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In vitro production of *Phalaenopsis* orchids

Phalaenopsis orkidelerinin *in vitro* üretimi

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Anahtar Kelimeler: Besin ortamı, bitki büyüme düzenleyicileri (PGR), doğrudan organogenez, gibberellik asit (GA3), sitokinin.

ABSTRACT

Phalaenopsis – known as moth orchids – are the most popular orchids cultivated indoors as decorative house plants. This makes propagation and cultivation of *Phalaenopsis* important for commercial growers. Enhancements to the micropropagation of *Phalaenopsis* would have pronounced economic benefits through reduced losses and wastage. We examined the effects of several nutrient media and specific plant growth regulators (PGRs) belonging to the gibberellic acid and cytokinin groups on the *in vitro* germination of *Phalaenopsis* seeds, utilizing a single group pretest-posttest model. The effects of several nutrient media such as: Knudson C (KCM), Lindemann (LM), Orchimax (-OM), Orchimax + activated charcoal (+OM), Murashige & Skoog (MS), as well as various PGRs such as 6-Benzylaminopurine (6BA), 6-Furfurylaminopurine (KIN), Adenin hemisulfate (AHS), Thidiazuron (TDZ), 2-Isopentenyl adenine (2iP), and Gibberellic acid (GA3), on the process of germination were also investigated. The explants obtained from the germinating seedlings were subjected to direct organogenesis, and the optimal PGR and tissue fragments were determined. The +OM medium facilitated the shortest germination period (in days). An inverse relationship between the concentration of TDZ and the percentage of germination in the context of the employed PGRs was observed. Apart from TDZ, the remaining PGRs exhibited a positive correlation with concentration. However, no significant difference in germination was observed in comparison to the control. The findings of direct organogenesis investigations revealed that the medium that exhibited the highest productivity was enriched with 5.0 ppm of 6BA. The media containing TDZ exhibited a reduced level of efficiency. Particularly, the group treated with 1.0 ppm of TDZ exhibited reduced efficacy compared to the control group. All concentrations of cytokinin in root elongation stage exhibited a favorable impact in comparison to the control. The variance between these PGRs was not statistically significant.

ÖZ

Phalaenopsis, ev dekorasyonu için iç mekanlarda yetiştirilen en popüler orkidelerdir. Bu nedenle, *Phalaenopsis*'in çoğaltılması ve yetiştirilmesi ticari yetiştiriciler için büyük önem taşımaktadır. *Phalaenopsis*'in mikro çoğaltılmasındaki iyileştirmeler, kayıpların ve israfın azaltılmasını sağlayarak ekonomik faydalar sunabilir. Bu çalışmanın amacı, bir grup ön test-son test modeli kullanarak *Phalaenopsis* tohumlarının *in vitro* çimlenmesi üzerinde çeşitli besin ortamlarının ve bazı bitki büyüme düzenleyicilerinin (PGR'ler) etkilerini incelemektir. Araştırmada kullanılan besin ortamları arasında Knudson C (KCM), Lin-demann (LM), Orchimax (-OM), Orchimax + aktif kömür (+OM) ve Murashige & Skoog (MS) yer alırken, PGR'ler olarak 6-Benzilaminopurin (6BA), 6-Furfurylaminopurin (KIN), Adenin hemisülfat (AHS), Thidiazuron (TDZ), 2-İsopentenil adenin (2iP) ve Gibberellik asit (GA3) incelenmiştir. Çimlenen fidelerden elde edilen eksplantlar, doğrudan organogenez işlemine tabi tutularak optimal PGR ve doku fragmanları belirlenmiştir. Sonuçlar, +OM ortamının en kısa çimlenme süresini (gün cinsinden) sağladığını göstermiştir. TDZ konsantrasyonu ile çimlenme yüzdesi arasında ters bir ilişki gözlenmiş, diğer PGR'ler ise konsantrasyon ile pozitif bir korelasyon göstermiştir. Ancak, kontrol ile karşılaştırıldığında çimlenme açısından önemli bir fark bulunmamıştır. Doğrudan organogenez araştırmalarının bulguları, en yüksek üretkenliğe sahip olan ortamın 5.0 ppm 6BA ile zenginleştirilmiş olduğunu ortaya koymuştur. TDZ içeren ortamlar düşük düzeyde bir verimlilik sergilemiş, özellikle 1.0 ppm TDZ grubu, kontrol grubuna kıyasla azalmış etkinlik göstermiştir. Kök uzatma aşamasındaki tüm sitokinin konsantrasyonları, kontrole kıyasla olumlu bir etki göstermiş, ancak bu PGR'ler arasındaki fark istatistiksel olarak anlamlı bulunmamıştır.

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1. INTRODUCTION

Ornamental plants, especially *Phalaenopsis* orchids, constitute a significant sector in the realm of plant life, as they exert a considerable influence on human health

