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In vitro asymbiotic germination of mature seed of medicinal orchid (*Orchis simia* Lam.)

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Abstract: The sexual reproduction of orchids is a notably slow process. This is due to their seeds lacking endosperm, which necessitates a fungal elicitor for germination in natural conditions. In the current study, we evaluated seed germination and the initial development of the protocorm of *Orchis simia*, an important medicinal orchid species, using a completely randomized design with three replications. The tetrazolium test revealed that 35% of the seeds were viable. Subsequently, we investigated the influence of casein, activated charcoal, indole acetic acid (IAA), photoperiod, and temperature on the germination of *O. simia* seeds. The analysis of variance demonstrated varying responses in terms of seed germination percentages, with photoperiod and temperature treatments having a more pronounced impact on germination. The optimal conditions for asymbiotic orchid seed germination in this experiment were achieved using Murashige and Skoog's (MS) medium supplemented with one-fifth of nitrate concentration, casein (2.0 g/l), activated charcoal (2.0 g/l), and an IAA growth regulator (1.0 mg/l), resulting in a germination rate of 31%. After a three-month period, the nodes underwent transformation into protocorms. The findings presented in this report can serve as valuable insights for the production of orchid plants and the conservation of this medicinal species.

Keywords: germination factors, growth regulator, orchid, protocorm.

Introduction

The Orchidaceae family stands out as one of the largest and most diverse families of flowering plants, encompassing an impressive 28237 species (Willis, 2017). These orchids are found in various regions, including the tropical humid forests of India, Sri Lanka, South Asia, South and Central America, and Mexico (Singh et al., 2019). Notably, the Orchidaceae family is included in the International Union for Conservation of Nature (IUCN) list of endangered plants, highlighting their ecological significance.

Within the realm of monocots, orchids represent one of the most advanced families, boasting around 850 genera (Stewart and Griffiths, 1995; Gutiérrez, 2010). Orchids are valued not only for their aesthetic beauty but also for their medicinal properties attributed to the presence of alkaloids, flavonoids, glycosides, and various other plant compounds (Gutiérrez, 2010). Owing to the persistent destruction of their natural habitats, excessive harvesting for medicinal applications, illegal trade, and overzealous cultivation by orchid farmers, orchid populations are facing a rapid and alarming decline. Additionally, the premature harvesting of orchids during their flowering stage, before the physiological ripening of seeds, underscores the need for mass *in vitro* propagation of this plant.

Micro orchid seeds face a natural limitation as they lack the ability to germinate independently. However, the germination process in these plants can be accelerated by a range of biotic and abiotic factors. Understanding the ecology of orchid reproduction, including issues such as non-germination or seed dormancy in natural conditions, represents a significant aspect of orchid growth characteristics in temperate climates (Butcher and Marlow, 1989). The seeds of terrestrial orchids have a special form of morphophysiological dormancy, which consists of morphological (the presence of an undifferentiated embryo and a strong seed coat) and physiological factors (the embryo does not have enough growth potential to penetrate the seed coat and germinate). In addition, seeds do not have endosperm, and the nutrients of mature seeds are concentrated in the embryo cells. Nutrients in seeds include lipids, proteins, and carbohydrates, and their amounts vary from species to species (Rasmussen, 1995). Although the seed embryo in these plants contains carbohydrates, the amount of sugar in the embryo is not usually enough to support germination completely or even to initiate germination (Manning and Van, 1987). In their natural habitat, orchid seed germination hinges on a specific symbiotic relationship with mycorrhizal fungi (Marks et al., 2013). This dependence poses a challenge to the sustainability of viable plant populations, particularly in regions grappling with habitat loss caused by forest degradation (Tremblay et al., 2005).

