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Enhancing *Phalaenopsis* Orchid Mass Regeneration: Evaluating Culture Medium Efficiency

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ABSTRACT

The *Phalaenopsis* orchid holds the largest share of the global orchid market and contributes 8.5% to the floriculture trade, offering significant economic potential for developing countries. Cost-effective mass propagation methods are essential to meet the increasing demand for in vitro *Phalaenopsis* plants. A particularly efficient method involves generating plantlets from flower stalks. This study evaluates the effectiveness of four culture media in producing plantlets from *Phalaenopsis* flower stalk buds: Vacin & Went (V&W) medium supplemented with 5 mg L^{-1} 6-benzylaminopurine (BAP) and 15% coconut water (CW); New Dogashima Medium (NDM) with 1.4 mg L^{-1} adenine hemi-sulfate; modified Hyponex/Kyoto medium containing 5 mg L^{-1} 6-benzyladenine (BA) and 5 mg L^{-1} potato juice (PJ); and a control group using half-strength Murashige and Skoog (MS) medium. Parameters such as plantlet count, leaf count, and phenolic secretion were analyzed. NDM exhibited the lowest phenolic symptoms at the base of the node explants, whereas the half-strength MS medium (1/2 MS) showed the highest phenolic secretion. The Hyponex/Kyoto medium yielded the highest average number of plantlets (17 per node) and leaves (32), surpassing all other media. Given the increased production of both plantlets and leaves from each stem node, the Hyponex/Kyoto medium was considered the most effective for large-scale *Phalaenopsis* clone production, optimizing the propagation process for commercial cultivation.

Introduction

Orchids are important species of cut flowers and potted plants, valued for their considerable ornamental and economic significance. Among them, *Phalaenopsis* orchids, especially hybrid varieties, have become particularly prominent in global markets. These orchids dominate the orchid industry due to their widespread popularity. Renowned for their elegant and long-lasting blooms, *Phalaenopsis* orchids are favored not only for their beauty but also for their ease of care and ability to thrive in indoor environments. Their availability in a diverse array of colors and patterns further enhances their appeal, making them a preferred choice for both experienced orchid enthusiasts and beginners alike.

In 2024, the commercial value of *Phalaenopsis* orchids reached an impressive USD 121.0 million (Deore et al., 2024). Notably, the Netherlands has emerged as a leading producer of in vitro embryos, seedlings, artificial seeds, and potted *Phalaenopsis* hybrids. Other significant producers include Japan, which accounts for 15% of global production, followed by Thailand (8%), Singapore (6%), Italy (4%), and various other countries collectively contributing 14% (Yuan et al., 2022; Moradi et al., 2017; Mahdavi et al., 2023). Despite their global success, *Phalaenopsis* orchid growers face substantial economic challenges, particularly with the high costs associated with importing tissue-cultured plants. These challenges highlight the need

for developing cost-effective and efficient protocols to enable the mass production of high-quality *Phalaenopsis* specimens. Although in vitro culture remains the primary method of orchid propagation, its relatively slow growth rates and high operational costs make the process financially demanding. Therefore, optimizing growth conditions at each stage of the propagation process is essential to lowering overall production expenses.

The culture of flower stalk nodes represents a critical initial step in the vegetative propagation of *Phalaenopsis* orchids. This technique is primarily aimed at inducing the formation of protocorm-like bodies (PLBs), which are structurally similar to somatic embryos and can be treated as such in propagation systems (Naderi Boldaji et al., 2023; Vahdati et al., 2006). While the induction of PLBs from the shoot apex, or shoot apical meristem, has been successfully demonstrated in other Orchidaceae species, the unique biological characteristics of monopodial *Phalaenopsis* orchids require alternative propagation strategies. In *Phalaenopsis*, only a single shoot apex is available per plant, and its removal would lead to the loss of the mother plant. As a result, researchers have focused their efforts on utilizing buds from the flower stalk for vegetative propagation (Rotor et al., 1950). Flower stalk (or spike) culture involves stimulating dormant buds located on the flower stalk to develop into new plantlets. Despite numerous attempts to establish vegetative propagation systems that do not compromise the integrity of the mother plant, flower stalk culture remains a valuable and practical approach for addressing the unique propagation challenges presented by *Phalaenopsis* orchids.