and psychology, thereby enhancing the overall quality of life. The use of plants in interior spaces enhances the aesthetic appeal and olfactory experience, while also promoting a sense of connection between nature and the built environment (Akça, 2021). Moreover, the longevity of these plants further contributes to the liveliness of the designated areas. According to estimates, orchids comprise 10% of the global floriculture industry, serving as both potted plants and cut flowers. The plant *Phalaenopsis* holds a huge portion of over 500 million USD in the worldwide production and consumption markets (Yuan et al., 2021). The popularity of orchids stems from the diverse range of colors, sizes, shapes, and scents exhibited by their flowers. Furthermore, there has been a notable rise in commercial demand for orchids in recent years. The Orchidaceae family comprises approximately 800 genera and nearly 30,000 species, exhibiting a broad geographical distribution across the globe (Arditti & Ghani, 2000). The Orchidaceae family is classified under the order Asparagales and is considered the second most extensive group of angiosperms. Hinsley et al. (2017) estimated 31,000 species of this plant, based on 880 genera and 29,199 species. Despite its ornamental nature, *Phalaenopsis* exhibits a high concentration of chemical metabolites, including polysaccharides and alkaloids. Consequently, they are employed in diverse regions of the globe by the food and pharmaceutical industries as well (Aytar & Kömpe, 2021). The in vivo propagation of *Phalaenopsis* orchids is limited by the capacity of their seeds to undergo germination. Consequently, the horticultural industry necessitates the utilization of *in vitro* germination (Park et al., 2018). Therefore, the use of *in vitro* techniques for the propagation of *Phalaenopsis* orchids has shown significant reliability when compared to conventional methods, especially in the successful cultivation of a wide range of cultivars (Paek & Murthy, 1977).The utilization of *in vitro* propagation with the involvement of various PGRs and nutrient media enables the possibility of largescale production of valuable commercial hybrids and the conservation of endangered species through clonal reproduction.

The aims of the study were:

i) to initially examine the impact of basic nutrient media and PGRs, specifically gibberellic acid and cytokinin, on the *in vitro* germination of *Phalaenopsis* sp. plant seeds. ii) to determine the optimal concentrations of PGRs and nutrient media for the *in vitro* root proliferation and shoot elongation of *Phalaenopsis* orchids. iii) to investigate the potential for direct organogenesis from shoot tip explants of *Phalaenopsis* orchids.

2. MATERIALS AND METHODS

The research was conducted at the Tissue Culture Laboratory of the Department of Plant Production and Technologies at Konya Food and Agriculture University. A plant growth facility was utilized for the purposes of seed germination, tissue culture, and the micropropagation process. The ambient temperature within the room was maintained at 25°C ± 2°C, and ventilation was provided by a 24000 BTU air conditioning system. Experimental conditions included setting the illumination to 2500 Lux and implementing an 8/16 photoperiod alternating between periods of darkness and light.

2.1. Plant materials

The plant materials, from which the seeds for this investigation were assessed, were procured from Koçtaş Store. Botanical specimens, whose floral structures remained in the prebloom developmental stage, were obtained and subsequently transported to a controlled laboratory setting, where they were maintained at a constant temperature of 25°C until the flowers reached full bloom. Fertilization was conducted after the complete expansion of the flowers. The stamen was extracted from its filaments and subsequently inserted into the ovary after incising the stylus. This procedure was executed on identical floral specimens. Upon the completion of the manual fertilization procedure, the plants were transferred to a growth medium and subjected to regulated environmental conditions to facilitate their growth. This procedure was sustained until viable capsules were produced through the process of fertilization (Figure A4). Upon the capsules' transition from a green hue to yellow, the process of seed extraction was initiated by separating them from the maternal plant.

2.2. Basic nutrient media

In the initial phase of the study, the germination experiments involved the evaluation of four distinct nutrient media that varied in their inorganic and organic compositions as given elsewhere (Bektaş, 2014).

2.3. Method

2.3.1. Determination of plant growth regulators

The germination and micropropagation investigations of *Phalaenopsis* sp. seeds exclusively employed Thidiazuron as source of cytokinin-like growth regulator. Gibberellic acid (GA3) was also employed in the germination experiments.

2.3.2. Preparation of nutrition media

All the basic media were purchased from Duchefa Biochemie, The Netherlands. In addition to Orchimax, a 2% sucrose solution was incorporated into the medium. The media were solidified using 0.6% agar (Phytoagar, Duchefa Biochemie, Netherlands) and enriched with 20 g/L sucrose as the primary carbon source. Following the addition of PGRs, the solution's volume was augmented to 1000 mL and the pH was subsequently modified to a range of 5.7-5.8. The sterilization process for the media involved placing them in capped bottles that were capable of withstanding autoclaving. The autoclaving process was conducted at a pressure of 1.5 atm and a temperature of 121 °C for a duration of 15 min. The agar containing media retrieved from the autoclave was subjected to gentle agitation to promote uniform distribution, after which it was allowed to cool to room temperature. The efficacy of PGRs was found to diminish under high temperature conditions. To mitigate this issue, sterilization of the regulators was conducted using filters with a diameter of 0.22 µm. Following sterilization, the regulators were added to the media after autoclaving and prior to cooling. The media, which underwent a cooling process to approximately 40°C, were subsequently transferred to culture dishes within a sterilized cabinet. In the subsequent micropropagation and rooting stages, either Orchimax activated charcoal or charcoalfree media were employed as the fundamental media.

2.3.3. Determination of viability percentages of seeds

The viability of seeds was assessed both pre- and post-sterilization using a methodology outlined in Lauzer et al. (1994)'s study. The seeds from each experimental group were immersed in a 1% solution of TTC (2,3,5-Triphenyltetrazoliumchloride, Sigma-Aldrich, Austria) and incubated in the dark at a temperature of 30 °C for a period of 48 hours. After this time frame, the seeds were subjected to scrutiny using a binocular microscope, whereby seeds exhibiting an orange-red hue were deemed to possess the capacity to germinate. Our study determined the impact of various concentrations of surface sterilization chemicals on seed viability, based on the data obtained.

2.3.4. Activated charcoal powder

The utilization of activated charcoal powder is employed for the purpose of eliminating phenolic compounds that could potentially arise within the culture media. Only 1% of activated charcoal was employed in the pertinent segments of this investigation.

