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Microclonal propagation of the orchid *Vanda* (*Orchidaceae Vanda*)

Oksana Kliachenko*
Doctor of Agricultural Sciences, Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-4087-4082

Oleksandr Subin
PhD in Biological Sciences, Associate Professor
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0001-6209-2439

Olha Oliinyk
PhD in Agricultural Sciences
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0000-0002-3591-3518

Diana Shyshkina
Student
National University of Life and Environmental Sciences of Ukraine
03041, 15 Heroiv Oborony Str., Kyiv, Ukraine
https://orcid.org/0009-0001-2486-7691

Abstract. Biotechnological methods of propagation of *Vanda* orchids represent an effective approach for obtaining large quantities of genetically uniform and disease-free plant material. This study aimed to investigate the specific features of microclonal propagation of *Vanda* orchids. To obtain planting material of different *Vanda* genotypes, namely 'Vanda coerulea' and 'Vanda sanderiana', direct and indirect morphogenesis, as well as *in vitro* rhizogenesis followed by adaptation to *in vivo* conditions, were applied. A stepwise method for obtaining aseptic material was developed, involving treatment with 70% ethyl alcohol for 1 minute followed by the application of the main sterilising agent, 2.5% NaClO, for 5-10 minutes. This protocol made it possible to obtain 70-80% sterile and viable explants while reducing the level of fungal and bacterial contamination. The results of direct and indirect morphogenesis, callus formation, and rhizogenesis in *in vitro* cultures of *Vanda* explants are presented. No significant differences in callus formation were observed between the studied cultivars. The frequency of callusogenesis for both genotypes reached 100%, indicating a high capacity for callus tissue formation. The best results in growth and shoot formation were achieved on Murashige and Skoog (MS) nutrient medium supplemented with 0.5 mg/L 6-benzylaminopurine (6-BAP). For rooting, the most effective medium was MS with halfstrength macro- and microelements supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA). This medium is recommended for the induction of rhizogenesis in regenerated plants of different

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*Corresponding author

Vanda cultivars. For the adaptation of regenerated plants, a substrate composed of sphagnum moss, peat, crushed beech leaves, and pine bark in a ratio of 1:1:1:1 was used. The survival rate of 'Vanda coerulea' plants under *in vivo* conditions reached 87.5%, whereas under the same conditions, the survival rate of 'Vanda sanderiana' plants was 83%. The obtained results may serve as a basis for the development of propagation protocols for *Vanda* orchids, ensuring a high level of control at all stages of cultivation. The implementation of such protocols is important for breeding programmes, the conservation of rare cultivars, and the improvement of plant material quality for commercial floriculture

Keywords: *in vitro*; callusogenesis; morphogenesis; rhizogenesis; *in vivo* adaptation

INTRODUCTION

Orchids of the genus *Vanda* are among the most striking representatives of the family *Orchidaceae*, distinguished by their high ornamental value, diversity of morphological forms, and long-lasting, intense flowering. Owing to these characteristics, they have gained popularity among amateur and professional growers not only worldwide but also in Ukraine. These plants are epiphytes or lithophytes native to tropical regions, naturally occurring in South-East Asia, the Philippines, Indonesia, northern Australia, and the Himalayas. The particular interest in *Vanda* orchids is due not only to their aesthetic appeal but also to their high commercial value, which makes them an important object for ornamental horticulture and large-scale propagation. However, traditional methods of propagation, such as seed propagation and vegetative reproduction, have significant limitations, including a long developmental cycle and an increased risk of viral infection, which restricts their efficiency for industrial cultivation. Therefore, the development of modern biotechnological approaches for the propagation of *Vanda* orchids is highly relevant. In particular, microclonal propagation makes it possible to obtain large quantities of genetically uniform and virus-free plant material, which in turn contributes to biodiversity conservation, improves cultivation efficiency, and ensures a stable supply of high-quality planting material. The study of biotechnological characteristics of orchids of the genus *Vanda* and the improvement of methods for their microclonal propagation represent key directions in the development of modern plant science, biotechnology, and the conservation of ornamental flora (Liu *et al*., 2020; Pathak *et al*., 2023).

One of the important research directions highlighted in the literature is the optimisation of *in vitro* culture conditions to enhance the efficiency of microclonal propagation of *Vanda* orchids. K. Nowakowska *et al*. (2022) demonstrated that the efficiency of microclonal propagation of *Vanda brunnea* Rchb.f. primarily depends on the mineral composition of the nutrient medium and is not influenced by the addition of 0.5 mg/L 6-BAP. L. Tikendra *et al*. (2025) reported that the highest multiplication coefficient (5.62) was achieved on MS medium supplemented with 1.2 mg/L kinetin and 0.6 mg/L indole-3-butyric acid. In the studies by X.-f. Liu *et al*. (2020) and P. Pathak *et al*. (2023), the effects of plant growth regulators on seed germination and protocorm formation in orchids were investigated. X.-f. Liu *et al*. (2020) emphasised the effective use of growth regulators to stimulate seed germination of *Vanda falcata*, which significantly enhances the success of *in vitro* propagation of this species. At the same time, P. Pathak *et al*. (2023) examined the influence of similar compounds on vegetative propagation and the phytochemical composition of *Vanda cristata*, an endangered orchid species. The investigation of these aspects is of considerable importance for the biopharmaceutical industry. A.N. Laily *et al*. (2024) studied the effect of organic components on the rate of protocorm formation and their viability. Another important aspect is the maintenance of the genetic uniformity of regenerated plants during *in vitro* cultivation. In the study by R. Baby *et al*. (2019a), it was determined that, using RAPD, SSR, and ISSR markers, the level of genetic polymorphism in regenerated plants did not exceed 5% regardless of the duration of cultivation; however, this value increas ed after six subcultures.

