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COMPARATIVE GROWTH MEDIA PERFORMANCES ON *IN VITRO* PROPAGATION OF SOME SALEP ORCHIDS

Yasemin KEMEÇ HÜRKAN [1,*], Kaan HÜRKAN [2], Cüneyt AKI [3]

[1] Department of Biology, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[2] Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[3] Department of Biology, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

ABSTRACT

Due to the increasing demand and over-collection of orchids from nature to produce salep, scientists have been led to search for more efficient ways to propagate these specific orchids *in vitro*. This present study compares germination performances of two commercial (Orchimax and Knudson C) and one specially prepared orchid growth media (SV) on economically and medicinally important orchids used to make salep; *Anacamptis pyramidalis* (L.) L.C. Rich, *Anacamptis morio* (L.) R.M. Bateman, Pridgeon & M.W. Chase subsp. *morio*, *Dactylorhiza romana* (seb.) Soo, *Neotinea tridentata* (Scop.) R.M. Bateman, Pridgeon & M.W. Chase and further aims to obtain a mature orchid plant by following the natural environmental cycle.

Significant differences in seed germination and protocorm development were observed. Asymbiotic germination tests showed that the specially prepared growth media performed better than the commercial media by 79.11% germination rate. Also, that *A. morio* subsp. *morio* had the best germination rate by 88.91%. Protocorms developed in the sixteenth week after sowing. Soil was collected from the natural habitat of each species and was used as a potting substrate, and this helped orchids to pass their initial acclimatization stage. Regeneration success of orchids for *in vitro* conditions could be increased by using SV growth medium, following their natural seasonal cycle and using specific substrates from their respective habitats.

Keywords: Micropropagation, Salep orchids, Orchimax, Knudson C, Svante Malmgren

BAZI SALEP ORKİDELERİNİN *IN VITRO* ŞARTLARDA YETİŞTİRİLMESİ ÜZERİNE FARKLI BESİN ORTAMLARININ VERİMLİLİKLERİNİN KARŞILAŞTIRILMASI

ÖZET

Orkidelerin, salep üretmek için doğadan aşırı miktarda toplanmaları, bilim insanlarını bu orkidelerin *in vitro* şartlarda üretilebilmesi için daha verimli yollar aramaya yönlendirmiştir. Bu çalışmada iki adet ticari olarak üretilen (Orchimax ve Knudson C) ve bir adet özel olarak hazırlanan (SV) besin ortamlarının, salep elde edilen *Anacamptis pyramidalis* (L.) L.C. Rich, *Anacamptis morio* (L.) R.M. Bateman, Pridgeon & M.W. Chase subsp. *morio*, *Dactylorhiza romana* (seb.) Soo ve *Neotinea tridentata* (Scop.) R.M. Bateman, Pridgeon & M.W. Chase orkidelerinin çimlenme performansı üzerine etkileri, çimlendirme boyunca doğadaki iklimsel şartlar taklit edilerek karşılaştırılmıştır. Yapılan çalışma sonucunda tohum çimlenmesinde ve protokorm gelişiminde, besin ortamlarının sebep olduğu önemli farklılıklar belirlenmiştir. Çalışmadaki asimbiyotik çimlenme denemeleri, en iyi çimlenme ortamının %79.11'lik oran ile SV besin ortamı olduğunu göstermiştir. Aynı zamanda *A. morio* subsp. *morio* orkidesinin, %88.91 oran ile en iyi çimlenme performansına sahip olduğu görülmüştür. Tohumların besin ortamlarına ekimlerinden 16 hafta sonra protokorm yapısına dönüştüğü gözlenmiştir.