2.3.5. Sterilization of capsules

Upon completion of a six-month duration, the capsules, which exhibited a chromatic alteration, were extracted from the rootstock, and subjected to a one-minute rinse in tap water. Subsequently, the capsules were subjected to immersion in a solution of 70% ethanol for a duration of 60 seconds. Subsequently, the capsules' seeds were longitudinally dissected using a sterile scalpel and then transferred onto the pre-prepared media in the most diluted form feasible. A meticulous approach was employed to ensure that approximately 200 seeds were present in each culture dish (magenta) during microscopic observation.

2.3.6. Initiation of *in vitro* cultures

The objective of our study was to examine the effects of nutrient media and PGRs on seed germination throughout the germination process (Figure A5). Accordingly, the task at hand can be outlined in the following two distinct stages:

2.3.6.1. Determination of the effects of various media on germination of seeds

During the preliminary phase of the study, the seeds extracted from the capsules were meticulously sown in the nutrient media. The magentas, which contained seeds, underwent a three-month cultivation period within a plant growth chamber utilizing the physical medium outlined in the initial section of the Materials

and Methods. Following the process of seed sowing, a quantitative analysis was conducted to determine the number of seeds present in the growth medium. Subsequently, the cultures were subjected to germination and monitored for a period of one week. After two months, the quantity of germinated seeds within the culture pots was assessed, and subsequently, the germination percentages were calculated based on the number of viable seeds. Based on the findings, the optimal conditions for seed germination were identified. The rates of germination and protocorm formation are expressed as percentages (%).

2.3.6.2. Determination of the effects of various PGRs on the germination of seeds

The second stage of the study utilized Orchimax activated charcoal medium as the foundational medium, based on an assessment of the outcomes of the prior investigation. This medium was assessed using solely three distinct concentrations of cytokinins, TDZ, and gibberellic acid (GA3). The concentrations of the growth regulators were selected based on the recommended, low, medium, and highest effective doses for each respective regulator. The growth regulators selected for this study and their respective concentrations are as follows: 6BA at 1.0, 2.5, and 5 ppm, 2iP at 1.0, 2.5, and 5 ppm, KIN at 1.0 2.5, and 5 ppm, AHS at 10, 50, and 100 ppm, TDZ at 0.1, 0.5, and 1.0 ppm, and GA3 at 0.5, 2.0, and 5.0 ppm. The control group involved in the study was orchimax activated charcoal medium that lacked a growth regulator.

2.4. Statistical Analysis

The statistical analysis of the data was conducted utilizing Microsoft Excel (Office 2007, Tool Pack Analyzer) and SPSS, Version 17.0 (SPSS Inc., Chicago, IL, USA). The statistical disparities among the means and the consequentiality of the disparities were computed at $P<0.05$ through the utilization of the Duncan test for one way ANOVA variable analysis. The Spearman two tail test was employed to establish the correlation between the fluctuations in PGR concentrations and the examined parameters.

3. RESULTS

3.1. Vitality percentages of seeds

The study determined that the seeds' viability percentage and contamination rate acquired through direct capsule opening were 72%±4.0% and 6±0.4%, respectively. Prior to conducting each experiment, assessments of seed viability were conducted. The viability percentages obtained were then utilized to determine the germination rates of the seeds.

3.2. Effect of basic nutrient media on *in vitro* seed germination

The study assessed the growth of mature *Phalaenopsis* seeds that were cultivated in five distinct nutrient media, each with its own specific composition. The seeds were observed after a period of approximately four months in these respective media. The germination rates and durations of the seeds were assessed utilizing a stereomicroscope (Table 1).

Table 1. Germination percentages of *Phalaenopsis* sp. and durations of seeds in different media

Germination	Nutrient media				
	KCM	**LM**	**OM**	**OM+**	**MS**
Percentage	22.40±1.8^{d}	29.20±2.4^{c}	49.90±3.9^{b}	57.90±3.10^{a}	36.20±3.0^{b}
Duration (days)	62	57	32	28	42

*KCM: Knudson Culture Medium; LM: Lindemann Medium; OM: Orchimax Medium; OM+: Orchimax Medium with active charcoal; MS: Murashige and Skoog Basal Medium. Similar letters in the same line are not different according to Duncan's multiple comparison test ($P<0.05$).

It is evident that the Orchimax basic nutrient medium (OM+) supplemented with activated charcoal results in the shortest germination time (in days). Subsequently, a period of 32 days was observed during which Orchimax (OM) was administered in the absence of activated charcoal. The onset of germination was noted to occur

on the 62nd and 57th days in Knudson C (KCM) and Lindemann media, respectively. Germination was observed on the 42nd day in the commonly utilized MS media. The emergence of root rhizoids in all experimental trials indicates the initiation of germination. Upon comparison of the germination percentages at the fourth month, it was observed that the Orchimax activated charcoal media exhibited the highest germination percentage (57.90%). The medium exhibiting the highest germination percentage (49.90%) was observed to be the one devoid of activated charcoal, namely Orchimax medium. The present study revealed that the MS medium exhibited a moderate level of germination percentage in comparison to the other tested media. Conversely, Lindemann medium exhibited the least germination percentage (22.40%). The germination rate of Knudson C media was observed to be low, measuring at 29.40%. As a result of dividing the highest germination value with the percentage of viability, the germination rate was calculated as 80.04% on OM+ medium that does not contain growth regulators.