It should be noted that the natural propagation of *Vanda* orchids occurs through seed reproduction, which is a highly complex process because the seeds lack endosperm and therefore cannot germinate independently. Seed germination is possible only in the presence of mycorrhizal fungi, which supply the embryo with essential nutrients. This characteristic is typical of all orchids and makes natural seed-based reproduction an expensive and relatively unreliable method. H.N. Rasmussen & F.N. Rasmussen (2014) examined the evolution of mycorrhiza in orchids, which is essential for understanding their development, particularly in the context of microclonal propagation. Mycorrhiza plays a key role in seed germination and seedling development, thereby confirming the effectiveness of *in vitro* cultivation methods for orchids. B.J. Davis *et al*. (2015) investigated the distribution of mycorrhizal fungi, which is important for the biogeography of orchids, especially with regard to their rooting and development. The study by A. Setiaji *et al*. (2021) demonstrated the effectiveness of biotechnological methods of microclonal propagation of *Vanda* orchids, making it possible to obtain genetically uniform and disease-free planting material. The most effective approach involved the use of Murashige and Skoog medium supplemented with 6-BAP for growth and shoot formation, as well as media supplemented with α-naphthaleneacetic acid for rooting. The developed sterilisation and adaptation methods ensured high plant survival rates and can be applied in industrial orchid production.

In this context, microclonal reproduction techniques, such as *in vitro* meristem culture, have become extremely popular in ornamental horticulture. These biotechnological approaches enable the production of large numbers of genetically identical, healthy plants that retain the morphological and decorative characteristics of the parent plant. This is of critical importance both for the conservation of rare cultivars and for their large-scale commercial propagation. This study aimed to investigate the specific features of microclonal propagation of *Vanda* orchids and to obtain healthy, virus-free planting material under *in vitro* conditions.

MATERIALS AND METHODS

The research was conducted in the Biotechnology and Bioengineering Educational and Research Laboratory of the NULES of Ukraine from 2023 to early 2025. The orchid cultivars 'Vanda coerulea' and 'Vanda sanderiana', characterised by high ornamental value and long-lasting flowering, were used for introduction into *in vitro* culture. Generally accepted biotechnological research methods were applied (Kolomiets *et al*., 2022). The study was carried out in several directions: investigation of the callusogenic capacity of *Vanda* orchids, analysis of direct and indirect morphogenesis, assessment of shoot formation intensity under *in vitro* conditions, rooting of regenerated plants, and their adaptation to *in vivo* conditions.

For the experiments, explants were collected from healthy donor plants. Before introduction into sterile culture, particular attention was paid to the sterilisation stage in order to prevent fungal and bacterial contamination during subsequent cultivation. Initially, the explants were thoroughly washed in a soap solution for 10-15 minutes to remove surface contaminants. They were then rinsed with running tap water, followed by distilled water, and finally twice with sterile distilled water. All subsequent procedures were performed exclusively under a laminar airflow cabinet in strict compliance with aseptic techniques. Protocorms and young flower shoots not exceeding 2 cm in length were used as primary explants.

Sterilisation was carried out using stepwise treatment of the explants according to the following schemes: 70% ethyl alcohol (1-2 min) → 2.5% NaClO (exposure for 5, 10, or 15 min); 70% ethyl alcohol (1-2 min) → 1% AgNO3 (exposure for 5, 10, or 15 min); 70% ethyl alcohol (12 min) → 2.5% NaClO + 1% AgNO3 (15 min). The sterile explants were rinsed three times for 10 minutes each with sterile distilled water. Afterwards, they were placed in Petri dishes on sterile filter paper for drying. Using sterile forceps and observing aseptic conditions, the explants were transferred into test tubes containing hormone-free Murashige and Skoog (MS) nutrient medium (Murashige & Skoog, 1962) and cultured in a thermostat in the absence of light at a temperature of 25°C ± 1°C. On the seventh day after the initiation of the experiment, the cultures were examined for the presence of microbiological contamination. Infected samples were removed from the thermostat, as they represented a potential source of infection. The sterilisation efficiency (Es), expressed as a percentage, was calculated using the following formula:

$$Es = \frac{Ne - Ndme}{Ne} \times 100\%, \qquad (1)$$

where N_e is the total number of planted explants (units), and N_{dme} is the number of damaged (infected) explants (units). For callus induction, the explants were transferred to nutrient media MSK1–MSK3 supplemented with α-naphthaleneacetic acid (NAA) at concentrations of 2-5 mg/L and 0.2 mg/L 6-benzylaminopurine (6-BAP), as well as 100 mg/L myo-inositol, 500 mg/L casein hydrolysate, and 30 g/L sucrose. For subculturing of *Vanda* orchid callus tissue, the average fresh weight of the inoculum was 2.0 ± 0.10 g. The frequency of callus formation was determined as the percentage ratio of explants that successfully formed callus to the total number of samples. The average monthly increase in callus fresh weight was calculated as the difference between the final mass after cultivation and the initial mass placed on the nutrient medium, in accordance with the methodology described by Yu.V. Kolomiets *et al*. (2022).

For the induction of indirect morphogenesis, callus tissue of *Vanda* orchids with an average mass of 2.0 ± 0.10 g was subcultured onto MSH1–MSH3 nutrient media enriched with phytohormones: 6-BAP at concentrations of 0.1-3.0 mg/L, kinetin at 0.5-2.0 mg/L, and 2,4 dichlorophenoxyacetic acid (2,4-D) in the range of 0.1-1.0 mg/L. Cultivation was carried out under controlled conditions at a temperature of 25°C ± 1°C, light intensity of 2.0-3.0 klx, a 16-hour photoperiod, and relative humidity of 70-75%. At the shoot formation stage, apical shoot segments and microcuttings 1-2 cm in length, containing a pair of leaves and part of the aerial roots where meristematic tissues are located, were used, as these tissues possess a high capacity for new shoot formation. Owing to the appropriate selection of the phytohormonal composition, particularly the balanced combination of cytokinins and auxins, positive results were achieved, whereby several new plants were obtained from a single explant. Subsequently, rhizogenesis was stimulated, taking into account that a well-developed root system ensures plant survival after transplantation to *in vivo* conditions. For this purpose, regenerated shoots were transferred to a medium with a reduced content of macronutrients supplemented with auxins. Indole-3-acetic acid (IAA) and indole3butyric acid (IBA) were most frequently applied at concentrations of 0.5-2.0 mg/L. These conditions promoted the formation of root primordia within the first two weeks of cultivation, which is consistent with the recommendations reported by T.D. Thomas (2008), B. Bhattacharjee & S.M.S. Islam (2014b), and A. Setiaji *et al*. (2021).

