Scientific Papers. Series B, Horticulture. Vol. LXVIII, No. 1, 2024
Print ISSN 2285-5653, CD-ROM ISSN 2285-5661, Online ISSN 2286-1580, ISSN-L 2285-5653

ASYMBIOTIC SEED GERMINATION AND *IN VITRO* SEEDLING DEVELOPMENT OF PHALAENOPSIS ORCHID HYBRIDS

Ioana CIOBANU, Adrian George PETICILĂ, Cătălina NICOLAE, Oana VENAT

University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: ioana.ciobanu@abonamenteservice.ro

Abstract

The commercial exploitation of Phalaenopsis, an epiphytic orchid, is widespread, but due to its challenging vegetative propagation, there is a necessity to enhance micropropagation techniques. This study aims to determine the optimal medium and growth regulators for the successful asymbiotic seed germination, development, and multiplication stages of Phalaenopsis. Seeds from 4-month-old-hand-pollinated orchid were sown on ½ Murashige and Skoog (MS). The resulting PLBs were transferred on 4 types of culture media:1/2 MS (1), Malmgren (MLG) media (2), ½ MS with MLG vitamins (3), ½ MS with MLG vitamins, microelements and iron at full strength (4).The fourth medium was found most suitable as it provided the highest length and overall best conditions for evolution. In the multiplication stage a variation of explants: protocorms, leaves, stems and roots were put on 3 culture media, with a variation of light condition, dark and 16/8 photoperiod. The first media did not contain growth regulators and the other two containing also 2 mg/l BAP +0.5 mg/l NAA+100 mg/lPVP+1 g/l CA, respectivly 2.5 mg/l BAP+0.75 mg/l NAA + 100 mg/l PVP + 1 g/l CA.

Key words*: Phalaenopsis, in vitro tissue culture, asymbiotic seed germination, seedling development, multiplication.*

INTRODUCTION

Comprising 62 species, Phalaenopsis stands as a multifaceted orchid genus primarily harnessed for commercial purposes, serving as both a cut flower and a potted plant. This orchid variety proves to be a lucrative venture in flower markets globally. The popularity of Phalaenopsis within the Orchidaceae family stems from its enduring flowers and the ease with which it can be cultivated in home environments (May, 2018).

Hybridization or cross-pollination within breeding programs has consistently proven to be a dependable method for generating a diverse array of successful cultivars. These cultivars exhibit appealing combinations of characteristics such as spray length, bud number, flower color and type, fragrance, seasonality, and compactness (Trevor, 2011). Over time, the orchid market has transformed from a hobbyist's pursuit to a highly commercialized industry, with considerable potential for further expansion (Edy Setiti, 2019). The horticultural sector is now witnessing a shift towards large-scale cultivation of orchid cut flowers and potted orchids. The ability to mass-cultivate orchids became feasible with a significant breakthrough in orchid seed germination, laying the groundwork for extensive breeding and the creation of new commercially viable orchid hybrids (Zheng, 2010).

Knudson's groundbreaking work in 1922 on asymbiotic germination of orchid seeds played a pivotal role in advancing plant tissue culture techniques for orchid micropropagation (Knudson, 1922). The development of asymbiotic seed germination and clonal propagation in vitro has not only facilitated large-scale orchid cultivation but has also established a viable economic market.

Given the minuscule size of orchid seeds, with a single fruit or capsule capable of producing up to a million seeds depending on the species, the potential for propagation is immense. However, due to their limited food storage, orchid seeds rely on a specific type of fungus from orchid mycorrhizae for germination and development. *In vitro* germination using nutrient media containing sugars has become crucial, particularly considering the low survival rate in the wild habitat (under 5%).

Orchid seeds stand apart from most flowering plants due to their tiny embryos. Unlike many plants, orchid seeds lack apical meristems and cotyledons at the time of seed dispersal, and they

exhibit diverse embryo developmental patterns, particularly in suspensor morphology. Orchid seeds have a unique germination process as they rely solely on fungal infection. Consequently, a practical method for orchid seed germination involving fungi has been employed for an extended period (Lee, 2007).

Following Knudson's revelation in 1946 that orchid seeds could successfully germinate on a straightforward medium consisting of minerals and sugar, the adoption of asymbiotic procedures became widespread, supplanting the previously favored symbiotic methods.

The efficacy of in vitro seed germination is affected by various factors, encompassing the nature of the culture media, seed maturity, plant growth regulators (Paudel et al., 2012), carbohydrates (Huh et al., 2016), and organic amendments.

The aim of this study was to determine the optimal medium and growth regulators for the asymbiotic seed germination, development, and multiplication stages of Phalaenopsis-type hybrids.