Research has demonstrated that the addition of specific organic and inorganic compounds to culture media can significantly influence plant tissue growth and morphology. In *Phalaenopsis* orchids, flower stalks exhibit three types of buds: dormant, vegetative, and reproductive. Buds located at the upper nodes tend to produce reproductive structures, while those at the lower nodes typically give rise to vegetative shoots (Urata et al., 1965). Adenine hemi-sulfate is widely used in plant tissue culture to enhance growth and stimulate multiple shoot formation. For instance, supplementing Murashige and Skoog (MS) medium with 15 mg L^{-1} adenine sulfate resulted in 79.9% multiple shoot formation in *Clitoria ternatea* after just 15 days of incubation (Rency et al., 2018). In orchid propagation, the success of tissue culture largely depends on the careful selection of nutritional components and plant growth regulators (PGRs). Alongside macro- and micronutrients, vitamins, carbon sources, and PGRs, the incorporation of complex organic additives plays a crucial role in improving culture outcomes. Additives such as potato homogenate, coconut milk, banana homogenate, and potato juice have shown significant benefits for seed germination and micropropagation across various orchid species. Potato juice, in particular, supplies essential nutrients including proteins, amino acids, and vitamins, all of which support robust cellular and organismal growth. However, its application must be optimized and validated for specific experimental contexts (Murdad et al., 2010).

Given these considerations, vegetative nodes on *Phalaenopsis* flower stalks serve as suitable explants for *in vitro* culture studies. Numerous compounds, including PGRs, amino acids, plant extracts, and organic substances such as coconut water (CW) and dry yeast, have been investigated for their potential to enhance in vitro plant development (e.g., Al-Khayri et al., 2010). PGRs act as signaling molecules that regulate growth and development by improving photosynthetic efficiency and overall physiological function in plants (Zhang et al., 2015; Mohammadpour Barough et al., 2024). Coconut water, a nutrient-rich, colorless liquid endosperm, is frequently added to culture media to stimulate rapid cell proliferation and growth (Shekarriz et al., 2014). In fact, its inclusion has proven essential for the successful development of young *Datura stramonium* embryos. As the effects of organic compounds can vary significantly depending on their concentration and combination, this study is designed to evaluate the impact of two distinct culture media, each containing CW and PGRs, on the induction of plant growth from *Phalaenopsis* vegetative nodes (Movahed et al., 2021).

Materials and Methods

Plant material

Phalaenopsis 'Nagasaki' flower stalks were collected from greenhouse-grown plants and transported to the laboratory. The stalks were cleaned by washing with a solution of water and soap to remove surface contaminants. They were then immersed in a 20% sodium hypochlorite solution for 20 min, followed by thorough rinsing with sterile distilled water three times, with each rinse lasting 2, 5, and 10 min, respectively. After sterilization, the flower stalks were transferred to a sterile environment and cut into 1 cm segments, each containing a node.

Culture media

Various solid culture media were prepared, including:

A control medium containing 1/2 MS with 5 mg L^{-1} Benzyladenine (BA) (Ghahremani et al., 2021).

New Dogashima Medium (NDM) enriched with 1.4 mg L^{-1} adenine hemi-sulfate (Tokuhara et al., 1994).

Modified Hyponex/Kyoto medium comprising 5 mg L^{-1} BAP (6-Benzylaminopurine) and 30 mL L^{-1} potato juice (PJ) (Zhang et al., 2022).

Vacin & Went (V&W) medium containing 5 mg L^{-1} BAP and 150 mg L^{-1} coconut water (CW) (Utami et al., 2019).

The pH of all media was adjusted to 5.7 ± 0.1. The media were sterilized by autoclaving at 121 °C and 15 pounds per square inch (psi) for 20 min, after which they were dispensed into jars in 50 mL volumes.