Anahtar Kelimeler: Mikroçoğaltım, Salep orkideleri, Orchimax, Knudson C, Svante Malmgren

1. INTRODUCTION

Orchids (Orchidaceae) are the second largest group of flowering plants with 27801 species [1]. They are an economically and aesthetically important plant group and have been used in many different branches of industry, such as food, pharmaceutical floriculture and cosmetics. The whole plant, as well as parts

*Corresponding Author: kemecyasemin@gmail.com

have been used as medicine and also used in several food products for centuries [2]. Moreover, orchids are very popular among plant enthusiasts from all over the world due to their various flower shapes, mimicry capabilities, germination strategies, habitats and etc. Many amateur orchidists organize orchid photography tours and travel long distances to search out orchids in their natural habitat. Although being popular, biodiversity and population of orchids have dramatically decreased due to global habitat loss, deforestation and urbanisation. Native orchids used in the preparation of salep and "Maras" ice cream are over-collected (20 tons ≈ 40 million tubers per year in Turkey) from their natural environment in Asia-Minor and some Mediterranean and Arabic countries [3, 4]. Hence there is an urgent need to propagate orchids on *in vitro* conditions to be able use them in medicine and within the food industry as well as re-establishing native populations of these endangered species.

Anacamptis pyramidalis (L.) Rich., *Dactylorhiza romana* (Sebast.) Soó, *Neotinea tridentata* (Scop.) R. M. Bateman, Pridgeon & M. W. Chase, and *Anacamptis morio* (L.) R. M. Bateman, Pridgeon and M. W. Chase subsp. *morio* orchids are some of the most collected orchids used in salep production in Turkey. Annually, on average 36.5 tons (73 million tubers) of these species of orchids are collected [5]. Therefore, it is necessary to propagate these orchids artificially *in vitro* conditions to be grown commercially and used to produce salep and re-establish the natural population from over collection.

In vitro tissue culture studies of orchids began in 1922. Knudson [6] with *Catteya*, *Laelia* and *Epidendrum* seeds successfully germinated on *in vitro* conditions by using agar and sucrose.

Hatipoglu et al. [7] used modified Burgeff, Fast and Voth growth media to germinate terrestrial salep orchids (*A. pyramidalis* and *Anacamptis sancta* (L.) R.M.Bateman, Pridgeon & M.W.Chase). They reported that the seeds germinated on Burgeff medium on the 20th day after sowing and the leaves emerged on 50th day. Although germination was successful, plantlets died during acclimatisation or hardening off period which is critical for young plantlets when taken from flask. Waes & Debergh [8] studied germination factors on 23 European orchids. One of the most important factors during seed germination is the duration of the pre-treatment of orchid seeds with calcium hypochlorite + Tween-80. They found the best germination occurred in continuous darkness at 23°C on dilute media. Vejsadová [9] found that the treatment of 7.2% of sodium and calcium hypochlorite improved the germination percentage of 3 critically endangered species of terrestrial orchids (*Dactylorhiza incarnata* (L.) Soó subsp. *serotina* (Hausskn.) Soó & D.M.Moore, *Dactylorhiza maculata* (L.) Soó and *Liparis loeselii* (L.) Rich.).

In nature, successful germination of orchid seeds depends on several factors (i.e. soil, humidity, temperature, mycorrhizal fungi, etc.). The best germination period for terrestrial orchids is September – October in the Mediterranean region. Once the seeds germinated, the protocorms begin develop in November – December, and the first leaves emerge in April [10].

Önal [11] studied the artificial germination of 21 terrestrial orchid species on Knudson C growth media and found tuber formation of *Orchis sancta* L. and *Orchis laxiflora* C.A.Mey. in dark conditions at 5°C was higher than standard tissue culture conditions (25°C and 16h light). Similarly, Szendrak & Read [12] applied 10-12°C in dark conditions for 4 weeks, on the orchid seeds in order to break their dormancy state. Kitsaki et al. [13] studied the propagation of 13 orchid species on Knudson C medium enriched with coconut milk and pineapple juice. They found the highest germination rate was on the coconut milk enriched medium. Valletta et al. [14] successfully germinated *Orchis mascula* Crantz seeds on Benzilamino and active charcoal with additional Orchimax growth medium added.

Hoque & Al-Forkan [15] studied 2 epiphytic orchids (*Bulbophyllum lilacinum* Ridl. and *Cymbidium aloifolium* Wall.*)*, which are medicinally important in Bangladesh. They successfully produced protocorms on 3 media (Phytomax, Vacin and Went) and Murashige and Skoog supplemented with auxins and sucrose. The survival rate was 50% for *B. lilacinum* and 36% for *C. aloifolium* at the hardening off stage.

