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Research Article

Optimizing micropropagation of Indonesian conserved orchid *Vanda celebica* using organic compounds and a temporary immersion system

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Abstract

Vanda celebica is an orchid species endemic and protected in North Sulawesi, Indonesia. To overcome such difficulties, micropropagation methods have been widely used, incorporating modified media and culture systems to improve success rates. Therefore, this study aimed to evaluate the viability of dry seeds stored at -20 °C for five years and the micropropagation of their protocorms using organic compounds, enhanced by a temporary immersion system (TIS) bioreactor. The genetic similarity between regenerants and the parental plant was also assessed using inter-sequence simple repeats (ISSR) markers to support conservation efforts. The results showed that *V. celebica* seeds stored for five years retained viability, with germination rates ranging from 56.16% to 73.45%. The addition of 40 g L^{-1} potato puree to the germination media significantly enhanced protocorm development. For further proliferation, ½-MS media supplemented with 150 ml L^{-1} coconut water and 50 ml L^{-1} carrot juice was found to be the most effective, resulting in a protocorm-like body (PLB) induction rate of 2.22 per explant, with an initiation time of 14.43 weeks, a 96.54% survival rate, and 30.56% of explants successfully induced. Dividing stage-4 protocorms yielded the best response, producing an average of 4.54 induced explants with an initiation time of 8.18 weeks. The TIS bioreactor system showed optimal results for shoot regeneration, achieving 64.84% and 37.07% regeneration rates. Genetic analysis indicated a similarity coefficient of 0.74-0.89 between regenerants and the parental plant, suggesting a genetic diversity comparable to *Vanda* species in their natural habitat.

Keywords: conservation (SDG 15); germination; *in vitro* culture; ISSR; protocorm-like body; seed storage

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Introduction

Vanda celebica Rolfe is an orchid species endemic to North Sulawesi, Indonesia. The orchid species is characterized by its reddish-brown crown with greenish-yellow petals. As an epiphytic plant, it thrives at an altitude of 500-600 meters above sea level (Motes *et al*., 2015). Due to its limited population, *V. celebica* is protected under Indonesian law through the KLHK Ministerial Regulation No. 106 of 2018, which amended PP No. 7 of 1999 on Protected Plant and Animal Species (RMERFI, 2018). The species' scarcity is largely attributed to direct collection from the natural habitat for commercial and personal use, coupled with limited propagation capacity due to its monopodial branching type (Widyastoety and Santi, 2012). Efforts to conserve *V. celebica* have been undertaken by the Bogor Botanical Garden study team. These include herbarium creation and the collection of *V. celebica* plants from nature. Seeds obtained through artificial pollination of collected plants were dried and stored at -20 °C. Their germination was periodically assessed, while *in vitro* shoot cultures were also maintained. However, as presented in the case of *Phalaenopsis amabilis*, seeds germination tends to decline with extended storage durations (Puspitaningtyas and Handini, 2021). To date, no evaluation has been conducted on the germination of *V. celebica* seeds stored for five years, categorized as medium-term storage. This assessment is essential to determine the optimal storage duration and methods, ensuring effective conservation of the species. Despite ongoing seed storage and *in vitro* culture efforts, propagation, and conservation methods have not been optimized to produce sufficient quantities of *V. celebica* seedlings. Addressing this issue can help reduce the need for natural seedling collection, thereby alleviating pressure on wild populations.

Micropropagation presents a viable solution to overcome the limitations of natural propagation. This *in vitro* method, which often uses embryogenesis, is widely adopted in orchid cultivation. Specifically, the development of somatic embryos known as a protocorm-like body (PLB) has been enhanced through media modifications enriched with plant growth regulators (PGRs) and organic compounds (Nugroho et al., 2024). Organic compounds are particularly favored by orchid farmers engaged in small-scale businesses, as they help reduce production costs (Hapsoro *et al*., 2018). Although the use of organic compounds has been shown to enhance orchid germination and growth, their optimal type and concentration for *V. celebica* remain unexplored. Previous investigations on other orchids, such as *Vanda* 'Kasem's Delight', have shown that adding 150 mL L^{-1} coconut water (CW) and 20% tomato extract promotes PLB proliferation (Gnasekaran *et al*., 2011). Similarly, the combination of 2 g L^{-1} yeast extract and 100 mL L^{-1} CW has been effective in the proliferation and development of *Dimorhorcis rosii* (Azlan and Jawan, 2015).

A bioreactor is a valuable tool for scaling up orchid micropropagation, enabling efficient propagation of various species. The bioreactor system designed for large-scale culture has been successfully applied to the proliferation of embryonic callus in *Paphiopedilum rothschildianum* and the shoot multiplication of *Epipactis flava* using a temporary immersion system (TIS) (Kunakhonnuruk *et al*., 2019). Among these, the TIS bioreactor is particularly advantageous as it closely mimics natural propagation conditions. By preventing continuous immersion of cultures in media, TIS reduces the adverse effects of prolonged exposure, which can negatively impact plant morphogenesis (Watt, 2012). The application of TIS in the micropropagation of *Vanda* species has shown promising results. For instance, several investigations on *Vanda tricolor* using shoot explants cultured on ½ MS media supplemented with 15 ml L^{-1} CW showed superior growth when the cultures were immersed for five minutes every 12 hours, compared to cultures grown in liquid media with shaker agitation (Nurillah *et al*., 2014). However, these results remain suboptimal and require further refinement. No investigations have been reported on the use of TIS in the micropropagation of *V. celebica*. Therefore, optimizing the micropropagation of *V. celebica* through TIS, particularly with the incorporation of organic compounds, is essential to support conservation and ensure sustainable utilization.