Explant culturing

The flower stalk nodes were used as explants and cultured under controlled conditions at a temperature of 25 ± 1 °C with a photoperiod of 16 h of light and 8 h of darkness for a total duration of 120 d. Explants were sub-cultured monthly to maintain culture growth and development.

Data analysis

The experimental setup was in a completely randomized design. Data analysis was performed using SAS 9.1 software, and mean values were compared using Duncan's multiple range test with a significance level set at 5%. The evaluated parameters included the number and length of plantlets (keikis) and the number of leaves.

Results

In this study, four distinct culture media were evaluated for their effectiveness in inducing plantlet formation from *Phalaenopsis* flower stalks. Each medium incorporated a cytokinin, 6-benzylaminopurine (BAP), alongside organic additives such as potato juice (PJ) and coconut water (CW). While the different media formulations did not produce a statistically significant effect on shoot (plantlet) length, they did have a significant impact on both the number of plantlets (keikis) and the number of leaves per plantlet, with significance observed at the 1% level ($\alpha = 0.01$) (Table S1). Among the media tested, the modified Hyponex/Kyoto culture medium demonstrated a statistically significant advantage beginning in the first month of cultivation, as shown in Figures 1 and 2. This medium consistently outperformed the others, yielding the highest number of plantlets, up to 23 plantlets per node, over the six-month experimental period. In contrast, the NDM culture medium resulted in the lowest plantlet production (Fig. 1).

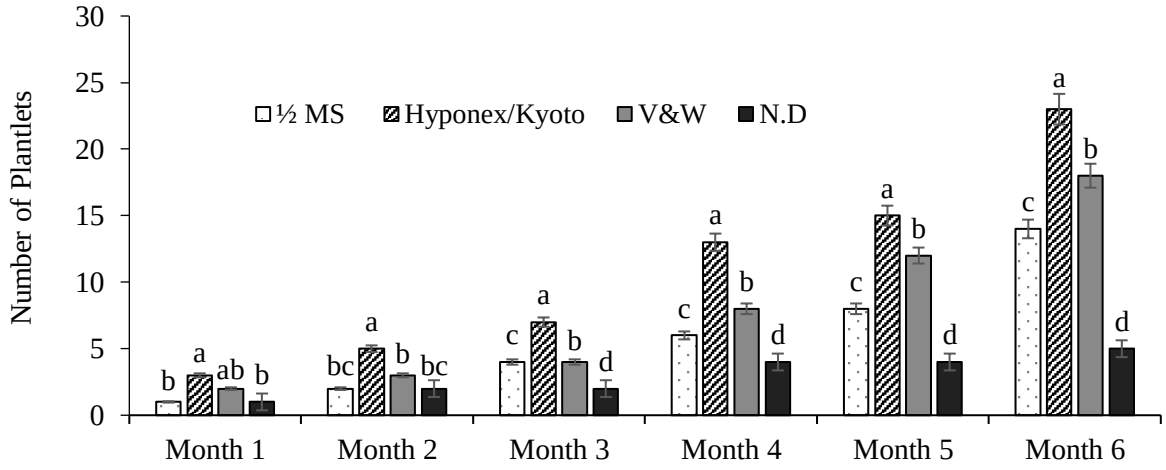

Fig. 1. Plantlets (keikis) generated from the culture of *Phalaenopsis* spike nodes on four different culture media over a six-month period. Data were collected monthly, and the average values were compared separately for each month.