MATERIALS AND METHODS

1. Plant Material: Surface Sterilization of Capsule and *In Vitro* Asymbiotic Germination

The Phalaenopsis capsule, obtained from hybridization was kindly provided by Cătălina Nicolae, University of Agronomic Sciences and Veterinary Medicine of Bucharest (USAMV). The four-month-old light-green capsule, hand-pollinated, underwent a process of immersion in 70% alcohol for 2 minutes, repeated four times, with intermittent rinsing using tap water.

Under the laminar-flow hood the capsule was immersed for 2 minutes in 70% ethyl alcohol, rinsed with distilled water. In the next stage, work was done with the help of a stirrer, for 15 minutes using a solution of 2% sodium dichloroisocyanurate (NaDCC) plus a jet of Domestos (cleaning solution based on chlorine, Romania). The capsule was left unrinsed, it was removed on a sterile filter paper to absorb the club. Later, it was cut transversely and longitudinally with a scalpel with a sterile blade. Work was done without touching the extremities. The small, powder-like seeds were sown with the help of a sterile scalpel on 1/2 Murashige and Skoog (MS) medium without added hormones, poured into plastic Petri dishes (Figure 1).

The seeds on the culture medium were maintained under a photoperiod of 16 hours of light per day, accompanied by 8 hours of darkness, at a temperature of 23 degrees Celsius ± 2.

Figure 1. Sowing the seeds with the help of a sterile scalpel on 1/2 Murashige and Skoog (MS) medium

2. *In Vitro* Protocorm-like Bodies (PLB) Development on Different Media

We employed protocorm-like bodies (PLBs) obtained from the preceding asymbiotic in vitro germination stage. Four different types of medium, devoid of added hormones, were assessed to determine the most advantageous for protocorm development: (1) half-strength Murashige and Skoog (Murashige & Skoog, 1962) (½ MS macro and micronutrients); (2) Malmgren medium (MLG) (Malmgren, 1996); (3) ½ MS with MLG vitamins and (4) ½ MS with MLG vitamins, microelements and iron at full strength. All media were supplemented with 30 g·L-1 sucrose, 1 g·L-1 Activated charcoal (AC) and solidified with 7 g·L-1 agar powder. pH was adjusted to 5.75 with 0.1M NaOH or HCl. Media were sterilized at 121°C for 20 minutes. All the cultures were maintained under a 16 and 8 h light and darkness, respectively, at 23 ± 2°C.

We chose to include activated charcoal in the medium as it enhances the growth of Phalaenopsis, as suggested by Ernst R (1975). The beneficial effects of charcoal on orchid seedlings or tissue culture-derived plantlets could be attributed to improved aeration. Another potential explanation is that charcoal absorbs ethylene and phenolic inhibitors known to impede growth and development.

Following a 4-week culture period, seedlings, measuring 0.5 cm in length with 1-2 leaves and

one or two roots, derived from the in vitro protocorm-like bodies (PLBs), were transferred to fresh media. Monthly replating was conducted to sustain the development rate of the plantlets. Each month, observations were recorded, including the count and length of leaves and roots, the dimensions of the plantlet, and the maximum width of leaves.

3. ***In Vitro*** **Multiplication**

During the multiplication stage, a variety of explants, including protocorm-like bodies (PLBs), leaves, stems, and root tips obtained from 6-month-old plants cultivated in vitro, were transferred to three different culture media under varying light conditions—both in darkness and in a 16/8 photoperiod. The first media selected was the most succesfull from the previous stage, (1) the ½ MS with MLG vitamins, microelements and iron at full strength, it did not contain growth regulators, it was used as control, while the other two had the same medium containing also

(2) 2 mg/l 6-Benzylaminopurine (BAP) + 0.5 mg/l 1-Naphthaleneacetic acid (NAA) + 100 mg/l Poly(vinylpyrrolidone) (PVP) + 1 g/l Activated Charcoal (AC), respectivly

(3) 2.5 mg/l BAP + 0.75 mg/l NAA + 100 mg/l PVP + 1 g/l AC.

All media were supplemented with 30 g·L-1 sucrose and solidified with 7 g·L-1 agar powder. pH was adjusted to 5.75 with 0.1M NaOH or HCl. Media were sterilized at 121°C for 20 minutes. Half of the cultures were maintained under a 16 and 8 h light and darkness, respectively and the other half were kept in darkness for the first 2 weeks/ when showing signs for the first leaf, both treatments at 23 ± 2°C.