At the final stage, the orchid plants were adapted to *in vivo* conditions by transplanting them into a moist, well-drained substrate composed of sphagnum moss, peat, crushed beech leaves, and pine bark in a ratio of 1:1:1:1, followed by placement under conditions of high humidity. The cultivation temperature was maintained within the range of 23°C-25°C, and irrigation was carried out only after partial drying of the upper layer of the substrate. The experimental data obtained during the study were subjected to statistical analysis using the MS Excel software package. All experiments were performed in triplicate, and 30 explants were used in each experimental variant. The tables present arithmetic mean values and their standard errors. Experimental studies involving plants, including the collection of plant material, complied with institutional, national, and international guidelines. The authors adhered to the standards of the Convention on Biological Diversity (1992).

RESULTS AND DISCUSSION

Selection, preparation of explants, and introduction into *in vitro* culture for obtaining aseptic explants of *Vanda* orchids

The stage of explant selection is one of the most critical steps in the process of microclonal propagation, as the quality of the initial material largely determines the subsequent success of cultivation. Various plant parts may be used as explants, including apical or axillary buds, stem fragments, leaves, aerial roots, or meristematic tissues, each of which has specific characteristics. The choice of explant type directly influences the efficiency of the process (Chugh *et al*., 2009; Bhattacharjee & Islam, 2014a). For *Vanda* orchids, the use of young, non-lignified aerial roots or apical buds is considered appropriate, as these tissues exhibit high regenerative capacity and are less susceptible to pathogen infection. In addition, the physiological condition of the plant and the environmental conditions under which the material is collected are of great importance (Rahman *et al*., 2009; Nowakowska *et al*., 2022; Tikendra *et al*., 2025).

A necessary prerequisite for the successful application of microclonal propagation methods is strict adherence to specific rules. First of all, the tissues intended for cultivation must be aseptically isolated from the parent plant. Obtaining sterile material for cultivation is a challenging task, as the plant surface is naturally colonised by epiphytic bacteria, fungi, and their spores. The correct selection of sterilising agents aims to neutralise the epiphytic microflora without damaging plant tissues. In addition, the sterilising substance should not penetrate deeply into the tissues and must be easily removed by washing. To obtain an aseptic culture of *Vanda* orchids, visually healthy, morphologically normal plants without external symptoms of bacterial, fungal, or viral infection were selected. The plants were thoroughly washed under running water to remove surface contaminants from the inflorescences, aerial roots, and leaves. To prevent microbial contamination, subsequent sterilisation procedures were performed in a laminar airflow cabinet.

The sterilisation methodology was selected experimentally for each object, depending on tissue sensitivity. Furthermore, the same organ from different plants requires different sterilisation conditions. Therefore, to achieve the research objectives, several commonly used sterilising agents with varying exposure times were applied. The sterilisation variants for explants of 'Vanda coerulea' and 'Vanda sanderiana', and the corresponding results are presented in Tables 1 and 2. For cultivation, the basal Murashige and Skoog nutrient medium was used. Following sterilisation, the explants were cultured on hormone-free MS medium at a temperature of 25°C ± 1°C, relative humidity of 70-80%, and a 16-hour photoperiod.

Table 1. Sterilisation efficiency of 'Vanda coerulea' explants

No.	Sterilising agent	Concentration (%)	Exposure (min)	Number of explants introduced *in vitro* (pcs)	Number of aseptic explants (pcs)	Number of viable explants (pcs)	Sterilisation efficiency (%)*
1	NaClO	2.5	5	30	26	24	80
2	NaClO	2.5	10	30	28	21	70
3	NaClO	2.5	15	30	30	10	33
4	$AgNO_3$	1	5	30	17	15	50
5	$AgNO_3$	1	10	30	25	18	60
6	NaClO $AgNO_3$	2.5 1	15 15	30	30	3	10
LSD_{05}					1.3	0.8	2.5

Note: * – calculated using formula 1

Source: developed by the authors

It should be noted that fungal contamination of 'Vanda coerulea' explants was observed on days 8-10, whereas bacterial and mixed fungal-bacterial contamination occurred between days 5 and 15 of cultivation. The experimental data indicate that the most effective method for sterilising explants was the stepwise treatment: 70% ethyl alcohol (1 min) → 2.5% NaClO (5-10 min), which allowed the production of 70-80% sterile and viable explants (Fig. 1). Overall, a positive outcome (~50-60% aseptic and viable explants) was obtained using 1% $AgNO_3$ for 5-10 minutes. However, the use of sterilising agents such as 2.5% NaClO and 1% $AgNO_3$ with a 15-minute exposure negatively affected explant viability due to tissue oxidation, and the sterilisation efficiency decreased to 10%.

The results of sterilising the inflorescences of 'Vanda sanderiana' are presented in Table 2.

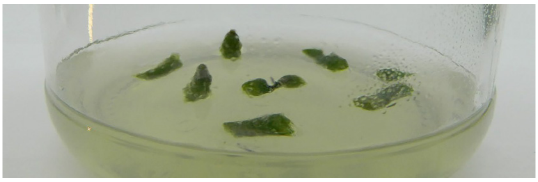

Figure 1. Aseptic protocorms of 'Vanda coerulea' (sterilisation variant No. 1)

Source: provided by the authors

Table 2. Sterilisation efficiency of 'Vanda sanderiana' explants

No.	Sterilising agent	Concentration (%)	Exposure (min)	Number of explants introduced *in vitro* (pcs)	Number of aseptic explants (pcs)	Number of viable explants (pcs)	Sterilisation efficiency (%)*
1	NaClO	2.5	5	30	26	25	80
2	NaClO	2.5	10	30	28	20	70
3	NaClO	2.5	15	30	30	9	31
4	$AgNO_3$	1	5	30	15	13	45
5	$AgNO_3$	1	10	30	25	18	60
6	NaClO $AgNO_3$	2.5 1	15 15	30	30	3	10
LSD_{05}					1.3	0.7	2.5

Note: * – calculated using formula 1
Source: developed by the authors

From the data presented in Table 2, it can be seen that, for 'Vanda sanderiana' explants, the most effective sterilisation method, as with 'Vanda coerulea', was the stepwise approach: treatment with 70% ethyl alcohol for 1 minute followed by immersion in 2.5% NaClO for 5-10 minutes. This protocol allowed the production of 70-80% sterile and viable explants (Fig. 2). This sterilisation regime did not damage the tissues or inhibit explant development, while ensuring maximal sterility. A positive result (45-60% aseptic and viable explants) was also obtained using 1% $AgNO_3$ for 510 minutes. Exposure to 2.5% NaClO or 1% $AgNO_3$ for 15 minutes adversely affected viability due to tissue oxidation. The lowest number of aseptic and viable explants was observed under sterilisation variant No. 4.