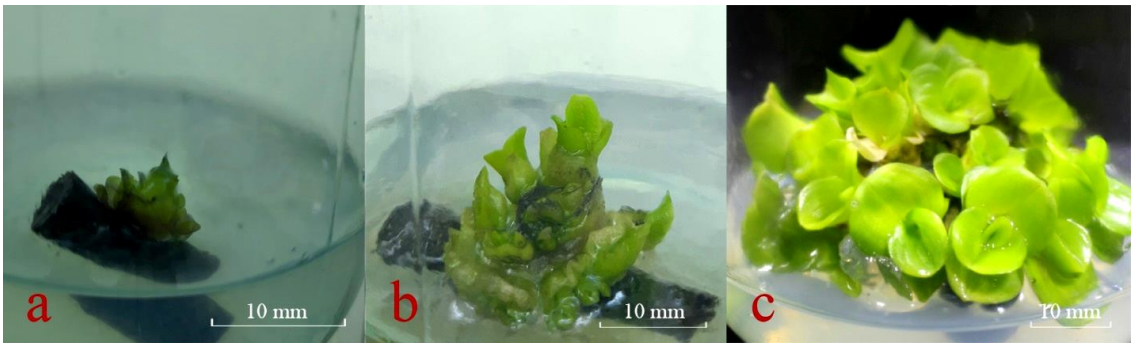

Fig. 2. Formation and growth of plantlets (keikis) from *Phalaenopsis* 'Nagasaki' spike nodes on modified Hyponex/Kyoto at the second (a), fourth (b), and sixth (c) months of culture.

The number of leaves was significantly affected ($P \leq 0.001$) as a result of supplying PJ and CW in conjunction with the Hyponex and V&W media. Although the V&W medium produced the highest number of leaves from the first to the third month, with a statistically significant difference compared to the other media. The Hyponex/Kyoto medium showed significantly greater positive effects on leaf production after the third month (Figs. 3 and 4).

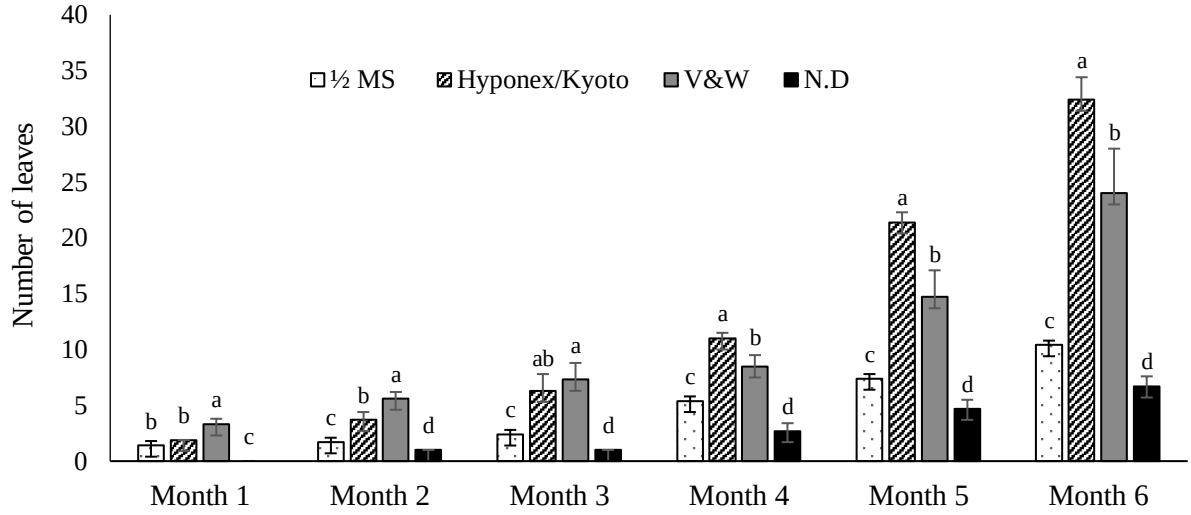

Fig. 3. Increase in the number of leaves over six months, corresponding to the growth of plantlets. Data were collected monthly, and the average values were compared separately for each month.

