu 2021, Wang et al. 2021, Zhao et al. 2021). The success of asymbiotic and symbiotic methods varies according to the orchid species, the nutrient media used, and the appropriate fungus (Rasmussen 1995). *A. laxiflora* is a wet meadow orchid found throughout Central Europe and the Mediterranean, including Turkey. There are some studies in which symbiotic and asymbiotic methods are applied to germinate seeds of this species (Mead and Bulard 1975, 1979, Özkoç and Dalcı 1993, Deconninck and Gerakis 2021, Kömpe et al. 2020). However, these studies mostly remained within the laboratory limits.

In this research, first, the efficiency of various organic substrates and sucrose on symbiotic seed germination and seedling growth was determined *in vitro*. Then *ex vitro* symbiotic germination was performed on the most effective substrate.

It is known that the basic functions of compatible fungi effective in the germination process of orchid seeds in nature are to carry carbon, nitrogen, and some other basic nutrients to the embryo (Jacquemyn et al. 2012). Detailed studies on the effect of carbon sources in *in vitro* replication studies were also conducted (Hadley and Perombel 1963, Smith 1966, Yam and Arditti 2009, Mehra et al. 2017). In addition to soluble carbon sources (such as monosaccharides, disaccharides, and polysaccharides), the effects of various organic substrates (milled oats, rotted leaves, cockroaches, peat moss, etc.) were also tested and different effects emerged (Mala et al. 2017). It was found that a compatible fungus and symbiotic method and substrates such as oat and peat moss were more effective in the germination of seeds of tropical exotic orchids (Mala et al. 2017). Orchid seeds do not contain endosperm, so they need all essential nutrients, especially carbon, to be transported by fungi until the photosynthetic stage. This occurs more abundantly and faster in the presence of rich substrates. Although the effect of hazelnut leaves has not been tested on the germination of any orchid seed before, Mala et al. (2017) suggested that natural substrates can be more effective in the development of symbiotic seedlings than oat in MOM.

In our study, it was revealed that different natural substrates significantly promoted germination and seedling development, and that sucrose increased this stimulation. This is not surprising since sucrose is a carbon source that plant cells use until the photosynthetic stage is established. The use of sucrose as a carbon source in the culture medium has been shown to be very important in orchid seed germination and protocorm development for both mature and immature seeds (Arditti 1967, Rasmussen et al. 2015, Gupta 2016).

Reintroduction studies have shown that if the transferred seedlings are well developed, the success and continuity of survival in nature are also higher (Aewsakul et al. 2013; Kömpe et al. 2022; Deniz et al. 2022). Therefore, in this study we performed an *ex vitro* experiment with the substrate that proved the most effective in order to obtain strong seedlings for reintroduction under *ex vitro* symbiotic conditions. *Ex vitro* symbiotic propagation studies conducted so far using different substrates have revealed very remarkable results. The *ex vitro* symbiotic method was first applied by Quay et al. (1995). These researchers inoculated fungi into a mulch obtained from *Allocasuarina fraseriana* trees. Although the germination rate was low, this was pioneering research for *ex vitro* germination (Quay et al. 1995). Aewsakul et al. (2013) inoculated two Epulorhiza isolates (isolated from *Dendrobium anosmum* and *Paphiopedilum sukhakulii*) onto different substrates (soil, coconut powder, peat moss) and planted *Spathoglottis plicata* seeds in these substrates. They achieved successful results with the best germination obtained in peat moss (Aewsakul et al. 2013).

The results obtained in this study with natural substrates different from those used by Aewsakul et al. (2013) are promising for both conservation and economic purposes. We observed that hazelnut leaves (young and old) had a remarkably positive effect on germination and seedling development compared to other substrates. This situation may arise from the structural or functional molecule content of the substrates. Various secondary metabolites present in straw and oak leaves may have negatively affected fungal activity and/or germination process. It is well known that many phenolic compounds have antifungal or allelopathic activity (Wang et al. 2017), so they may have negatively affected fungal activity under symbiotic germination conditions. The reason why specifically young hazelnut leaves are more effective than other substrates is most likely because during the active photosynthetic phase leaves contain more carbon (including soluble carbon), nitrogen, and other essential elements. Since most of the nutrient elements are transferred to younger leaves during the aging process, old leaves are poorer in terms of nutrient content (Taiz and Zeiger 2010). However, old leaves also contain structural polysaccharides, cellulose, which can be degraded into glucose monomers and transported to the embryo during orchid seed germination (Gong et al. 2023). This can explain satisfactory germination results obtained in our study with substrate from old hazelnut leaves, even without the presence of sucrose. The chemical profiles of these substrates should be evaluated in detail in future studies to determine their potential effects on symbiotic germination.

Conclusions

The addition of organic materials as a supplementary substrate to the nutrient medium was found to enhance the germination and seedling development of *A. laxiflora* in *in vitro* conditions. The highest germination and seedling growth was obtained using fresh hazelnut leaves. Sucrose, together with natural organic additives, strongly supported germination and seedling growth. Fresh hazelnut leaves also proved successful in *ex vitro* conditions providing high

percentage of seedlings with developed leaves and roots. Given the importance of orchids and their status as a threatened species, it is critical to develop cheap, easy, and fast methods for their mass production. The use of these substrates for this purpose is promising, as it would not only reduce the cost but also decrease the laboratory dependency, thereby providing new avenues for future research. Further detailed studies are needed to optimize these methods and fully understand their potential for application in orchid production.

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