3.3. Effects of some PGRs on *in vitro* seed germination

In this phase of the research, the seeds extracted from the capsules of the second group were evaluated for their viability and contamination rate. The results indicate that the percentage of viability was 70% ± 3.6, while the contamination rate was 6 ± 0.6%. The control group in this study was treated with Orchimax (OM+) containing activated charcoal but without the use of any growth regulator. Table 2 presents the impact of specific concentrations of certain cytokinins, cytokinin-like TDZ, and gibberellic acid on the duration and timing of germination. The germination percentages are recorded at the onset of the fourth month. The results indicate that the highest percentage of germination was observed at a concentration of 0.1 ppm, with a value of 58.75%, while the lowest percentage was recorded at a concentration of 1.0 ppm, with a value of 50.55%. The impact of KIN on seed germination suggests that the maximum concentration has a favorable influence, albeit lacking statistical significance when compared to the control cohort. The germination percentages were determined to range from 59.8% to 60.50%. The application of gibberellic acid (GA_3) had a positive impact on germination, with no significant difference in the statistical analysis. The results indicate that using 5.0 ppm of GA_3 resulted in a germination rate of approximately 90% compared to the proportion of viable seeds.

Table 2. Effects of some PGRs on germination time and rate of *Phalaenopsis* seeds

Treatments	Concentration (ppm)	Germination (day)	Germination (%)
TDZ	**0.1**	30	58.75±1.5[a]
	0.5	32	54.60±2.7[b]
	1.0	32	50.55±1.8[b]
KIN	1.0	30	58.9±2.9[a]
	2.5	29	59.8±3.4[a]
	5.0	30	60.50±3.6[a]
6BA	1.0	32	58.8±3.1[a]
	2.5	33	59.5±4.7[a]
	5.0	30	60.6±4.9[a]
2iP	**1.0**	32	57.8±4.2[a]
	2.5	30	58.9±2.8[a]
	5.0	30	58.7±1.5[a]
AHS	10.0	30	57.8±2.4[a]
	50.0	30	59.4±3.6[a]
	100.0	30	59.5±4.0[a]
GA_3	0.5	29	58.9±3.6[a]
	2.0	29	60.00±2.7[a]
	5.0	28	62.55±3.5[a]
Control	N/A*	29	59.10±2.35[a]

*Similar letters in the same column are not different according to Duncan's multiple comparison test (P<0.05). Not applicable.

3.4. Shoot formation studies

To facilitate the cultivation of seedlings intended for direct organogenesis as explant sources, the final phase of our investigation involved the cultivation of robust plants that had germinated in various growth media. These plants were individually cultivated in media supplemented with 5.0 ppm of 6BA and 5.0 ppm of KIN. The plants were subcultured at 28 days intervals. At the conclusion of the fifth month, explants measuring approximately 10 mm were obtained from the shoot tip, root, and leaves of the juvenile seedlings that had developed shoots, leaves, as well as roots (Figure 1 & Figure A1).

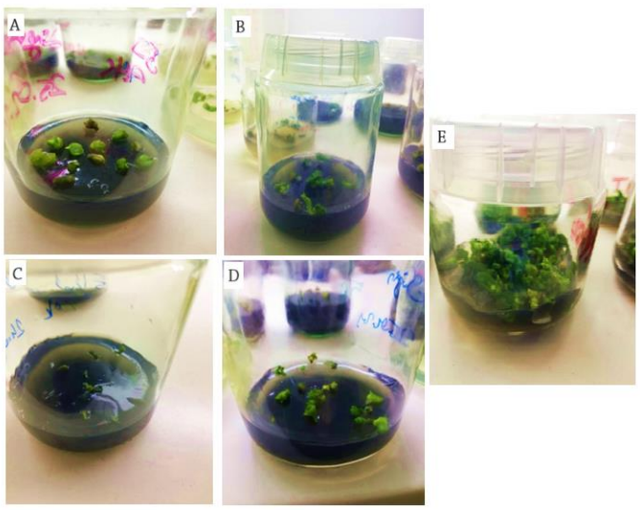


Figure 1. Transfer of shoot explants (A); Development of explants in culture media (B); Leaf explants status (C); Development of shoot explants (D); Elongation of shoot explants (E).

3.5. Direct organogenesis from shoot tip explant

The shoot tips yielded a positive response exclusively. The root and leaf explants exhibited complete blackening, with a 100% response rate. Hence, Table 3 denotes data exclusively derived from shoot tip explants. The initial parameter being assessed in this study pertains to the response observed in shoot formation. In alternative terms, it refers to the ability of each explant to engage in a shoot out. The percentage yield values obtained from a sample of 10 explants, which were selected from a larger population of 100 explants. These explants were transferred to nutrient medium. Based on the observations, it was found that the media supplemented with a concentration of 5.0 ppm of 6BA exhibited the highest level of productivity. Conversely, media containing concentrations of 0.5 and 1.0 ppm of TDZ resulted in lower levels of efficiency. Specifically, the group treated with a concentration of 1.0 ppm of TDZ exhibited decreased efficiency in comparison to the control group, with a recorded efficiency of 41%. The effectiveness of KIN and 2iP in the obtained shoot yield from the explant has also been observed. While the efficacy of the alternative PGR, the AHS, was found to be lower compared to other treatments, it still exhibited a statistically significant improvement when compared to the control group. TDZ treatment resulted in a significant increase in shoot elongation, while the impact on explant yield was comparatively limited. Particularly at a concentration of 0.5 ppm, TDZ exhibited superior efficacy compared to the other experimental groups. Increased TDZ concentration of 1.0 ppm had a detrimental impact on shoot elongation, resulting in a decrease of 62.40 mm. A positive correlation was observed between the concentration of the substance and the elongation of the shoot. Application of 200 ppm of AHS resulted in a significant increase in shoot elongation, with a recorded measurement of 53.55 mm. The observation indicates that lower concentrations of KIN have a favorable impact on shoot elongation, whereas higher concentrations have an adverse effect. The optimal concentration of KIN for promoting shoot elongation was determined to be 5.0 ppm. The experimental groups with varying concentrations of 6BA exhibited significantly more favorable outcomes compared to the control group. While the concentration of 5.0 ppm exhibited the highest yield, there was no statistically significant difference observed when compared to the other concentrations of 6BA.