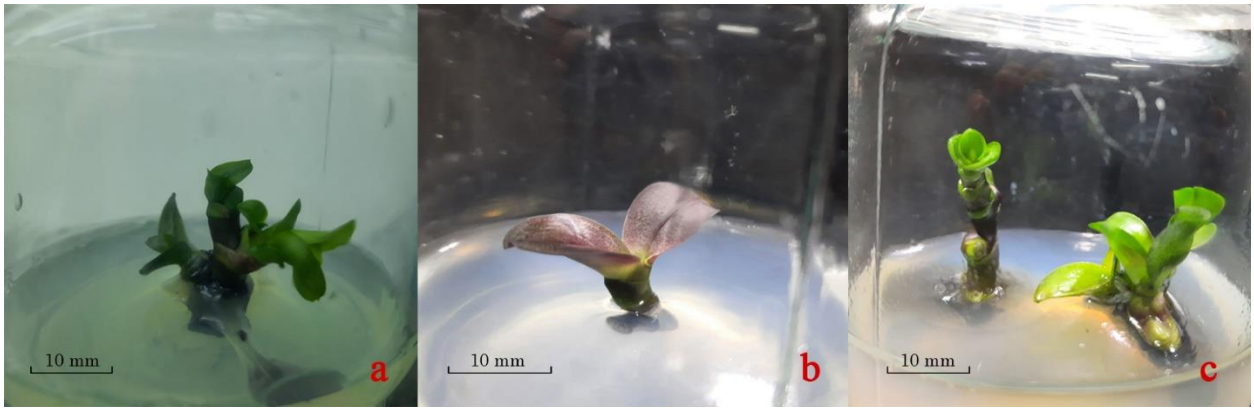

Fig. 4. Comparison of leaf number resulting from the growth of plantlets (keikis) of *Phalaenopsis* 'Nagasaki' after six months on 1/2 MS (a), NDM (b), and V&W (c) media, compared with the leaf number observed on modified Hyponex/Kyoto medium in Fig. 2 (c).

Discussion

Our findings are consistent with previous observations on flower stem culture, where the addition of BAP (6-Benzylaminopurine) led to enhanced shoot multiplication, resulting in the highest recorded number of shoots. This highlights the importance of cytokinins, particularly BAP, in promoting the growth and proliferation of *Phalaenopsis* orchid explants, further validating their efficacy for *in vitro* plant regeneration processes. In a similar study on Oncidium spp., culture on a medium supplemented with 0.2 mg L^{-1} BAP resulted in enhanced shoot multiplication, achieving the highest number of shoots recorded (Kalimuthu et al., 2017).

In a comparable investigation on *Paphiopedilum callosum* orchids, a solitary shoot measuring 2 cm in height, with three leaves, was cultured on Hyponex N016 medium. The medium was further enriched with 1.0 mg L^{-1} NAA (1-Naphthaleneacetic acid), 30 g L^{-1} sucrose, 170 mg L^{-1} NaH_2PO_4 (Sodium dihydrogen phosphate), and 1.0 g L^{-1} peptone, resulting in the production of 12 plantlets per node (Huy et al., 2019). In comparison, our study showed a 78.26% increase in plantlets with the Hyponex medium (compared to the NDM, which produced the

fewest plantlets). The role of cytokinins in the culture medium is primarily associated with enhanced cell division and the subsequent induction of shoot growth, as observed in *Oncidium* (Posast et al., 2018). In our research, we also found that BAP significantly influenced the growth and induction of plantlets (keikis).

Furthermore, successful regeneration of *Phalaenopsis amabilis* (L.) Bl. flower stalks was achieved using a V&W medium enriched with 5 mg L^{-1} BAP, 30 mg L^{-1} thiamine, and 20% coconut water (CW) (Seeni et al., 2000). This highlights the importance of selecting an appropriate culture medium to optimize these outcomes and underscores the effectiveness of cytokinins, particularly BAP, in promoting seedling growth and production. In other studies, shoot tip culture on a medium supplemented with 8.8 mM BAP and 4.4 mM NAA resulted in the rapid multiplication of *Vanda coerulea* and the successful establishment of clonal plants (Mohr et al., 1995). Relative to our study, these findings reported a 22.15% increase in seedlings, although the seedlings produced had 18.48% fewer leaves than those observed in our research.