Advancements in orchid seed germination techniques, particularly *in vitro* germination, have significantly enhanced the reliability of germination and the propagation of numerous orchid species. This approach offers an ideal system for investigating the growth and development of orchid seeds and seedlings (Kauth et al., 2008). Propagation of orchids using tissue culture methods is suitable because there is a good opportunity to improve and increase the number of seedlings through mass propagation of important orchids, hybrids, and or a new variety in a short period of time (Goh and Wong, 1990; Chen and Chang, 2000; Pathak et al., 2001; Chen et al., 2004; Bhattacharjee and Hossain, 2015; Borah et al., 2015; Sibin and Gangaprasad, 2016; Bhatti et al., 2017; Mohanty and Salam, 2017; Decruse and Gangaprasad, 2018). One of the major obstacles to the mass propagation of economically important orchids for commercial purposes and to prevent the risk of extinction is the unavailability of efficient and reliable instructions for the germination of this plant species. In general, the biology of seed germination is the same for all orchid species and includes two developmental stages-embryo swelling and protocorm formation. However, several factors such as seed maturity, seed dormancy, seed sterilization, composition and content of the nutrient medium, light, and temperature have a great influence on seed germination (Arditti, 1967; Kauth et al., 2008; Zeng et al., 2014; Dulić et al., 2019). Hence, it is imperative to develop specific guidelines for each orchid species to expedite and optimize the germination and growth of these plants.

In the case of most terrestrial orchids, the inclusion of organic additives rich in amino acids within nutrient media significantly influences seed

germination. Commonly employed organic additives in *in vitro* culture include coconut water, banana powder, peptone, hydrolyzed casein, yeast extract, and pineapple juice. Notably, the organic content of casein has been found to have a beneficial impact on the germination rate of medicinal orchid seeds, such as those of *Eulophia nuda*, which face extinction (Nanekar et al., 2014). *Rhynchostylis retusa*, *Cymbidium elegans*, *Cypripedium calceolus*, and *Epipactis helleborine* species also showed higher germination speed and shorter period length for germination in a casein-enriched nutrient medium (Nandi et al., 1999).

In order for symbiotic and asymbiotic orchid seed germination to be effective, many conditions such as photoperiod, temperature, and nutrition should be considered. Lighting is one of the most important environmental factors in orchid seed germination, which was less studied. In most orchid species, light has an inhibitory effect on seed germination. However, there are species whose seeds germinate under light conditions, and *in vitro* culture, germination responses to the length of the light period often depend on the type of species (Arditti et al., 1981; Kauth et al., 2008). However, germination responses to photoperiods is often species-specific, regardless of growth habit (Kauth et al., 2008). darkness is often considered to stimulant germination of terrestrial orchids. For example, seeds of terrestrial orchids may not germinate until do not be under the soil (Rasmussen and Rasmussen, 1991). Also, many terrestrial orchids grow in shadow environments better than their epiphytic counterparts (Rasmussen, 1995), While the light may not reach the floor of the habitat easily (Rasmussen and Rasmussen, 1991). Van Waes and Debergh (1986) reported that even a small increase in light intensity from complete darkness to 1.2 µmol/m2/s reduced the germination of several European terrestrial orchids. Asymbiotic germination of *Cypripedium acaule*, a North American terrestrial orchid, was lower when seeds were incubated in a photoperiod of 16 h (6.7% germination) compared to complete darkness (96.7%) (ST-ARNAUD, 1992).

The use of growth regulators stimulates the zygote embryo to form protocorms that grow into seedlings (Pant and Gurung, 2005). Cytokinin treatments showed increased asymbiotic germination in many orchid species. Miyoshi and Mii (1988), and Stewart and Kane (2006) also reported increased germination levels of several terrestrial orchids.

Vegetative propagation is a time-consuming process for generating a substantial quantity of orchid clones. Consequently, tissue culture represents an alternative method for mass-scale propagation and conservation of rare and endangered orchids. This technique can significantly expedite the identification and protection of new plant species. The current study seeks to explore the *in vitro* asymbiotic germination of medicinal orchid seeds. It investigates the influence of factors such as light conditions, temperature, casein, activated carbon, and plant growth regulators to accelerate the germination and propagation of this plant within a shorter timeframe compared to natural conditions.

Materials and Methods

Plant materials

Orchid seedlings (*Orchis simia* Lam.) were collected from the Ramyan region in Golestan province, Iran, during flowering stage. These seedlings were transplanted into pots, with soil carefully placed around their roots, and they were nurtured until the formation of capsules. By the end of May, mature capsules were harvested and stored in a refrigerator at 5°C, awaiting tissue culture.