The study revealed that the growth regulator 2iP, which belonged to the cytokinin group evaluated in this investigation, induced shoot elongation at a comparatively lower rate compared to the remaining groups. The shoot elongation values range from 36.96 to 39.40 mm. The value fluctuates within the range. The values observed in this study corresponded to the lowest yields among all growth regulators examined. There was no observed positive correlation between the presence of 2iP and the elongation of shoots.

Regarding root elongation, all cytokinins and their respective concentrations exhibited a favorable impact compared to the control group. However, no statistically significant difference was observed between these

growth regulators. Regarding the formation of roots, all cytokinins and their respective concentrations tested exhibited favorable outcomes. Statistical analyses revealed that the number of root formations decreased with increasing concentrations, except for the increased 2-rope. All *in vitro*-grown seedlings were prepared to be transferred to the external environment. (Figure 2; Figure A2; Figure A3).

Table 3. Effects of PGRs on shoot and root formation of *Phalaenopsis* shoot tip explants

Treatments	Concentration (ppm)	PS[1] (%)	AE[2] (mm)	RL[3] (mm)	NR[4]	NL[5]
TDZ	0.1	54^{c}	52.50±4.60^{b}	12.28±1.10^{a}	3.8^{a}	4.8^{b}
	0.5	47^{d}	62.40±3.70^{a}	12.95±0.90^{a}	3.1^{b}	5.7^{a,b}
	1.0	41^{d}	50.00±3.60^{b}	12.75±0.80^{a}	3.0^{b}	5.2^{b}
AHS	50.0	58^{c}	44.60±2.68^{c}	12.86±0.80^{a}	3.7^{a}	4.2^{c}
	100.0	64^{b}	49.50±3.60^{b,c}	13.65±0.70^{a}	3.3^{b}	3.9^{c}
	200.0	70^{a,b}	53.55±3.00^{b}	12.29±0.40^{a}	3.2^{b}	4.0^{c}
KIN	1.0	72^{a,b}	50.83± 3.40^{b}	10.05±0.9^{a,b}	3.5^{a,b}	6.6^{a}
	5.0	77^{a}	52.24± 3.0^{b}	11.55±0.90^{a}	3.5^{a,b}	6.8^{a}
	10.0	69^{a,b}	42.31± 2.80^{c}	10.20±0.7^{a,b}	3.0^{b}	6.6^{a}
6-BA	1.0	78^{a}	46.60±2.75^{b,c}	12.63±0.80^{a}	3.0^{b}	5.9^{a,b}
	5.0	80^{a}	49.15±2.80^{b,c}	13.41±0.95^{a}	2.8^{b,c}	6.8^{a}
	10.0	72^{a,b}	46.74±2.50^{b,c}	13.86±0.90^{a}	2.7^{b,c}	6.0^{a}
2-iP	5.0	68^{a,b}	39.40±3.40^{c}	9.56±0..70^{b}	3.2 ^{b,c}	4.2^{c}
	10.0	74^{a,b}	36.96±3.10^{c}	9.95±0.50^{b}	3.7^{a}	4.3^{c}
	20.0	70^{a,b}	38.00±2.80^{c}	10.83±0.60^{a,b}	2.8^{c}	4.0^{c}
Control	N/A*	45^{d}	19.80±1.72^{d}	8.31±0.5^{c}	2.0^{d}	2.7^{d}

***PS:** Percentage of Shooting, **AE:** The amount of elongation. The differences between the final height and the initial height of the 4-month-old seedlings were taken, and the averages of these differences were given with ± standard deviations. Measurements were taken from 10 samples for each media., **RL:** Root length. Root lengths were given with ± standard deviations by taking the averages of the roots of the 4-month-old seedlings with the longest length. Measurements were taken from 20 samples for each media, NR: Number of roots. The average root numbers of 4-month-old seedlings were calculated with ± standard deviations. Measurements were taken from 20 samples for each media, **NL:** Number of leaves. The average of the leaf numbers of the 4-month-old seedlings was given with ± standard deviations. Measurements were taken from 25 samples for each media.*Not applicable. Similar letters in the same column are not different according to the Duncan multiple comparison test ($P<0.05$).

Figure 2. *In vitro* propagated seedlings are ready to be transferred to *in vivo*

4. DISCUSSION

The elimination of mycorrhizal fungi is achieved by supplementing the *in vitro* medium with the essential nutrients needed for seed germination. In our investigations, the germination of mature seeds from *Phalaenopsis* sp. was examined under varying nutrient compositions in the growth media. Notably, it was observed that the seeds exhibited a higher germination rate (57.90%) when subjected to the OM+ media. Orchimax, lacking activated charcoal at a concentration of 49.90%, exhibits the second highest germination percentages among the various nutrient media investigated in this study. The MS media exhibited a moderate germination percentage in comparison to the other tested mediums. The germination rate in the OM+ medium without growth regulator was determined to be 80.04% by proportioning the highest germination value with the percentage of viability. Merely a fraction of 5% of orchid seeds exhibit the ability to undergo germination within their native habitat, as they patiently await the arrival of optimal climatic conditions conducive to this process. Based on these data, the values associated with OM+ media exhibit a considerable degree of significance, particularly with regards to the duration and speed of germination. The orchid species *Orchis sancta L.* and *Serapias vomeracea* exhibit similarities to the subject of our study. The highest rate of germination was observed in the OM+ media, as reported by Bektas (2014).