These results emphasize the potential of BAP and CW in enhancing orchid micropropagation while addressing the challenge of phenolic secretion, which often hinders successful tissue culture efforts. In another study on *Phalaenopsis hybrid* 'Pink,' different concentrations of sucrose and CW were added as sources of carbon and natural additives in various media. Four months after culturing, seedling growth was significantly affected by the combination of sucrose and CW in both 1/2 MS and V&W media (Zahara et al., 2017). Similarly, flower stalks of *Dendrobium nobile* were successfully proliferated using a medium supplemented with BAP and kinetin (Kin), along with CW. Among the different concentrations of CW (5, 10, 15, 20, 25, and 30% v/v), the treatment containing 150 mL L^{-1} CW produced the highest number of shoots, while higher concentrations resulted in reduced shoot numbers and lengths (Asghar et al., 2011). Our findings were consistent with these results, as CW supplementation in the media proved effective in promoting shoot proliferation.

Moreover, the most substantial increase in the proliferation percentage of trimmed protocorms from *Phalaenopsis gigantea* was observed in a culture medium enriched with 15% (v/v) coconut water (CW) and 2.5 g L^{-1} activated charcoal (AC) (Murdad et al., 2016). Potato juice (PJ) is renowned for its composition, which includes soluble sugars that serve as a natural carbon source, as well as a variety of amino acids, vitamins (such as thiamine, pyridoxine, and ascorbic acid), and essential minerals (such as phosphorus, magnesium, potassium, calcium, iron, and manganese). These constituents collectively contribute to its suitability for promoting germination (Gnasekaran et al., 2009). In our study, the Hyponex medium containing 30% (w/v) PJ was identified as the most effective treatment, suggesting the beneficial impact of PJ on plantlet production.

Furthermore, CW is rich in organic ions and contains phytohormones like indole-3-acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA), and zeatin, which are commonly used as growth supplements in plant tissue culture (Santoso et al., 2000). The inclusion of CW in the V&W culture medium facilitated optimal plantlet growth and induction, making it a viable treatment in our study. Additionally, incorporating activated charcoal into the Hyponex/Kyoto medium proved to be an effective method for controlling phenolic secretion, demonstrating exceptional proliferation and regeneration efficiency. Comparative analysis of the four culture media revealed that an increase in organic additives, particularly those with a vitamin and carbohydrate base, correlated with enhanced plantlet production efficiency.

Our findings suggested that the incorporation of organic substances into the Hyponex culture medium resulted in a 15% increase in plantlet production without thidiazuron (TDZ), compared to the study by Zanello (2022) using the Hyponex/Kyoto medium. By evaluating various media for cultivating *Phalaenopsis* orchid inflorescence stem nodes and comparing them with modified Hyponex medium (without activated charcoal), we observed a 28.4% increase in plantlet production (12.47 ± 1) and a 35.6% increase in leaf count (20.38 ± 0.62).

Conclusions

Several culture media have been identified as effective for the commercial propagation of *Phalaenopsis* orchids. In this study, we compared three widely used commercial media with the control medium employed in our laboratory to determine the most effective medium for the vegetative propagation of *Phalaenopsis* orchids via inflorescence stem node culture. Among the tested media, the modified Hyponex/Kyoto medium, supplemented with 5 mg L^{-1} BAP and 30 mg L^{-1} potato juice (PJ), demonstrated the highest efficacy, yielding the greatest number of plantlets and leaves after five months of cultivation. This result underscores the synergistic effect of BAP and PJ in promoting optimal growth and development. These findings offer new insights into the role of specific medium components in enhancing the propagation efficiency of *Phalaenopsis* orchids, supporting the hypothesis that targeted modifications to culture media can significantly improve micropropagation outcomes.

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Conflict of Interest
The authors indicate no conflict of interest in this work.

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Supplemental Materials

Table S1. Variance analysis of different culture medium treatments on plantlet count, leaf number, and shoot length on nods explants of *Phalaenopsis* 'Nagasaki' flower stalks.

S.O.V	df	Plantlet number	leaf number	shoot length	Media Phenolic degree
media	3	179.2**	428.2**	2.61ns	2.7**
error	8	2.00	16.00	0.16	0.8

** and * are significant at the 1 and 5 percent levels, respectively, and ns: not significant.