Figure 2. Aseptic and viable explants of 'Vanda coerulea' on day 30 of cultivation (sterilisation variant No. 1)
Source: provided by the authors

There is a trend towards minimising the use of highly toxic compounds as sterilising agents. In the study by R. Baby *et al*. (2019b), a stepwise method was employed for sterilising *Vanda* orchid explants using 0.1% $HgCl_2$ as the primary sterilant with exposure times ranging from 1 to 10 minutes. The percentage of sterile explants obtained varied from 0% to 80%. With longer exposure, the toxic effect of 0.1% $HgCl_2$ on explant tissues became apparent. Therefore, the approach used in the present study, employing 2.5% NaClO, is both safer and sufficiently effective for working with inflorescence shoots and protocorms. As a result, aseptic and viable explants of 'Vanda coerulea' and 'Vanda sanderiana' were obtained and subsequently used in studies on callusogenesis, morphogenesis, and plant regeneration.

Features of callusogenesis in *Vanda* orchids

In higher plants, callus tissue is defined as tissue formed through uncontrolled cell proliferation in explants following their initial subculture. This process is widely applied in biotechnological research as well as in microclonal propagation of plants. Studies have shown that the process of callusogenesis *in vitro* largely depends on the external culture conditions and the composition of the nutrient medium, particularly the presence of plant growth regulators. Effective callus induction is generally observed when using the classical auxin-to-cytokinin ratio of 10:1 (Kushnir & Sarnavska, 2005). It should be noted that plant growth regulators play a key role in stimulating cell proliferation and structural changes within tissues, thereby enabling the formation of callus tissue with high potential for subsequent regeneration. In *Vanda* orchids, callus tissue is typically induced from protocorms and inflorescence shoots by culturing them on modified MS medium supplemented with growth regulators, specifically α-naphthaleneacetic acid and 6 benzylaminopurine, at various concentrations. Three variants of nutrient media were used for callus induction (Table 3).

Explants were placed in sterile flasks containing the nutrient medium and cultured in the dark at 25°C ± 1°C for 14 days. An increase in explant size was observed, indicating active uptake of carbohydrates and mineral nutrients from the medium. Subsequently, the explants were transferred to a light regime with an intensity of 3,000 lx and a 16-hour photoperiod, and cultured at 24°C ± 1°C. On day 30, the frequency of callus formation and the biomass of the callus were recorded, with the results presented in Table 4.

Table 3. Growth regulator composition in callus-inducing media

Variant	Growth regulators
1	2 mg/L NAA + 0.2 mg/L 6-BAP
2	5 mg/L NAA + 0.2 mg/L 6-BAP
3	2.5 mg/L NAA + 0.4 mg/L 6-BAP

Source: developed by the authors

Table 4. Dependence of callusogenesis in *Vanda* orchids on the content of growth regulators in the nutrient medium

Variant	Medium composition	Number of explants (pcs)	Number of explants forming callus	
			(%)	Callus fresh weight (mg)
'Vanda coerulea'				
1	2 mg/L NAA + 0.2 mg/L 6-BAP	30	90 ± 4.5	270 ± 13.5
2	5 mg/L NAA + 0.2 mg/L 6-BAP	30	97.5 ± 4.9	540 ± 24.3
3	2.5 mg/L NAA + 0.4 mg/L 6-BAP	30	100 ± 5.0	690 ± 30.2
'Vanda sanderiana'				
1	2 mg/L NAA + 0.2 mg/L 6-BAP	30	88 ± 4.4	300 ± 15.0
2	5 mg/L NAA + 0.2 mg/L 6-BAP	30	95 ± 4.8	490 ± 24.5
3	2.5 mg/L NAA + 0.4 mg/L 6-BAP	30	99 ± 4.9	655 ± 32.8

Source: developed by the authors

From the data presented, it can be seen that the highest callus mass and the greatest percentage of callus formation were recorded on MS medium variant 3, where the concentrations of NAA and 6-BAP were 2.5 mg/L and 0.4 mg/L, respectively. This high regenerative capacity aligns with the findings of A.N. Laily *et al*. (2024), who reported that the correct auxin-to-cytokinin ratio is critical for callus induction in *Vanda celebica*. In general, the optimal ratio of auxins to cytokinins for callus formation is quite variable and depends on the specific culture. In the study by S.A. Dar *et al*. (2021), callus induction in *Atropa acuminata* was most effective at a 1:1 ratio of 6BAP to NAA, while A.F. Likhanov *et al*. (2017) observed callus induction in *Aesculus hippocastanum* using kinetin and 2,4-D at a 1:6 ratio. A similar trend is generally observed across the *Orchidaceae* family (Sheyko & Musatenko, 2013; Shemediuk & Skip, 2014). In addition to quantitative characteristics, the density and colour of the callus tissue were also assessed. In *Vanda* orchids, the callus was typically cream-white or yellowish in colour, with a loose to medium texture and pronounced meristematic activity, indicating a high regenerative potential of the tissue (Fig. 3).

Figure 3. Callus tissue of 'Vanda coerulea'
Source: provided by the authors

Thus, according to the results of the conducted studies, the most effective callus-inducing medium for both *Vanda* orchid cultivars was MS medium supplemented with 2.5 mg/L NAA and 0.4 mg/L 6-BAP. Under these conditions, 99-100% of the explants produced callus, demonstrating the high efficiency of this medium for inducing callusogenesis. The fresh callus mass obtained on this medium was 690 mg for ‘Vanda coerulea’ and 655 mg for ‘Vanda sanderiana’.

Features of indirect morphogenesis in *Vanda* orchids

One of the least explored aspects of cellular differentiation is morphogenesis. The main challenge in studying the physiological, biochemical, and molecular processes underlying it lies in the asynchrony of cell differentiation within tissues that later form organs. Several researchers have shown that morphogenetic centres are spatially distinct, and the cells involved exhibit varying levels of metabolic activity. During the cultivation of unorganised callus tissue, diverse morphological structures arise, eventually forming shoots, roots, leaves, and in some cases, complete plants (Palama *et al*., 2010; Lee *et al*., 2013; Hardjo *et al*., 2021). In Vanda orchids, indirect morphogenesis typically involves two main stages: first, callus tissue is formed from the explant, and subsequently, morphogenetic structures develop within the callus, either through organogenesis or somatic embryogenesis. The formation of organs within the callus culture is closely associated with the ratio of plant growth regulators.