Tetrazolium viability test

Seed viability was evaluated using 2, 3, 5-triphenyltetrazolium chloride solution. A small sample of seeds (5 mg) was pretreated with 10% sucrose solution for 24 h at room temperature. Then the solution was drained with a sampler, 0.1% tetrazolium solution was added, and the tubes were incubated in the dark for 24 h in a 40°C water bath, according to the method described by Hosomi et al. (2011). Seed viability was assessed using a light microscope with 100 seeds replicated three times. Red seeds were considered alive and brown seeds or no embryos were considered dead. The percentage of live seeds was calculated by dividing the number of live embryos by the total number of tested embryos.

Surface sterilization of capsules

Initially, the capsules underwent surface disinfection using a 70% ethanol solution for 1 minute, followed by rinsing with sterile distilled water. Subsequently, they were disinfected in a 20% sodium hypochlorite solution for 15 minutes, after which they were transferred within a laminar hood and subjected to two additional washes with sterile distilled water.

Culture media

MS culture media (control), MS plus 0.2% casein, $MS_{N_{\frac{1}{5}}}$ (MS culture medium containing one-fifth of nitrate plus 0.2% casein), and $MS_{N_{\frac{1}{5}}}$ supplemented with (0.2% casein and 1.0 mg/l IAA) were employed for the cultivation and germination of orchid seeds. In addition, all these cultures contained 0.2% activated charcoal. The pH of the culture medium was adjusted to 5.8 by adding 1 N NaOH or 1 N HCl and then it was autoclaved at 121 °C with a pressure of 1.2 kPa for 20 min. The sterilized culture medium was transferred to the culture chamber (laminar hood) and 25 ml of culture medium was poured into 100 ml sterile flasks.

Cultivation of seeds

The sterilized capsules inside the laminar airflow hood, after washing with sterile distilled water and drying with sterile filter paper, were transferred to a sterilized Petri dish, and using a sterilized scalpel, a longitudinal slit was given to the capsules and the seeds were placed on the culture medium. About 200 seeds were cultivated in each culture container containing 25 ml of culture medium. The cultivated seeds were kept in the incubator under different light conditions (darkness and light/darkness 12/12 h) and temperature (20 and 25 °C).

Statistical method

The treatments studied include casein (2.0 g/l), activated charcoal (2.0 g/l), indole acetic acid (1.0 mg/l). The data relating to the germination percentage of mature seeds and the number of days until germination were analyzed using SPSS statistical software in a completely randomized design with 3 replications and mean comparison was done by Duncan's multi-range test method at 5% probability level.

Results

The mature seeds of *Orchis simia* used in this experiment as explants have a brown color and the embryos consist of a concentrated mass with a seed coat (Figure 2A). Physiological developmental stages from seed to early protocorm development are given in Figure 2. The tetrazolium (TZ) test before sterilizing the seeds showed that 35% of the seeds were alive, 40% of the seeds were without embryos, and 25% of the seeds were dead (Figure 2B). The effects of culture medium with different conditions on germination were evaluated. The results of the TZ test of *O. Simia* seeds confirmed the results of their *in vitro* germination in MS medium (N1/5) + IAA+ casein (31%) medium. Studies have questioned the use of the TZ test alone as an indicator of seed germination and show the importance of confirming *in vitro* germination results, as we determined. The TZ test showed that seed viability was significantly higher than the observed maximum germination. This discrepancy can be explained by the fact that the nutrients or other components required by the explant are not present or are not in the optimal concentration in the culture medium (Lauzer et al., 2007). The results of the analysis of variance showed that the percentage of germination of orchid seeds of *Orchis simia* in different culture media has a significant difference at the level of 1% (Table 1).