Although micropropagation of terrestrial orchids has been studied for some time now, only a few of the studies were carried out under seasonal climate conditions, thus most of the progeny failed to survive passed the acclimatisation stage.

In this study, we aimed to:

(1) Test germinative performances of two commercial and one specially prepared orchid growth media on four salep orchid species;
(2) Compare and decide the best germination media and compound ratio;
(3) Achieve success during the hardening off period by following seasonal climate conditions;
(4) Discuss the *in vitro* propagation capabilities of salep orchids by means of conservation.

2. MATERIALS AND METHODS

2.1. Collecting the Plant Materials

The whole plants of *A. pyramidalis*, *D. romana*, *N. tridentata* and *A. morio* subsp. *morio* species (ten samples of each) were collected from Çanakkale (NW–Turkey) (Figure 1.). *A. pyramidalis*, *D. romana* and *N. tridentata* plants were self-pollinated to obtain seeds and then collected after the eight-week old seed capsules had matured. The seed capsules were stored at 4°C until sown.

Figure 1. Sample collection sites

2.2. Preparing the Growth Media

Two commercial orchid growth media; Orchimax Orchid Medium "ORC" (O0257 Duchefa Biochemie BV, Haarlem, the Netherlands) and Knudson C Orchid Medium "KC" (K0215 Duchefa Biochemie BV, Haarlem, the Netherlands), and one special recipe of orchid growth medium "SV" [16] were used for *in vitro* experiment series. We added 20 g/L of sucrose and 1 g/L of activated charcoal as the carbon source to KC and ORC media. We also added 6 g/L of agar to solidify all the media. The medium SV was prepared according to the original recipe of Malmgren [16], 75 mg/L $Ca_3(PO4)_2$ (Duchefa – C0506.1000), 75 mg/L KH_2PO_4 (Sigma - 04243), 75 mg/L $MgSO_4$ (Sigma - 63140), 20 g/L sucrose, 1 g/L active charcoal, 6 g/L agar and 20 ml/L pineapple juice. The pH of the three growth media was adjusted to 6.0 before autoclaving. All 3 growth media were autoclaved at 121°C for 15 minutes.

2.3. Sowing the Seeds

Orchid seeds were surface sterilized by immersing them in 10% (v/v) sodium hypochlorite for 10 minutes and then rinsed 3 times in sterile distilled water. Inoculation was realized in three petri dishes containing three different growth media. The seeds were incubated in dark conditions at 18±1°C for 12 weeks. Four weeks after sowing process, germinated seeds were counted by using a stereo microscope (Olympus SD30). Twelve weeks after inoculation, 2-3mm sized protocorms were observed and transferred to the subculture media. Then protocorms were stored in the dark conditions at 8°C for 20 weeks. After leaf generation, we applied a photoperiod by using a fluorescent light (36W, cool daylight) at 40cm distance until formation of tubers for 16 weeks (Figure 2.).