Callus tissues of *Vanda* orchids exhibit the capacity for both somatic embryogenesis and organogenesis, leading to the formation of shoots, roots, and even floral structures. This developmental plasticity makes the genus highly promising for large-scale microclonal propagation. At the same time, the potential for organogenesis is higher in tissues closer to the plant base, whereas explants isolated from the stem show reduced morphogenetic activity. Furthermore, repeated subculturing of callus cultures gradually diminishes the capacity to form new organs, although root formation may remain stable over an extended period (Lee *et al*., 2013; Chen, 2018; Hardjo *et al*., 2021). In addition, studies by R. Baby *et al*. (2019a) and K. Nowakowska *et al*. (2022) have shown that callusogenesis carries a risk of somaclonal variation. This is particularly important for rare and endangered species, where maintaining the genotype is critical; thus, the use of molecular markers to monitor regenerant plants is necessary.

To stimulate indirect morphogenesis, callus tissues with a mass of 2.0 ± 0.10 g were cultured on modified MS medium supplemented with 6-benzylaminopurine, kinetin, and 2,4 dichlorophenoxyacetic acid at varying concentrations. Explants were maintained at 25°C ± 1°C under a light intensity of 2.0-3.0 klx, a 16-hour photoperiod, and 75-80% relative humidity. During the study, active organogenesis was observed in both Vanda cultivars under investigation (Table 5).

Table 5. Organogenic potential *in vitro* of different *Vanda* orchid cultivars

Passage No.	Organogenesis intensity		
	Organogenesis frequency (%)	Number of shoots (pcs)	Shoot height (cm)
‘Vanda coerulea’			
1-3	100 ± 5	4.0 ± 0.25	7.50 ± 0.10
5-6	55 ± 2.8	2.1 ± 0.18	5.20 ± 0.14
7-8	13 ± 0.7	0.4 ± 0.07	2.10 ± 0.09
‘Vanda sanderiana’			
1-3	98 ± 4.9	5.5 ± 0.3	7.0 ± 0.4
5-6	65 ± 3.3	3.2 ± 0.2	6.1 ± 0.3
7-8	15 ± 0.8	18 ± 0.9	2.8 ± 0.1

Source: developed by the authors

The results of the study indicate that the most intensive organogenesis in Vanda orchid cultivars occurs during passages 1-3 (each passage lasting 21 days) (Fig. 4). However, with further increases in the number of passages (beyond 5-6), shoot formation activity declined significantly and almost ceased after passages 8-9. This reduction is likely attributable to somaclonal variation arising from prolonged cultivation.

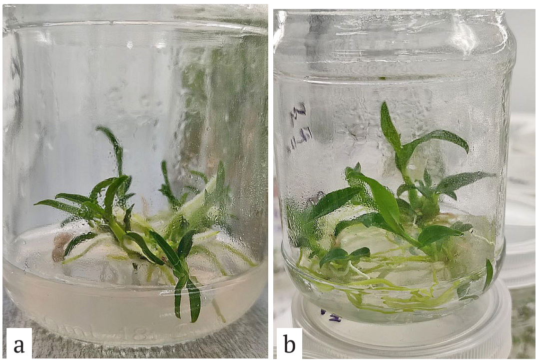

Figure 4. Shoots of 'Vanda coerulea' derived from callus tissue

Note: a – passages 5-6; b – passages 1-3

Source: provided by the authors

Thus, the study established that the efficiency of indirect morphogenesis in *Vanda* orchids depends on several factors, namely the genotype of the donor plant, the presence of growth regulators in the culture medium, and the number of callus subcultures. To maintain high morphogenetic activity, it is recommended to limit the number of passages to 5-6 and periodically renew the original callus. Induction of direct morphogenesis in orchid genotypes. Apical meristems are a key source of explants in the microclonal propagation of orchids, particularly in the genus *Vanda*. Their high meristematic activity and capacity to form secondary shoots under appropriate culture conditions highlight the importance of this type of explant material (Lang & Hang, 2006; Rahman *et al.*, 2009).

One of the decisive factors for successful plant regeneration is the carefully selected composition of the culture medium, particularly the optimal concentration of plant growth regulators. The interaction between cytokinins and auxins plays a crucial role in regulating morphogenesis. Research has demonstrated that the combination of 6-benzylaminopurine (6-BAP) with a low concentration of IAA promotes the intensive formation of new shoots (Maharjan *et al.*, 2019; Kolomiets *et al.*, 2022; Muharyati *et al.*, 2024). In the experiment, apical shoot segments measuring 0.6-1.2 mm in length were used. For morphogenesis induction, aseptic explants were transferred to MS media with varying compositions and concentrations of growth regulators (Table 6).

Table 6. Composition of culture media for organogenesis induction

No.	Medium component	Concentration in different medium variants (mL/L)				
		1	2	3	4	5
1	MS macronutrients	100	100	50	100	100
2	MS micronutrients	1	1	1	1	1
3	MS vitamins	1	1	1	1	1
4	Fe chelate	5	5	5	5	5
5	6-BAP	1.0	0.25	–	0.5	–
6	IAA	–	–	0.1	–	–
7	NAA	0.5	1	–	1	–
8	Myo-inositol (g/L)	0.1	0.1	0.1	0.1	0.1
9	Yeast extract (g/L)	0.5	0.5	–	1	–
10	Casein hydrolysate (g/L)	0.5	0.5	–	–	1
11	Sucrose (g/L)	–	30	30	–	30
12	Glucose (g/L)	20	–	–	15	–
13	Agar (g/L)	6.9	6.9	6.9	6.9	6.9

Source: developed by the authors

Explants were cultivated under a light intensity of 3-4 klx, with a 16-hour photoperiod, at 24°C ± 2°C and 70% relative humidity. The results were evaluated on the 30th day of *in vitro* cultivation. During this period, the growth of shoot length, the number of internodes, activation of meristems, and explant colour were monitored. An intermediate stage of microclonal propagation (MCP) involves producing a large number of microclones from a single initial explant and promoting their intensive growth *in vitro*. The main factor in MCP is achieving a high multiplication rate, which is largely influenced by plant growth regulators and synthetic growth stimulators. For active growth of orchid cultures, auxins such as IAA and IBA, alongside 6-BAP, are recommended. In addition, essential components of the culture media include casein hydrolysate and yeast extract, which enhance explant development (Chugh *et al.*, 2009). Between days 42 and 49 of cultivation, the formation of secondary shoots was observed. The number of shoots produced ranged from one to three per explant, with an average length of 2.6 cm (Fig. 5).