Throughout micropropagation, the release of phenolic substances is a common occurrence, often negatively impacting the response of explants. Browning or blackening of cultured explants occurs only after the tissues are wounded, initiating the oxidation of phenolic substances regulated by polyphenol oxidase. The addition of activated charcoal (AC) mitigates the release of phenolic exudates. The positive effects of AC may stem from its ability to positively stimulate various developmental processes and absorb phenolic compounds, as suggested by Dipika Sarmah in 2017. The inclusion of Poly(vinylpyrrolidone) (PVP) in the medium also diminishes phenolic exudates. PVP serves a crucial role in plant tissue culture by inactivating or binding polyphenols and alkaloids that emanate from explants.

When choosing the explants, various factors were taken into account. Protocorm-like bodies (PLBs) intended for multiplication should exhibit normal size and color, and they should not surpass 2 months in age. Older, stagnant, or deteriorated PLBs tend to yield abnormal plants with stunted growth, both in vitro and *in vivo*. Additionally, small or yellow-colored protocorms are deemed unfit and are consequently discarded.

In thin-leaf culture, it is imperative to choose young leaves, specifically the most recently formed leaf. The segment for culture is transversely cut from the basal portion of this leaf, with segments measuring 1 mm. These segments are then positioned in the culture medium with the cut portion facing down and the adaxial side facing up.

For root-tip culture, only the tips of the roots, measuring under 0.5 cm, should be utilized. These root tips are placed in the culture medium with the cut portion facing down.

RESULTS AND DISCUSSIONS

1. Plant Material, Surface Sterilization of Capsule, ***In Vitro*** **Asymbiotic Germination**

The surface sterilization process was effective, enabling the seeds to germinate in the tested medium with a germination rate of 87.3%.

Phalaenopsis hybrid seed germination commenced with embryo swelling approximately three weeks after sowing. By the sixth week, embryos were released from the testa, transforming into rounded, yellow protocorms. At this stage, a shoot apex became visible at one side of the protocorm (see Figure 2). The protocorm progressed into an elongated shape, transitioning to a green color. Subsequently, absorbing hair developed, and the first and second leaves formed in succession. Roots emerged from the seedlings approximately nine weeks after sowing. The regular replating of protocorm-like bodies (PLBs) derived from seeds holds significant importance in enhancing their survival rate.

In the future we want to evaluate if the introduction of plant hormones and/or organic

compounds can improve the success rate of the germination.

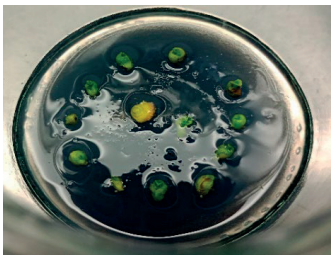

Figure 2. PLBs 7 weeks after sowing. Leaves forming, color changing from yellow to green

2. ***In Vitro*** **Protocorm-like Bodies (PLB) Development on Different Media**

Following the asymbiotic seed germination stage, the protocorm-like bodies (PLBs) were replated, and their development was assessed on four distinct media without plant growth regulators, as detailed in the Materials and Methods section 2.

The plantlets underwent relocation to fresh media every 30 days. Conclusions were drawn from measurements and observations made over the course of 4 months of cultivation (refer to Figure 3).

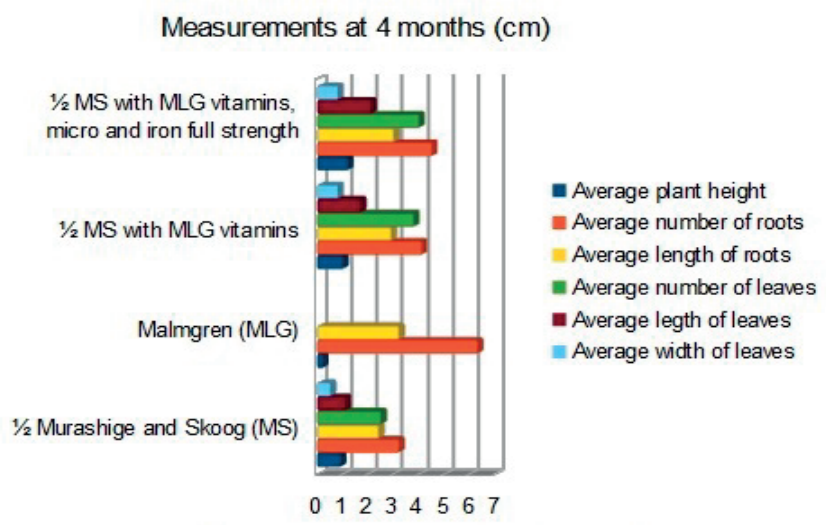

Figure 3. Measurements taken 4 months after starting the experiment

The 1/2 MS medium with MLG vitamins and microelements, including iron at a normal concentration (Option 4), demonstrated the highest suitability by providing the maximum number of leaves, along with optimal root and leaf length, offering overall favorable conditions for the development of protocorm-like bodies (PLBs) in general (see Figure 4).