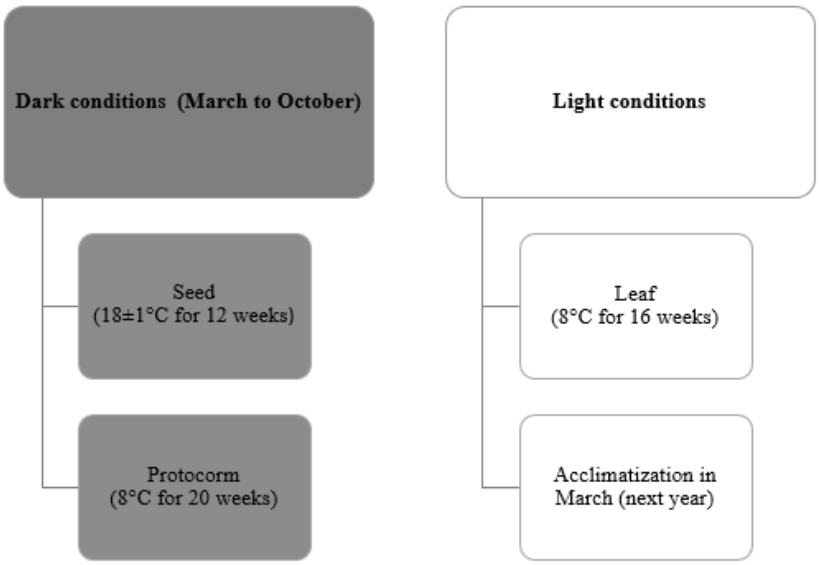

Figure 2. Scheme of experimental timing followed during the study

2.4. Acclimatisation

Clay pots were used for get the better drainage and ventilation. One-year-old plantlets were transferred to the clay pots which contained the soil collected from the habitat of each species, *Pinus brutia* Ten. bark and quartz sand (2:1:1 w/w/w) were used for the first year after sowing in March. The soil's humidity was continuously tracked and kept between 40% - 70% (Figure 3.).

Figure 3. *A. morio* subsp. *morio* transferred to pot

2.5. Statistical Analysis

Statistical analysis were realized with Marascuilo multiple proportions compared to test XLSTAT 2016 (Addinsoft, Paris, France) software for statistical analyses.

3. RESULTS

3.1. Germination of Seeds

Germination started on the 8th day after sowing the seeds for all 4 species (Figure 4.). We calculated germination percentages on the 30th day (Table 1).

Figure 4. Germination of *A. morio* subsp. *morio* on 34th day. The bar represents 1mm.

Table 1. Germination percentages of studied species without consideration of growth media

Species	**Germination (%)**
Anacamptis morio subsp. *morio*	88.91 d
Anacamptis pyramidalis	74.42 a
Dactylorhiza romana	66.30 c
Neotinea tridentata	55.03 b

The highest germination percentage (without considering growth media difference) was seen on *A. morio* subsp. *morio* (88.91%), followed by *A. pyramidalis* (74.42%), *D. romana* (66.30%) and *N. tridentata* (55.03%).

Overall germinative performances of the 3 growth media were calculated without considering species differences (Table 2). The best germination was seen on SV medium (79.11%), and there was no statistically significant difference between KC and ORC.

Table 2. Germinative performances of three growth media

Growth Medium	Germination (%)
KC	73.46 a
ORC	70.99 a
SV	79.11 b

We calculated germination percentage by means of species – growth media relation (Table 3). The highest germination percentage was seen on *Anacamptis morio* subsp. *morio* on SV medium (94%), and the least was seen on *N. tridentata* on KC medium 54.47%.

Table 3. Species – growth media relation

Species	Growth medium	Germination (%)
A. morio subsp. *morio*	KC	88.06 b
	ORC	82.39 a
	SV	94.00 c
A. pyramidalis	KC	71.24 a
	ORC	72.23 a
	SV	80.40 b
D. romana	KC	64.95 b
	ORC	56.18 a
	SV	79.10 c
N. tridentata	KC	54.47 a
	ORC	54.66 a
	SV	54.85 a

3.2. Emergence of Protocorms

Protocorms started to form 30 days after sowing on the all studied species (Figure 5). Protocorms were counted when they were 2-3mm size during the 4th month, just before transferring them to subculture. Although *N. tridentata* seeds germinated, protocorms did not form on this species.