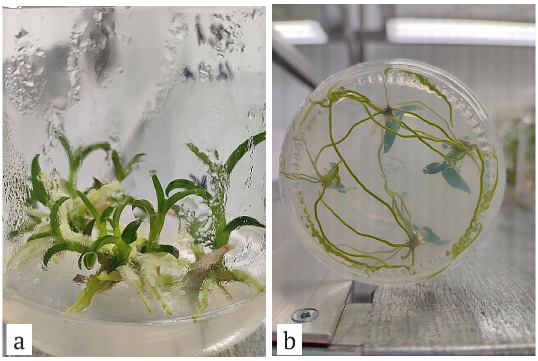

Figure 5. Regenerated plants from apical shoots of *Vanda* orchids
Note: a – 'Vanda coerulea'; b – 'Vanda sanderiana'
Source: provided by the authors

The results indicate that the optimal concentration of growth regulators, particularly 6-BAP and IAA, promotes the activation of apical meristems and the effective formation of new shoots, corroborating the findings of Y. Muharyati *et al.* (2024). This underscores the suitability of using apical shoots in microclonal propagation of orchids, as this approach enables the rapid production of a substantial quantity of high-quality, genetically uniform planting material. Studies of experimental morphogenesis *in vitro*, at various levels of organisation from single cells to complete organs, have provided the foundation for developing efficient microclonal propagation techniques (Kaeppler *et al.*, 2000; Podhaetskyi *et al.*, 2018; Kolomiets *et al.*, 2022). A particularly promising approach involves the use of leaf explants containing remnants of aerial roots and callus to initiate shoot formation in *Vanda* species (Lang & Hang, 2006; Rahman *et al.*, 2009). This method enhances the proliferation rate and accelerates the large-scale production of healthy planting material (Kaeppler *et al.*, 2000; Podhaetskyi *et al.*, 2018). The method of direct organogenesis involves the formation of new shoots directly from explant cells, bypassing the callus stage and thereby maintaining the genetic stability of the plant material. However, for certain *Vanda* orchid cultivars, the use of callus as an intermediate stage can enhance the morphogenetic potential of tissues, improving regeneration outcomes (Rahman *et al.*, 2009; Nongdam *et al.*, 2023).

Shoot formation in *Vanda* orchids occurs via two main mechanisms: the development of shoots from existing meristematic zones of the leaf near the base of the aerial root, or the formation of secondary meristems through the redifferentiation of specialised callus cells. Both mechanisms are influenced by growth regulators, particularly the cytokinin 6-BAP and the auxins IBA and NAA in appropriate ratios (Rahman *et al.*, 2009; Hardjo *et al.*, 2021). Leaf explants measuring 1.5-2 cm, including remnants of aerial roots, were used. Cultivation was carried out on modified MS medium supplemented with 1.5 mg/L 6-BAP and 0.2 mg/L IBA, under a temperature of 25°C ± 1°C, a 16hour photoperiod, light intensity of 3 klx, and relative humidity of 70-80%. By day 28, shoot formation was observed in 83% of explants, with the number of shoots per explant ranging from 1 to 5. By day 42, the average number of shoots per explant reached 66.7 ± 0.3, demonstrating the effectiveness of combined explants (leaf + aerial root) as a source of regeneration (Table 7 and Fig. 6).

Table 7. Shoot formation in 'Vanda coerulea' from combined explants (leaf + aerial root + callus)

Observation period	Shoots 1-2.9 cm	Shoots 3-4.9 cm	Shoots ≥5 cm	Total shoots
'Vanda coerulea'				
28 days	42 ± 2.1	18 ± 0.9	6 ± 0.3	66 ± 3.3
42 days	58 ± 2.9	32 ± 1.6	12 ± 0.6	102 ± 5.1

Source: developed by the authors

Figure 6. Regenerated 'Vanda sanderiana' plants from leaf explants with aerial roots and callus
Source: provided by the authors

The shoot formation process was accompanied by active proliferation of callus tissue at the base of the explants. Subsequent differentiation of these structures into apical organs indicates a high morphogenetic potential of these organogenic centres (Hardjo *et al.*, 2021; Nongdam *et al.*, 2023). Thus, the results confirm the efficacy of using leaf explants with remnants of aerial roots and callus for regeneration in *Vanda* orchids. This approach achieves high micropropagation efficiency, minimises the risk of somaclonal variation, and allows the mass production of virus-free planting material for industrial applications. The regenerated plants serve as material for further cloning, thereby increasing the multiplication coefficient.

Induction of morphogenesis in the introduced *Vanda* explants

For morphogenesis induction, sterile explants were transferred to fresh nutrient media containing varying concentrations of growth regulators and 2 g/L activated charcoal. Explants were cultivated under a light intensity of 3-4 klx, with a 16-hour photoperiod, at 24°C ± 2°C and 70% relative humidity. Results were assessed on day 30 of *in vitro* cultivation. During the cultivation period, the formation of shoot length, the number of internodes, the activity of meristems, and the colour of the explants were monitored. An intermediate stage in micropropagation involves generating a large number of microclones from a single initial explant and promoting their vigorous growth *in vitro*. Cultivation of orchids on media No. 3 and No. 5 produced negative outcomes due to tissue oxidation caused by phenolic compounds (Fig. 7). Media No. 1 and No. 2 are recommended for active growth, development, and propagation, as they support rapid protocorm enlargement and the formation of new leaves and roots by day 15 of cultivation (Fig. 8).