The comparison of the means shows the importance of the organic matter and growth regulator IAA in the germination of the desired orchid seed. MS culture medium together with one-fifth of nitrate and having casein organic matter 2 g/L plus IAA 1 mg/L was the most suitable culture medium ($\bar{Y}$= 31%) for the germination of these seeds (Figure 1A). About 4 weeks after placing the cultures in the incubator at a temperature of 20°C in the dark, some of the seeds swelled and formed small white dots with thin threads, and about two months later, they started to form protocorms (Figure 2D). Also, MS culture medium (control) showed the lowest percentage of seed germination ($\bar{Y}$= 8.66 %). The number of days until seed germination in MS medium (control) took longer than in other culture mediums ($\bar{Y}$= 31.66 days). There was no significant difference in the number of days until germination for other culture mediums, and the length of culture was about 25 days. By adding casein organic

material to the MS medium, the germination percentage increased by 19.33% compared to the MS medium (control), and the germination time decreased to 25 days (Figure 1B). The incubator temperature of 20°C and dark conditions played a very important role in seed germination so under the mentioned conditions, seed germination was observed after one month, but in the seeds incubated at 20°C and light conditions, seed germination was not observed. This indicates the importance of dark conditions for *orchis simia* seeds germination. Also, no seed germination was observed in the seeds incubated in the dark at 25°C. Therefore, dark conditions and a temperature of 20°C are very important for germination in this plant. The results of the mean comparison showed that among the tested culture mediums, MS medium (N1/5) + casein + IAA with the highest percentage of germination and the lowest number of days to germination was the most suitable medium compared to other tested mediums. Based on the obtained results, IAA growth regulator and casein had a positive and significant effect on the percentage of germination and the number of days until germination.

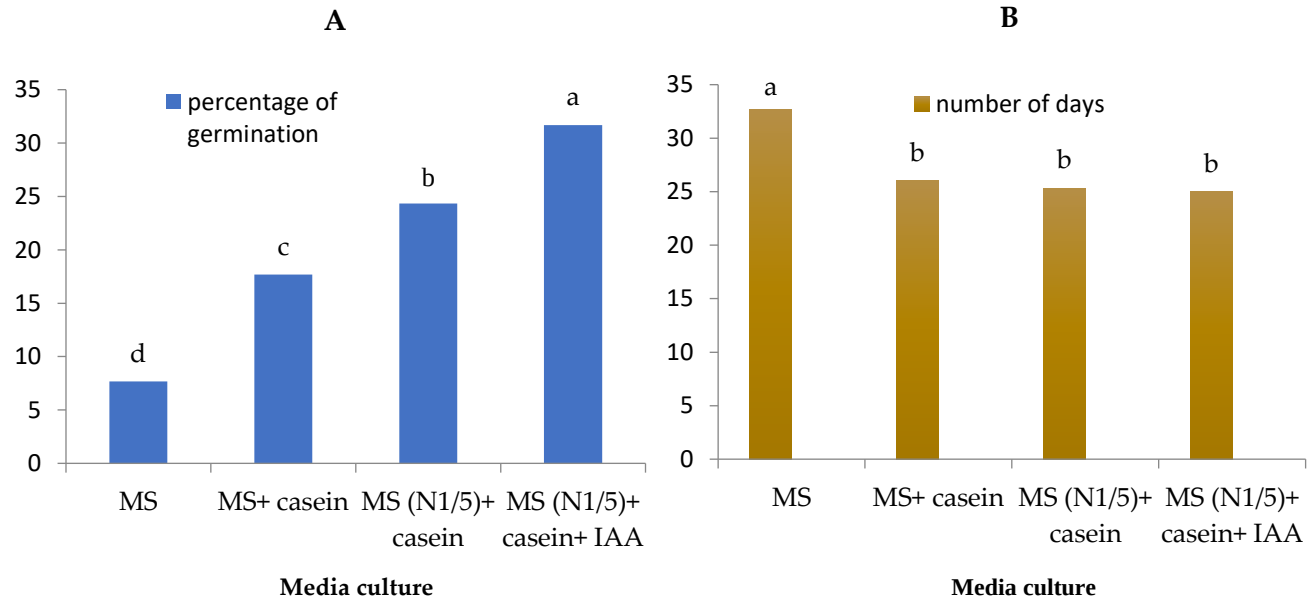

Figure 1- Comparison of the means percentage of germination (A) and the number of days to initial germination (B) in different culture media by Duncan's multi-range test method.