Numerous studies have documented that orchid seed germination rates are significantly higher when cultivated in a growth medium containing both organic and inorganic nitrogen sources (Stewart & Kane, 2006). In our study, the OM+ and OM- nutrient media, in addition to inorganic nitrogen, also contained tryptone as an organic nitrogen source. This suggests that the higher germination rates observed in these two specific environments, compared to others, can be attributed to the presence of an organic nitrogen source. Notably, previous research by Raghavan and Torrey (1964), and Van and Debergh (1986) found that the inclusion of an inorganic nitrogen source during orchid seed germination can impede the process, which aligns with our study's findings.

There is a discernible disparity in the rate of germination between the OM+ and OM-media. The inclusion of an additional 1% of activated charcoal in OM+ media is believed to create a light-restricted environment for the seeds within the medium, thereby enhancing the germination process. According to the study of Paek and Murthy (1977), it has been observed that the presence of activated carbon in the growth medium can enhance rooting by impeding the penetration of light. Nevertheless, it has been indicated that the utilization of activated carbon has the potential to elevate the pH level, thereby facilitating nitrogen absorption. Consequently, there was an observed increase in growth (Eymar et al., 2000). In contrast to findings in other studies, Waes (1987) reported a reduction in germination due to the presence of activated carbon in the environment. The pH levels of all the media used in our investigation were adjusted to a range of 5.7 to 5.8. Additionally, the cultured samples were incubated at a temperature of 23±2 °C with a photoperiod of 16/8 hours. These pH values and incubation temperatures align with those reported in previous research. Van Waes and Deberg (1986) reported the optimal pH level for orchid germination in temperate zones as 5.8. In contrast, research by Arditti (1967) and Arditti (1979) indicated that the most favorable temperature range for germination and subsequent seedling growth falls within 20–25 °C. Contrary to our findings, Mead and Bulard (1975) reported that orchid germination is optimal in a dimly lit environment. Valletta et al. (2008) found that *Orchis mascula* seeds have the most favorable germination process under a photoperiod consisting of 16 hours of light followed by 8 hours of darkness. In contrast, seeds incubated in continuous darkness did not germinate. The 16-hour light/8-hour darkness photoperiod, which we implemented in our study, proved highly effective in promoting germination.

In our investigation, viability assessments conducted on the seeds of *Phalaenopsis* sp. revealed that a portion of the seeds did not exhibit signs of viability. The study involved determining the viability percentages and contamination rates of seeds obtained through the direct opening of the capsule. The viability percentages were found to be 72 ± 4.0%, while the contamination rate was determined to be 6 ± 0.4%. The germination rates of the seeds were subsequently determined based on the viability percentages obtained. According to Crafts and Miller (1974), it has been asserted that the fungi generate cytokinins within this symbiotic association, consequently leading to the germination of the seeds.

Considering the observed elongation in seedlings at the age of four months of our study, it was observed that the application of TDZ resulted in a significant increase in shoot elongation, while not significantly affecting explant yield. Particularly at a concentration of 0.5 ppm, TDZ exhibited superior efficacy compared to the other experimental groups. Nevertheless, it was noted that the increase in shoot elongation was adversely impacted by higher concentrations of TDZ (1.0 ppm). The alternative group of individuals from the AHS also exhibited shoot elongation in comparison to the control group during the shoot elongation experiment. A significant correlation was observed between the concentration levels and the extent of shoot elongation.

In a study conducted by Chen and Chang (2000), it was observed that lower concentrations of TDZ exhibited favorable outcomes in terms of shoot formation. However, in our study higher concentrations of TDZ were found to have an impact on the formation of callus and adventitious shoots. The utilization of TDZ has demonstrated greater efficacy compared to 6BA, as indicated by Ernst (1994) and Chang and Chang (1998). Previous studies in the literature have documented the positive impact of TDZ on the promotion of shoot formation and reproductive processes in orchids (Chen & Piluek, 1995; Nayak et al., 1997; Chen et al., 2004; Ferreira et al., 2006). In our study, regarding the elongation of roots, it was observed that all cytokinins, regardless of their concentrations, exhibited a favorable impact when compared to the control group. Nevertheless, there was no statistically significant difference observed between these growth regulators. Regarding the quantity of roots generated, it was observed that all cytokinins tested, along with their respective concentrations, exhibited favorable outcomes when compared to the control group.

The statistical analyses of our study revealed that higher concentrations, apart from the increased 2-rope concentration, were associated with a decrease in the number of root formations. Previous studies have documented that the presence of auxins in the environment is essential for the promotion of root formation (Bhojwani & Razdan, 1986; Mansuroğlu & Gürel, 2001; Díaz & Álvarez, 2009).

The statistical analysis results also indicated that, overall, the use of 2-rope as a PGR had a smaller impact on leaf formation when compared to other regulators. Previous research has documented that cytokinin group growth regulators exhibit positive effects on shoot formation as well as inhibit leaf formation (Gaspar et al., 1996; Haberer & Kieber, 2002). Nevertheless, in a study conducted by Bektaş et al. (2013), it was observed that the concurrent application of 2.0 mg/L of Zeatin (ZEA) and 0.5 mg/L of Indole-3-butyric acid (IBA) resulted in a greater stimulation of leaf development. In our investigation, exclusively cytokinins and their varying concentrations were examined. In prospective investigations, it is hypothesized that the concurrent application of auxin and cytokinins will yield a more favorable outcome in terms of leaf development.