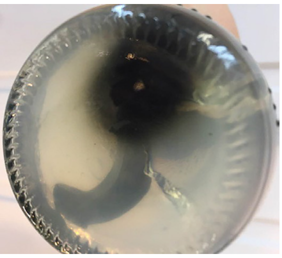

Figure 7. Phenolic auto-toxicity in a 'Vanda coerulea' explant on medium No. 3 on day 10 of cultivation
Source: provided by the authors

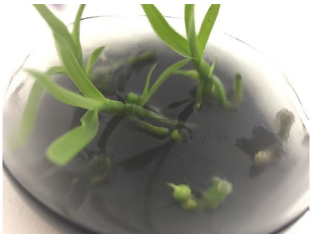

Figure 8. 'Vanda coerulea' on medium No. 2 on day 15 of cultivation
Source: provided by the authors

Thus, morphogenesis in both Vanda orchid cultivars is most intensive on media supplemented with 6-BAP (1.0 mg/L), NAA (0.5 mg/L), yeast extract (0.5 g/L), casein hydrolysate (0.5 g/L), and activated charcoal (2 mg/L). Under these conditions, explants reached 2.5-4.0 cm in size, acquired a deep green colour, and exhibited activation of dormant buds and protocorm growth. This combination of cytokinins and auxins is considered optimal for overcoming apical dominance in the *Orchidaceae* family. This aligns with the findings of L. Tikendra *et al.* (2025), who reported the most effective morphogenesis on MS medium supplemented with 1.2 mg/L kinetin and 0.6 mg/L IAA. On medium containing IAA (0.1 mg/L) without activated charcoal, phenolic exudation was pronounced, explants appeared weak, and dormant bud activation did not occur. When 6-BAP (0.5 mg/L) and NAA (1.0 mg/L) were added to the medium, explants remained small (0.5-1.0 cm), pale in colour, and exhibited only weak bud sprouting.

Rooting of regenerated *Vanda* orchids *in vitro*

Root formation in regenerated plants represents the final and simultaneously one of the most challenging stages of micropropagation for orchids, particularly for *Vanda* species. Successful development of a functional root system is critical for subsequent growth under *in vivo* conditions (Thomas, 2008; Kumar & Rao, 2012; Maharjan *et al*., 2019). A distinctive feature of *Vanda* orchids is the production of aerial roots, which are covered with velamen – a specialised tissue that absorbs moisture from the air and participates in photosynthesis. Therefore, successful rhizogenesis requires not only the correct medium but also adequate access to oxygen and light. Roots develop most effectively under diffuse natural light or cool artificial lighting at an intensity of 2-3 klx.

In the studies of T. Murashige & F. Skoog (1962) and G.P. Kushnir & V.V. Sarnatska (2005), the use of half-strength MS medium without added growth regulators was recommended to stimulate root system development. However, A. Setiaji *et al*. (2021) and Y. Muharyati *et al*. (2024) demonstrated the advantages of including auxins, such as NAA or IBA. The effect of different NAA concentrations (0.5-2.0 mg/L) on root formation in microshoots of various *Vanda* orchid cultivars was examined using agarised MS medium with half-strength mineral salts. Standard-sized shoots with well-developed leaf rosettes were selected for the experiment and placed on several nutrient medium variants. The results are summarised in Table 8. The highest rooting percentage (87.5%) was observed on the MSR2 medium supplemented with 0.5 mg/L NAA (Fig. 9). By contrast, MSR1 and MSR3 showed considerably lower rooting rates of 30% and 55%, respectively.

Table 8. Effect of nutrient medium composition on the rooting of *Vanda* ‘Vanda coerulea’ shoots

Variant	Medium composition	Number of shoots (pcs)	Number of rooted shoots (pcs)	Rooting, %
MSR1	½ MS	40	12 ± 0.6	30 ± 1.5
MSR2	½ MS + 0.5 mg/L NAA	40	35 ± 1.8	87.5 ± 4.4
MSR3	½ MS + 2.0 mg/L NAA	40	22 ± 1.4	55 ± 2.8

Source: developed by the authors

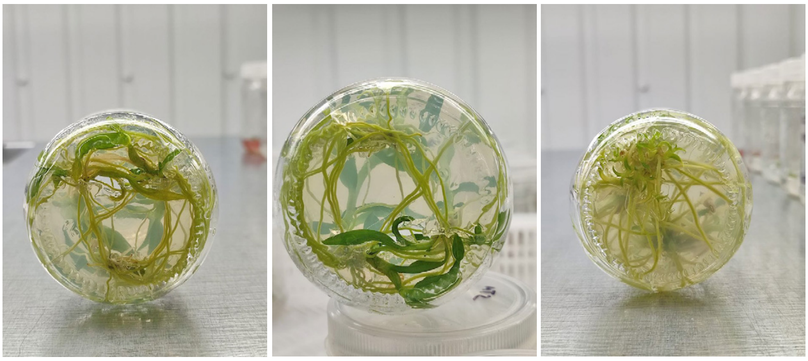

Figure 9. Rooted regenerated ‘Vanda coerulea’ plants on MSR2 medium
Source: provided by the authors

Based on the results of the study, the most effective medium for stimulating root system development in regenerated *Vanda* orchids was MS medium with half-strength macro- and microelements, supplemented with 0.5 mg/L NAA. This medium promoted a high rooting rate, which is critical for successful root system development and the subsequent adaptation of plants to *in vivo* conditions. Therefore, the use of this medium is recommended for integration into *Vanda* orchid micropropagation protocols, ensuring consistent and reliable outcomes.

Acclimatisation of regenerated *Vanda* orchids to *in vivo* conditions

Plants with well-developed root systems and healthy, dense green leaves were removed from the nutrient medium for subsequent acclimatisation. To facilitate adaptation to a non-sterile environment, the culture vessels were opened, and 2-3 mL of sterile distilled water was added, leaving the plants under these conditions for two days. The plants were then carefully removed, washed under running water to remove any residual agar medium from the roots, and disinfected with a 1% potassium permanganate solution to prevent potential infections. Prepared plants were planted in a substrate composed of sphagnum moss, peat, shredded beech leaves, and pine bark in a 1:1:1:1 ratio. Occasionally, additional components such as charcoal, fallen leaves, or dolomite flour are added to improve substrate properties; however, the core substrate typically consists of various proportions of bark, moss, peat, and fern roots. The pots with planted orchids were placed under conditions of high humidity to promote successful establishment. After 10-12 days, as the plants adapted, they established successfully and began to grow. The survival rates of regenerated plants of different *Vanda* orchid cultivars are presented in Table 9.