Table 1. Analysis of variance of studied orchid traits in different culture media

Sources of variation	Degree of freedom	Mean square	
		Germination %	Number of days until germination
Treatment	3	265.89**	29.56 **
Residual	8	1.75	1.67
Coefficient of variation (%)		6.35	4.78

** ؛Significant at the 1% probability level

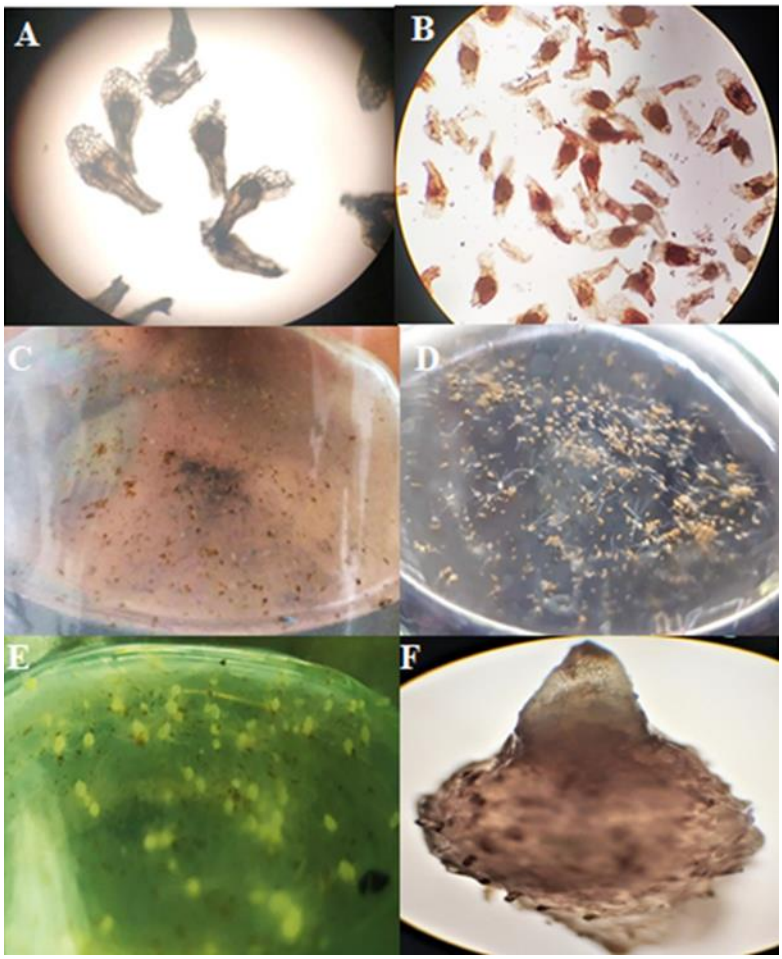

Figure 2. *In vitro* germination of *orchis simia*. A. Mature seeds. B. Seeds subjected to tetrazolium test. C. Seed germination in MS + casein medium. D. Seeds germinated in MS medium (N1/5) + IAA + casein. E. Protocorm formation. F. Protocorm with 10 x magnification

4. Discussion

Due to their unique characteristics, orchids do not reproduce via seeds in the same manner as other crops. Orchid seeds are incredibly small, almost microscopic in size, and they lack essential components such as endosperm, cotyledon, and a primary root. Their germination and initial growth depend on a symbiotic relationship with fungi (Seaton et al., 2013). Furthermore, orchid seeds contain an undifferentiated embryo that lacks the necessary enzymes for metabolizing polysaccharides and lipids. Despite the presence of sugars like sucrose, fructose, maltose, rhamnose, and glucose in the orchid embryo, these sugars are often insufficient for sustaining germination. Consequently, the presence of symbiotic fungi is crucial during the initial stages of seed germination, as they provide the embryo with essential resources such as water, carbohydrates, vitamins, and minerals after penetrating the embryo (Kauth et al., 2008). In nature, from the thousands to millions of microscopic seeds produced by an orchid pod, only a mere 2-3% of them undergo germination (Dutta et al., 2011). Vegetative propagation of this plant is very slow and time-consuming (Pradha and Pant, 2009). Therefore, this reproduction method cannot meet the needs of the people, the market, and different pharmaceutical companies (Basker and Bai, 2010). The *in vitro* culture method will solve these problems by reducing the time required for seed germination as well as plant propagation on a large scale (Pradha and Pant, 2009). Tissue culture has become a standard propagation method for orchid conservation. Seed germination *in vitro* is an advance in orchid propagation (Fay, 1996). In this