5. CONCLUSION

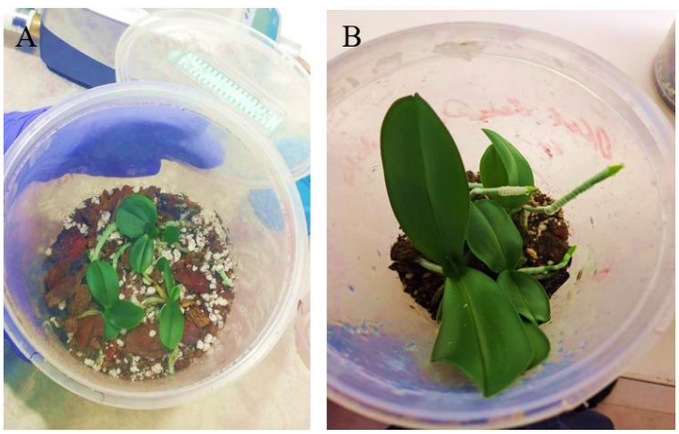
Since our study solely aimed to investigate the impact of basic nutrient media and specific growth regulators, namely gibberellic acid and cytokinins, on the *in vitro* germination of plant seeds belonging to the orchid species *Phalaenopsis* sp. therefore, as a continuation of the work we suggest the following stages for further investigations:

- Assessment of auxins along with cytokinins on leaf and root formation in determining the effects of plant growth regulators.
- Examination of callus formation, where cytokines and auxins are used equally for indirect organogenesis.
- Protocorm-like structures are structures that are composed of many meristematic cell centers capable of forming shoots and roots (Da Silva et al., 2000). *Phalaenopsis* sp. creates synthetic seeds by coating protocorm-like structures (PBY) with alginate. Therefore, more investigation of the germination capabilities of the synthetic seeds in the culture media and soil conditions should be conducted.
- It can be presented under new research titles such as adaptation studies of seedlings grown in culture medium to an external environment.

Appendix

Figure A1. *İn vitro* completed development of seedlings

Figure A2. *İn vitro* propagated plantlets transferred to the external environment



FigureA3. Seedlings transferred to in vivo (A); Orchids growing outdoors (B)

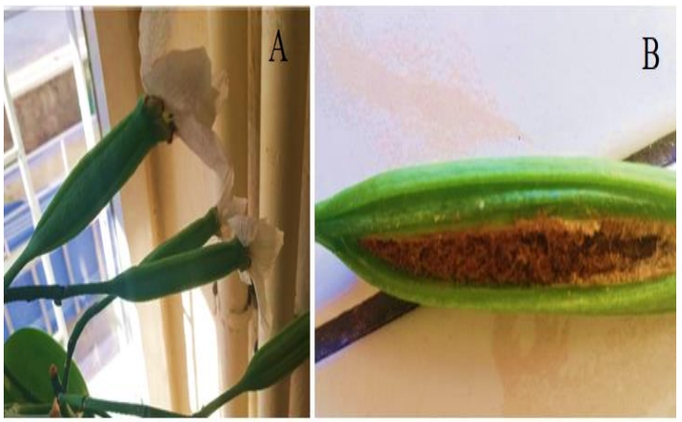


Figure A4. (A) Seed stubs formed after fertilization; (B) Seeds inside the seed pod

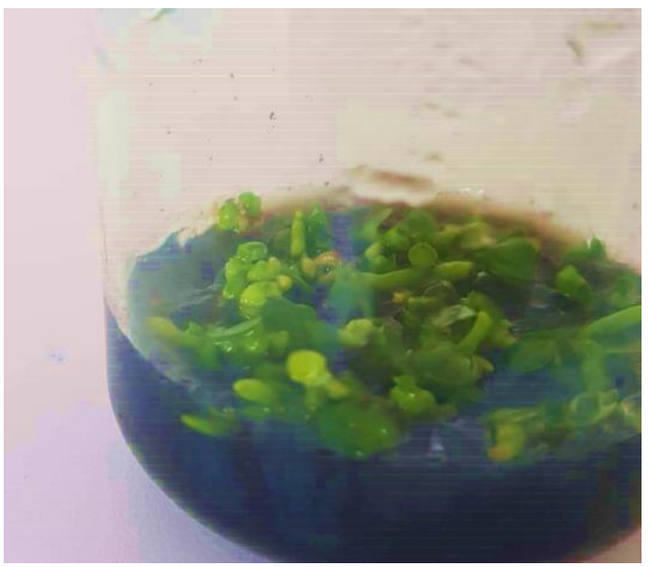


Figure A5. Germination of orchid seeds

Figure A6. Explant preparation from developing seedlings

REFERENCES

Akça Ş (2021). İç mekân süs bitkileri tüketici tercihlerinin belirlenmesi; Zonguldak kenti örneği. *Türk Tarım ve Doğa Bilimleri Dergisi* 427-435.

Arditti J (1967). Factors affecting orchid seeds, *Botanical Review* 33(1): 1-97.

Arditti J (1980). Aspects of the Physiology of Orchids. *Advances in Botanical Research* 7:421-655.

Arditti J, Ghani A (2000). Numerical and physical properties of orchid seeds and their biological implications. Tansley Review, *New Phytologist* 145(3): 367-421.

Aytar E, Kömpe Y (2021). Orkidelerin Geleneksel Kullanımları, Fitokimyasal İçerikleri ve Biyolojik Aktiviteleri. *Black Sea Journal of Engineering and Science* 4 (3):141-152.

Bektaş E (2014). *Orchis sancta* l. ve *Serapias vomeracea* (BURM. F.) briq. türlerinin bitki doku kültürü yöntemiyle üretimi. Doktora, Karadeniz Teknik Üniversitesi, Trabzon, Türkiye.