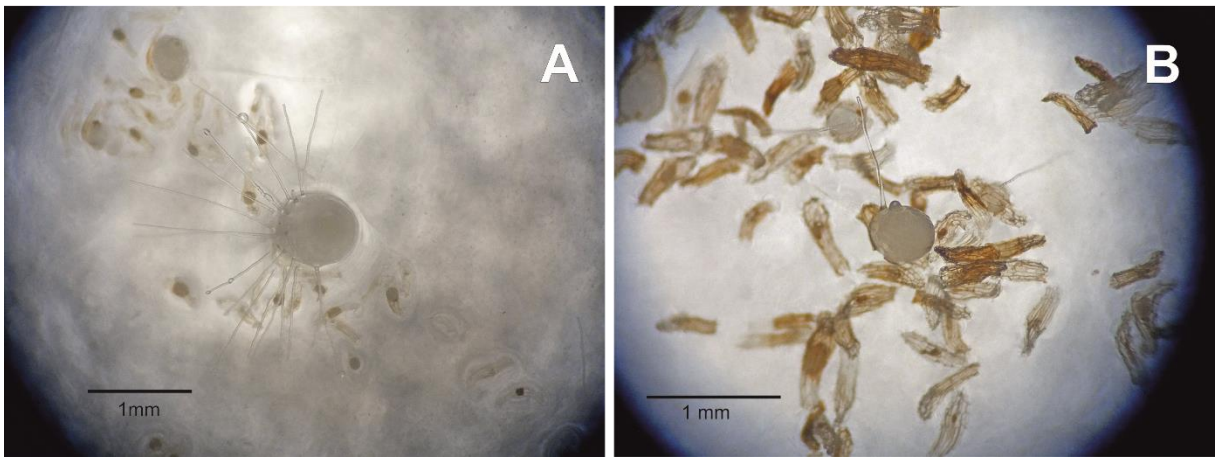

Figure 5. Protocorm formation on A) *A. morio* subsp. *morio* and B) *D. romana*

All 3 growth media affected protocorm development differently (Table 4). The highest protocorm development percentage was seen on SV (60.96%) followed by KC (40.29) and ORC (14.65).

Table 4. Growth media – protocorm developmen relation

Growth media	Protocorm development (%)
KC	40.29 b
ORC	14.65 a
SV	61.96 c

When species – growth media relation to protocorm development investigated, the highest percentage was calculated on *A. morio* subsp. *morio* on SV (70.42%), and the least was *A. pyramidalis* on ORC (3.69%) (Table 5).

Table 5. Species – growth media relation on protocorm development

Species	Growth media	Protocorm development (%)
A. morio subsp. *morio*	KC	53.82 b
	ORC	16.09 a
	SV	70.42 c
A. pyramidalis	KC	4.98 a
	ORC	3.69 a
	SV	12.90 b
D. romana	KC	13.94 a
	ORC	19.12 a
	SV	48.71 b

3.3. Raising of Plantlets

Plantlets were raised on all 3 species, in which protocorms developed, in different months after sowing; *A. morio* subsp. *morio* (6th month), *D. romana* (10th month) and *A. pyramidalis* (10th month) (Figure 6.). We counted the plantlets and calculated plantlet development percentage the first year after the sowing (Table 6). Although protocorm development was successful on KC medium, plantlet development failed. Thus, we could not transfer any plants from KC medium to pots.

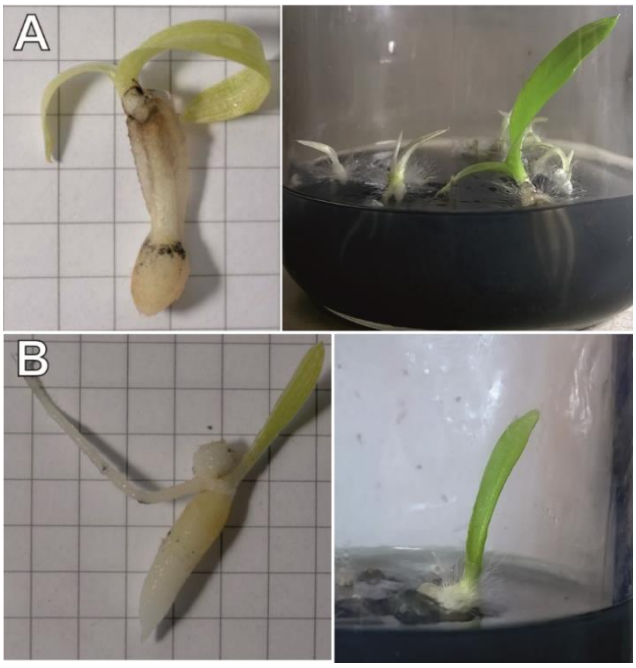

Figure 6. One-year-old plantlets of A) *A. morio* subsp. *morio* and B) *D. romana*. Background: 1x1mm milimetric paper