study, different MS media were used for the germination of mature orchid seeds. According to the observations, germination was seen in all investigated media, but the percentage of germination was different (Lal et al., 2020), in the study of the germination of mature seeds of two orchid species, observed the first germination in 4 weeks after sowing, and protocorm development was observed 7-9 weeks after sowing. Also, in the study of the germination of immature orchid seeds by Jain and Saxena (2009), the seeds started to germinate in Mitra medium with peptone in dark conditions for one week and formed protocorms in 6 weeks, and about 90% of the germinated seeds started to form a protocorm. In the study of Fatahi et al. (2022), the characteristics of asymbiotic seed germination and seedling growth *in vitro* conditions were significantly ($P<0.05$) influenced by two main types of organic compounds and nitrogen sources. So the seeds cultivated in media containing pineapple juice (PJ) and casein hydrolyzed (CH) had the highest germination percentage. In addition, germination was the most frequent in the MS medium with casein organic matter and indole acetic acid growth regulator. In the present study and the comparisons made, it is possible to understand the application and importance of cytokinin growth regulators on the germination rate of *Orchis Simia* orchids. The role of plant growth regulators (PGRs) in orchid germination is unclear and the response of growth regulators is often species-specific. A major obstacle in understanding the role of exogenous and endogenous PGRs in promoting/inhibiting orchid seed germination may be the small size of the seeds and the possible low levels of PGRs in the embryo. Research on the concentration of endogenous PGRs in orchid seeds, as well as when PGRs are active at germination, will greatly increase the knowledge of how PGRs affect orchid seed germination (Kauth et al., 2008).

Through an examination of the *in vitro* multiplication of mature seeds from *Orchis coriophora* L and the evaluation of various PGRs on their germination, the highest observed germination rate (44.2%) was achieved in the Orchimax culture medium supplemented with activated charcoal and 1 mg/liter of indole-3-acetic acid (Bektaş et al., 2013). Castillo-Pérez et al. (2021) investigated the germination of mature seeds of the orchid *Stanhopea tigrina* in Morashig and Skoog medium and the auxins indole-3-acetic acid (IAA) and indole-3-butyric acid singly or in combination with salicylic acid or coconut water were tested for rooting, and the combination of coconut water (100 ml/L) alone or in combination with indole-3-acetic acid (2.5 or 5 mg/L) was the best treatment. Based on the obtained results, the IAA growth regulator and casein had a positive and significant effect on the percentage of germination and the number of days until germination. Therefore, orchid seeds can germinate better in a medium with organic matter and IAA growth regulators. For many plant species, temperature is a major factor in initiating and breaking physiological seed dormancy (Baskin et al., 2004). Baskin et al. (2006) recommended an alternating temperature range to study the germination ecology of all seeds because constant temperatures are not common *in nature*. However, orchid seeds often germinate *in vitro* at a constant temperature. In the medicinal orchid species *Orchis simia*, mature seeds germinated at a constant temperature of 20°C, but no germination was observed at a temperature of 25 °C.

There are several valuable studies on orchid seed germination and temperature. Like many other species, orchid seeds germinate within a certain temperature range, but maximum germination occurs at a certain temperature. *Dactylorhiza majalis* seeds germinate between 10 and 30°C, but the optimum temperature range seems to be between 23 and 24.5°C (Rasmussen et al., 1990). The percentage of germination decreased below 15°C and above 27°C (Rasmussen and Rasmussen, 1991; Pradha and Pant, 2009). The effect of light and darkness on the germination of orchid seeds is debatable. Zettler and Hofer (1997) reported a significant reduction in germination when *S. odorata* seeds were exposed to a short light period. Germination in complete darkness for three weeks was higher than the germination of seeds that were exposed to 7 days of 12.12 and 16/8 h light period and then in darkness for 2 weeks. Stewart and Kane (2006) reported that light inhibited asymbiotic germination and the development of *Habenaria macroceratitis*. The aforementioned terrestrial orchids all grow in shady areas. If the orchid studied in this research also grows in shady areas. In the study of the seed germination of the orchid

species called Spiranthes by Zale et al. (2022), the seedlings in light/dark periods of 0/24 h and light/darkness of 16/8 h had significantly more fresh weight than the dark treatment group, and therefore for optimal growth need light. The impact of light and darkness on orchid seed germination remains a subject of debate. In line with our findings, another study reported the highest germination rate for *Cyrtopodium punctatum* seeds under continuous darkness (0/24 h) (Dutra et al., 2009).