Bektaş E, Cüce M, Sökmen A (2013). *İn vitro* germination, protocorm formation and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. *Turkish Journal of Botany* 37:336-342.

Bhojwani S S, & Razdan M K (1986). Plant tissue culture: theory and practice. Elsevier.

Chang C, Chang W (1998). Plant regeneration from callus culture of *Cymbidium ensifolium* var. *misericors*. *Plant Cell Reports* 17: 251-255.

Chen JT, Chang WC, (2000). Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae). *Plant Science* 160(1): 87-93. doi: https://doi.org/10.1016/S0168-9452(00)00367-8.

Chen TY, Chen JT, Chang WC (2004). Plant regeneration through direct shoot bud formation from leaf cultures of Paphiopedilum orchids. *Plant Cell, Tissue and Organ Culture* 76(1): 11-15.

Chen Y, Piluek C (2004). Effects of thidiazuron and N6-benzylaminopurine on shoot regeneration of *Phalaenopsis*. *Plant Growth Regulation* 16: 99-101.

Crafts C, Miller C (1974). Detection and identification of cytokinins produced by mycorrhizal fungi. *Plant Physiology* 54(4): 586-588.

Díaz M, Álvarez C (2009). Plant regeneration through direct shoot formation from leaf cultures and from protocorm-like bodies derived from callus of *Encyclia mariae* (Orchidaceae), a threatened Mexican orchid. *İn vitro Cellular and Developmental Biology-Plant* 45(2): 162-170.

Ernst R (1994). Effect of thidiazuron on *in vitro* propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). *Plant Cell Tissue and Organ Culture* 39: 273–275.

Eymar E, Alegre J, Toribio M, Lo' pez-Vela D (2000). Effect of activated charcoal and 6-benzyladenine on *in vitro* nitrogen uptake by *Lagerstroemia indica*. *Plant Cell Tissue and Organ Culture* 63(1): 57-65.

Ferreira W, Kerbauy G, Costa A (2006). Micropropagation and genetic stability of a *Dendrobium hybrid* (Orchidaceae). *İn vitro* Cellular & Developmental Biology-Plant 42(6): 568-571.

Gaspar T, Kevers C, Penei C, Greppin H, Reid D, Thorpe T (1996). Plant Hormones and Plant Growth Regulators in Plant Tissue Culture. *İn vitro Cellular and Developmental Biology-Plant* 32: 272-289.

Haberer G, Kieber J (2002). Cytokinins: New Insights into a Classic Phytohormone. *Plant Physiology* 128: 354-362.

Hinsley A, de Boer H, Fay M, Gale S, Gardiner L, Gunasekara R, Phelps J (2017). A review of the trade in orchids and its implications for conservation. *Botanical Journal of the Linnean Society* 186(4): 435-455.

Lauzer D, St-Arnaud M, Barabe D (1994). Tetrazolium staining and *in vitro* germination of mature seeds of *Cypripedium acaule* (Orchidaceae). *Lindleyana* 9(3): 197-204.

Mansuroğlu S, Gürel E (2001). Mikroçoğaltım. In: Babaoğlu, M., Gürel, E. ve Özcan, S. (eds.), Bitki Biyoteknolojisi I - Doku Kültürü ve Uygulamaları. pp: 262-281, Selçuk Üniversitesi Vakfı Yayınları, Konya.

Mead J, Bulard C (1975). Effects of vitamins and nitrogen sources on asymbiotic germination and development of *Orchis laxiflora* and *Ophrys sphegodes*. *New Phytologist* 74(1):33-40.

Nayak NR, Rath SP, Patnaik S (1997). *İn vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-Ham) Sw. through thidiazuron-induced high frequency shoot proliferation. *Scientia Horticulturae* 71(3-4): 243-250.

Paek K, Murthy H (1977). Temperate Oriental *Cymbidium* species. In: Kull, T., Arditti, J. (eds.), Orchid Biology: Reviews and Perspectives. VIII. Edition, 287, Kluwer Academic Publishers, Dordrecht.

Park SY, Huh YS, Paek KY, (2018). Common protocols in orchid micropropagation. Orchid Propagation: From Laboratories to Greenhouses-Methods and Protocols pp: 179-193. DOI: 10.1007/978-1-4939-7771-0_8.

Raghavan V, Torrey JG (1964). Inorganic nitrogen nutrition of the seedlings of the orchid, Cattleya. *American Journal of Botany* 51(3): 264-274.

Silva A, Fernandes MM, Fereira AG (2000). Ontogenetic events in androgenesis of Brazilian barley genotypes. *Revista Brasileira de Biologia* 60(2): 315- 319.

Stewart L, Kane M (2006). Asymbiotic seed germination and *in vitro* seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. *Plant Cell Tissue and Organ Culture* 86(2): 147-158. DOI: 10.1007/s11240-006-9098-y.

Valletta A, Attorre F, Bruno F, Pasqua G (2008). *İn vitro* asymbiotic germination of *Orchis mascula* L. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology* 142(3): 653-655. DOI: 10.1080/11263500802411205.

Van Waes J, Debergh P (1986). *İn vitro* germination of some Western European Orchids. *Physiologia Plantarum* 67(2): 253-261.

Waes J (1987). Effect of Activated Charcoal on *İn vitro* Propagation of Western European Orchids. Symposium on *İn vitro* Problems Related to Mass Propagation of Horticultural Plants. *Acta Horticulturae* 212:131-138.

Yuan S, Bolaños-Villegas P, Tsao C, Chen F (2021). The breeding of *Phalaenopsis* hybrids*. The Orchid Genome* pp: 29-40, Springer.