Table 9. Survival of regenerated plants of different *Vanda* orchid cultivars

Cultivar	Number of plants planted in substrate (pcs)	Number of plants established	
		pcs	%
'Vanda coerulea'	40	35	87.5 ± 4.4
'Vanda sanderiana'	40	33	82.5 ± 4.1

Source: developed by the authors

By the 30th day of cultivation, the seedlings had reached a height of 8-9 cm and developed 6-8 true leaves, making them suitable for transplanting into open-ground greenhouse conditions (Fig. 10). The substrate for *Vanda* orchids should be loose, moisture-retentive, and nutrient-rich, although nutrient content is less critical and can be adjusted through fertilisation. Most *Vanda* orchids do not have a clearly defined dormancy period, so watering should be moderate but regular throughout the year. The substrate should remain moist but not waterlogged, and water at room temperature should be used. Various types of containers can be used for planting, including ceramic or plastic pots, baskets, or mesh holders.

Figure 10. Regenerated 'Vanda sanderiana' adapted to *in vivo* conditions
Source: provided by the authors

Thus, the adaptation study of regenerated plants of different *Vanda* cultivars demonstrated that the survival rate of 'Vanda coerulea' under *in vivo* acclimatisation conditions was 87.5%, while under the same conditions, 'Vanda sanderiana' achieved 83%. These results indicate a high survival capacity for both cultivars, with 'Vanda coerule'a showing slightly better performance.

CONCLUSIONS

The conducted research yielded aseptic, viable explants of cultivars 'Vanda coerulea' and 'Vanda sanderiana', which were subsequently used for studies on callus induction, morphogenesis, and plant regeneration. It was determined that the most effective callus-inducing medium for both cultivars of *Vanda* orchids was MS medium supplemented with 2.5 mg/L NAA and 0.4 mg/L 6BAP. Under these conditions, 99-100% of explants formed callus, with a fresh callus mass of 690 mg for 'Vanda coerulea' and 655 mg for 'Vanda sanderiana'. The efficiency of indirect morphogenesis in *Vanda* orchids depends on several factors, including the genotype of the donor plant, the presence of growth regulators in the culture medium, and the number of passages of callus tissue. To maintain high morphogenetic activity, it is recommended to limit the number of passages to 5-6 and to periodically renew the initial callus. The results also confirm the suitability of using leaf explants with remnants of aerial roots and callus for regeneration in *Vanda* orchids. This approach allows for highly efficient micropropagation, minimises the risk of somaclonal variation, and enables the mass production of virus-free planting material for commercial purposes. The regenerated plants provide material for further cloning, thereby increasing the propagation coefficient. Morphogenesis in both Vanda cultivars was most vigorous on a medium supplemented with 6-BAP (1.0 mg/L), NAA (0.5 mg/L), yeast extract (0.5 g/L), casein hydrolysate (0.5 g/L), and activated charcoal (2 mg/L). The most effective medium for stimulating root system formation in regenerated *Vanda* plants was MS medium with half-strength macro- and microelements, supplemented with 0.5 mg/L NAA. This formulation ensures a high level of rooting, making it suitable for inclusion in micropropagation protocols for this genus. During the acclimation of regenerated plants, survival rates for the cultivar 'Vanda coerulea' reached 87.5%, while under identical conditions, 'Vanda sanderiana' achieved 83%. These results provide a basis for developing standardised propagation protocols for *Vanda* orchids, ensuring a high level of control at all stages of cultivation. Implementation of such protocols is critical for breeding programmes, the conservation of rare cultivars, and the improvement of plant material quality for commercial horticulture.

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CONFLICT OF INTEREST

None.

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Мікроклональне розмноження орхідеї *Vanda* (*Orchidaceae Vanda*)

Оксана Кляченко
Доктор сільськогосподарських наук, професор
Національний університет біоресурсів і природокористування України
03041, Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-4087-4082

Олександр Субін
Кандидат біологічних наук, доцент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0001-6209-2439

Ольга Олійник
Кандидат сільськогосподарських наук
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0000-0002-3591-3518

Діана Шишкіна
Студент
Національний університет біоресурсів і природокористування України
03041, вул. Героїв Оборони, 15, м. Київ, Україна
https://orcid.org/0009-0001-2486-7691

Анотація. Біотехнологічні методи розмноження орхідеї *Vanda* є ефективним інструментом отримання великої кількості генетичнооднорідного оздоровленого рослинного матеріалу. Метою роботи було вивчення особливостей мікроклонального розмноження орхідеї *Vanda*. Для отримання посадкового матеріалу різних генотипів орхідеї *Vanda*, а саме: ‘Vanda coerulea’ та ‘Vanda sanderiana’ методом мікроклонального розмноження використано прямий, непрямий морфогенез і ризогенез *in vitro* та адаптація до умов *in vivo*. Розроблено ступінчатий метод отримання асептичного матеріалу, який полягає у використанні 70 % етилового спирту протягом 1 хвилини з подальшим використанням основного стерилянту 2,5 % NaClO протягом 5-10 хв, що уможливлює отримання 70-80 % стерильних життєздатних експлантатів, знижуючи рівень контамінації грибами та бактеріями. Представлено результати прямого та непрямого морфогенезу, калюсогенезу і ризогенезу в культурі *in vitro* експлантатів орхідеї *Vanda*. Встановлено, що між сортами орхідеї не спостерігалося значних відмінностей у процесах калюсогенезу. Частота калюсогенезу для обох генотипів становила 100 %, що вказує на високу здатність до утворення калюсної тканини. Найкращі результати росту та пагоноутворення були досягнуті на живильному середовищі Мурасіге-Скуга (МС), доповнене 6-Бензиламінопурином (6-БАП) у концентрації

0,5 мг/л. Для укорінення найбільш ефективним виявилося середовище МС з половинною концентрацією макро- та мікросолей, доповнене 0,5 мг/л α-нафтилоцтовою кислотою (НОК), яке рекомендоване для ризогенезу рослин-регенерантів орхідеї *Vanda* різних сортів. Для адаптації рослин-регенерантів використано субстрат з сфангового моху, торфу, подрібнених листків бука та соснової кори у співвідношенні 1:1:1:1. Приживаність рослин орхідеї до умов *in vivo* сорту 'Vanda coerulea' становила 87,5 %, тоді як за однакових умов у рослин сорту 'Vanda sanderiana' – 83 % відповідно. Отримані результати досліджень можуть бути основою для створення протоколів розмноження орхідеї *Vanda*, що забезпечить високий рівень контролю на всіх етапах вирощування. Впровадження таких протоколів є важливими для селекційної роботи, збереження рідкісних сортів та поліпшення якості рослинного матеріалу для промислового квітникарства

Ключові слова: *in vitro*; калюсогенез; морфогенез; різогенез; адаптація *in vivo*