Conclusion

In this study, the MS (N1/5) + casein + IAA medium emerged as the most favorable culture medium for the germination of mature seeds. Notably, germination was observed four weeks after cultivation, with protocorms appearing within two months after seed cultivation. Dark conditions were found to be significantly conducive to the germination of the tested orchid species, as no germination occurred under bright conditions. It is advisable to explore the use of additional organic compounds such as coconut water and peptone to potentially enhance germination results. Furthermore, investigating the impact of varying IAA concentrations on the duration and germination percentage of the target orchid seeds would be a valuable avenue for future research.

Supplementary Materials:

No supplementary material is available for this article.

Author Contributions:

Conceptualization, Z.M.J. and N.B.; methodology, Z.M.J. and N.B.; software, N.B.; validation, Z.M.J.; N.B., N.B.J., G.R. and J.F.; formal analysis, Z.M.J. and N.B.; investigation, Z.M.J.; resources, Z.M.J.; data curation, Z.M.J and N.B.; writing—original draft preparation, Z.M.J. and N.B.; writing—review and editing, Z.M.J., N.B., and G.R.; visualization, N.B.J. and N.B.; supervision, Z.M.J. and N.B.; project administration, Z.M.J. and N.B.; funding acquisition, N.B., N.B.J., G.R. and J.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest:

The authors declare no conflict of interest.

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مقاله پژوهشی

جوانه‌زنی غیرهمزیستی بذر بالغ ارکیده دارویی (*Orchis simia* Lam.) در شرایط آزمایشگاهی

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چکیده: تکثیر جنسی ارکیده‌ها به دلیل فاقد آندوسپرم بودن بذرهای آن از روند کندی برخوردار بوده، ضمن اینکه جوانه‌زنی آنها در طبیعت نیز نیازمند به محرک‌های قارچی می‌باشد. در مطالعه حاضر، جوانه‌زنی بذر و توسعه اولیه پروتوکورم گونه *Orchis simia* ،یک ارکیده مهم دارویی، در قالب طرح کاملا تصادفی با سه تکرار مورد ارزیابی قرار گرفت. آزمایش تترازولیوم نشان داد ۳۵ درصد از بذرها زنده بودند. متعاقبا اثر کازئین، زغال فعال، ایندول استیک اسید، دوره نوری و دما بر جوانه‌زنی بذور *O. simia* بررسی شد. تجزیه و تحلیل واریانس پاسخ‌های متفاوتی از نظر درصد جوانه‌زنی بذر نشان داد، بطوری‌که تیمارهای دوره نوری و دما تاثیر بارزتری بر جوانه‌زنی داشتند. شرایط بهینه در این آزمایش برای جوانه‌زنی بذر گیاه ارکیده به صورت غیرهمزیست، محیط کشت موراشیگ و اسکوگ (MS) با یک پنجم نیترات همراه با کازئین (۲ گرم در لیتر)، زغال فعال (۲ گرم در لیتر) و تنظیم کننده رشد IAA (یک میلی‌گرم در لیتر) بوده، که در نتیجه آن سرعت جوانه‌زنی ۳۱٪ بدست آمد. پس از یک دوره سه ماهه، گویچه‌ها به پروتوکورم تبدیل شدند. یافته‌های ارائه شده در این گزارش می‌تواند درک جدیدی برای تولید گیاهان ارکیده و حفاظت از این گونه دارویی ارائه نماید.

کلمات کلیدی: ارکیده، پروتوکورم، فاکتورهای جوانه‌زنی، تنظیم‌کننده رشد.

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