Table 6. Development percentages of plantlets

Species	Growth media	Plantlet development (%)
A. morio subsp. *morio*	ORC	30.24 a
	SV	55.87 b
A. pyramidalis	ORC	15.79 a
	SV	27.78 b
D. romana	ORC	19.23 a
	SV	47.17 b

3.4. Acclimatisation and Dormancy Stages

A. morio subsp. *morio*, *A. pyramidalis*, and *D. romana* plantlets were transferred to the clay pots containing the soil collected from habitat of each species, *P. brutia* bark and quartz sand (2:1:1 w/w/w) were used after the 12th month after sowing. The first leaves dried out and the plantlets started their dormancy stage after the second week of transferring them to pots.

Early flowering *Ophrys* species require 2 of dormancy, while this stage takes 6 months for *Orchis*, *Anacamptis* and *Neotinea* species [10]. The basal leaves emerged from *A. morio* (SV: 58.82% and ORC: 22.67%) on the 8th month of the dormancy stage (Figure 7.). There was no rosette leaf development observed on *D. romana* and *A. pyramidalis*.

Figure 7. Leaf of *A. morio* subsp. *morio* 8 months after transferring to the pot

4. DISCUSSION

In the present study, we successfully germinated all 4 salep orchid species (*A. pyramidalis*, *D. romana N. tridentata*, and *A. morio* subsp. *morio*) on 3 different growth media (ORC, KC, and SV) by following the cycle of natural conditions. Terrestrial orchids germinate and spread better on calcareous and alkaline soils in nature [17]. On these types of soils $Ca_3(PO_4)_2$ (tricalcium phosphate) was observed at a higher rate [18].

Our results indicated that the best germination was observed on SV medium which has $Ca_3(PO_4)_2$ 75mg/l. According to Arditti and Ernst's [19] study, gibberellic acid (GA_3) inhibits germination of orchid seeds. Similarly, Gümüş [20] indicated that some plant growth regulators (GA_3) have inhibition effects on orchid seeds. Van Waes and Debergh, Önal, Kitsaki et. al., Çağlayan et al. [8, 11, 13, 21] indicated on their results that adding organic substrates to growth medium, increased the germination

ratio of orchid seeds. Thus, we decided not to add plant growth regulators but add pineapple juice as organic substrate to SV medium. This resulted in the increase of germination on SV medium.

There are many studies on propagation of terrestrial European orchids which used seeds of *A. pyramidalis* [7, 20, 22], *D. romana* [20, 23], *N. tridentata* [20, 22] and *A. morio* subsp. *morio* [20, 24, 25, 26, 27]. However, none of them considered the dormancy period after acclimatization, and finalised studies after the leaves died during acclimatization stage. The ideal time to start the acclimatization stage is when the young plants are in their dormancy period [16]. During the life cycle of orchids in nature, the plants need a 2-6 months dormancy period to generate basal leaves [10]. In this study, we attentively followed the dormancy period which took 6 months, and observed the emergence of basal leaves just after the dormancy period finished.

According to Gümüş [20], it is necessary to add fungus isolates to substrates collected from orchid habitats. In our experiments, instead using a standard acclimatisation substrate consisting of pearlite, vermiculite, and peat, we used a mixture of the soil collected from the habitat of each species, *Pinus brutia* bark and quartz sand (2:1:1 w/w/w) to provide rich nutrition, enough moisture, good ventilation, and added arbuscular mycorrhizal fungi to young plants. This application helped young plants to emerge their first basal leaves just after the dormancy period. Following their seasonal cycle is essential for orchids to develop roots and formation of a tuber during the winter and spring months [28]. Through our study, we imitate the conditions of the Mediterranean region's natural environment factors of temperature, moisture, and lighting.

5. CONCLUSIONS

Hereby, we successfully produce adult orchids after their dormancy period. Herewith, we recommend to use of SV growth medium by following the conditions of the natural cycle of the orchids to be propagated. Moreover, it is quite helpful to transfer young plants to soil collected from the orchids' habitats.

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