The 1/2 MS medium with MLG vitamins (Option 3) also supported plant growth, although measurements revealed inferior results compared to medium Option 4.

While the MLG medium (Option 2) produced the longest roots, it was deemed less suitable as it only induced root regeneration and restricted stem development. This medium could be employed for rooting or as a source for root-tip culture (refer to Figure 5).

Maintaining the 1/2 MS medium (Option 1) beyond the germination stage impeded the growth of plantlets, with measurements after 4 months indicating poor results. After this stage, the plants exhibited an increased demand for vitamins and iron, and failure to meet this demand led over time to halted development or dwarfism.

Figure 4. Six month old plants grown on 1/2 MS with MLG vitamins and microelements, including iron at normal concentration

Figure 5. MLG medium was the most unsuitable, as it induced only regeneration of roots and restricted the development of stem

3. *In Vitro* Multiplication

During the multiplication stage, we assessed three media, as detailed in the Materials and Methods section 3. The evaluation included four types of explants: protocorm-like bodies (PLBs), leaf-thin sections, root tips, and stems (6 months old), conducted under both light and darkness conditions (see Figure 6).

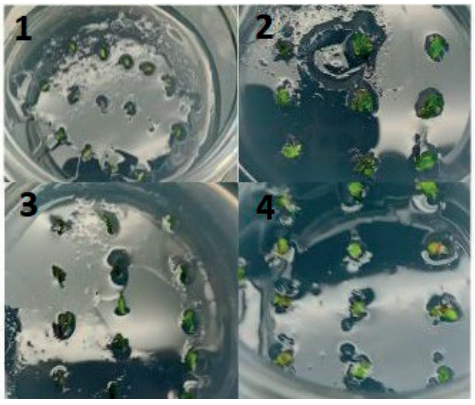

Figure 6. Explants at the time of initiating multiplication
1 - Root-tip culture; 2 - Stem culture;
3 - Thin-leaf culture; 4 - PLB culture

Phalaenopsis is recognized for its production of phenolic compounds. To mitigate the associated risks, we employed explants sourced from tissue culture, known for lower phenolic production. Additional measures, including Polyvinylpyrrolidone (PVP), Activated Charcoal (AC), initial darkness conditions for the first 2 weeks or until the emergence of the first leaf, and regular replating to fresh media, collectively contributed to the success of the experiment. These strategies effectively controlled the production of phenolic compounds, a common challenge in the multiplication phase. We recommend the use of PVP and AC specifically in root-tip culture and leaf-thin section culture.

In leaf and root-tip cultures, the presence of hormones is essential for eliciting an explant response. In the absence of hormones in the control media, regeneration of leaves and root-tips did not initiate. On the contrary, for all other media with hormones, regeneration commenced after 9 days, irrespective of light or dark conditions. Stems and protocorm-like bodies (PLBs) exhibited the fastest response in both conditions.

These conclusions are drawn from observing the explants for a duration of 3 weeks, and it's important to note that the experiment is still ongoing.

Protocorm-like bodies (PLBs) exhibited optimal development in the dark, particularly on medium version (2). Stems displayed varied responses based on light conditions; those kept in the dark developed into PLBs, while those in the light formed stems with new leaves. The most favorable overall development occurred in stems in the dark, specifically on medium version (3).

Leaf segments and root tips demonstrated accelerated development in the dark, especially on medium version (3). During this stage, explants exhibited a superior response in darkness, and we recommend this light regime for its efficiency and cost-effectiveness. However, extending the period of darkness beyond the initial phase, after eliciting a response, is known to inhibit their development.

CONCLUSIONS

Phalaenopsis orchids stand out as one of the most favored orchid varieties, valued for their

ease of cultivation in home environments and their significant economic importance as sought-after ornamental cut flowers. The swift and uncomplicated propagation through seed-derived methods has proven to be an efficient approach, providing hybridizers with extensive selection possibilities for establishing future valuable hybrids. This approach is made feasible through the adoption of tissue culture techniques.

Tissue culture methods, in comparison to traditional breeding, require less time for the development and maintenance of varietal purity. This allows for the creation of new varieties with desirable traits such as compact growth habits, variegated foliage, and peloric flowers, enhancing the potential for orchid improvement.

ACKNOWLEDGEMENTS

This research work was carried out with the support of University Professor PhD George Adrian Peticilă from the University of Agronomic Sciences and Veterinary Medicine of Bucharest. We give special thanks to PhD student Cătălina Nicolae from the Research Center for Studies of Food Quality and Agricultural Products, Bucharest, fellow orchid lover, for providing the Phalaenopsis capsule.

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