Micropropagation often leads to concerns about genetic changes in regenerants, making the evaluation of genetic stability crucial for conservation efforts. DNA analysis using molecular markers, such as inter-sequence simple repeats (ISSR), provides a reliable method for assessing genetic stability. For instance, the application of five ISSR primers to micropropagated regenerants of *Vanda cristata* showed a high level of genetic similarity to the parent plant, confirming the stability of the propagated plants (Pathak *et al*., 2022). Therefore, the study aims to evaluate the germination of *Vanda celebica* seeds stored for five years, identify the optimal media for seed germination, determine the most effective media and explant types for propagation through PLB induction, identify the most suitable TIS bioreactor conditions for shoot regeneration, and assess the genetic stability of micropropagated regenerants, with the intention of providing *Vanda celebica* seedlings to support conservation (SDG 15).

Materials and Methods

Material source

Vanda celebica seeds were sourced from the Bogor Botanical Garden collection in Bogor, West Java, Indonesia. These seeds, stored for five years (Figure 3B), originated from mature capsules produced through the artificial pollination of plants at the Bogor Botanical Garden (Figure 3A). Before storage, the seeds were air-dried in a desiccator for 5–7 days and then stored in small tubes at -20 °C to maintain their viability.

Seed germination with different supplementation of organic compounds on media

The seeds were surface sterilized through a step-by-step process to ensure a contaminant-free environment. Initially, they were immersed in 2.5 mL of sterilized distilled water containing two drops of Tween 20, followed by agitation for 10 seconds before being vacuumed for one hour. Subsequent steps were carried out inside a Laminar Air Flow Cabinet (LAFC). After removing the initial solution, the seeds were treated with 10% NaClO for 10 minutes, followed by 5% NaClO for five minutes. Between treatments, seeds were rinsed three times with sterilized distilled water. During the final rinse, approximately 0.7 mL of seeds were sprayed onto germination media using a pipette. Excess water on the media surface was carefully removed with a pipette, ensuring even seed distribution (Puspitaningtyas and Handini, 2014).

The seeds were cultivated in 90 × 20 mm Petri dishes containing 25 mL of modified Knudson C (1946) medium. This basal medium, known as KCA, included 100 mL L^{-1} CW and 1 g L^{-1} activated charcoal. Various organic compounds and plant growth regulator (PGR) treatments were added to optimize germination. They included P = KCA added with 40 g L^{-1} potato puree, PY = KCA supplemented with 40 g L^{-1} potato puree and 0.2 g L^{-1} yeast extract, BS = KCA added with 40 ml L^{-1} bean sprout extract, BSY = KCA with 40 ml L^{-1} bean sprout extract and 0.2 g L^{-1} yeast extract, T = KCA supplemented with 40 ml L^{-1} tomato juice, TY = KCA added with 40 ml L^{-1} tomato juice and 0.2 g L^{-1} yeast extract, NB = KCA supplemented with 0.1 g L^{-1} NAA and 3 g L^{-1} BAP, NBY = KCA added with 0.1 g L^{-1} NAA, 3 g L^{-1} BAP, and 0.2 g L^{-1} yeast extract, Ye = KCA with 0.2 g L^{-1} yeast extract, as well as O = KCA as the control.

The potato puree was prepared through the grinding of potatoes using a blender. The tomato and carrot were blended and filtered to obtain their juices. The bean sprout extract was prepared by boiling 150 g bean sprouts in 1000 ml water until the volume was reduced to 600 ml. The yeast extract was sourced from a commercial laboratory material. All media were further enriched with 20 g L^{-1} sucrose and 5 mL L^{-1} vitamin stock (niacin, pyridoxine, thiamine, and glycine) and solidified with 0.38% gellan gum. Each of the 10 treatments was replicated in three experimental units, with approximately 100–150 observational units per experimental group.

Germination and protocorm development were monitored every four weeks over a period of 16 weeks using a stereo microscope (Nikon SMZ1270). Observations were processed with Imaging NIS-ELEMENT D

software (Nikon Instruments). The developmental stages of germination and protocorms were assessed based on Wu *et al*., (2014), as follows, stage 0: ungerminated seeds with an embryo, stage 1: enlarging embryo and ruptured testa (germination), stage 2: the appearance of shoot and/or rhizoids, stage 3: emergence and elongation of first leaf, stage 4: the presence of one leaf and one or more roots, and stage 5: the presence of two or more leaves and roots. The germination percentage was calculated by dividing the total number of germinated seeds (stages 1-5) by the total number of seeds sown in each petri dish.

PLB induction with different explants, concentration of basal media, and organic compounds

Protocorms from *in vitro* germination were used as explants for the PLB induction experiments. These protocorms were derived from periodically germinated seeds stored at the Bogor Botanical Garden, using Knudson C (KC) as germination media based on (Handini *et al*., 2016) and the same seed accession as the germination experiment. Explants consisted of whole protocorm stage 3 (W-3), whole protocorm stage 4 (W-4), and protocorm stage 4 divided into apical and basal (D). Approximately 12 explants were cultured in 90 × 20 mm petri dishes containing 25 mL of PLB induction media. The media were prepared based on Murashige and Skoog (1962) (MS), modified with 150 mL L^{-1} coconut water (CWF), and supplemented with various organic compounds such as 50 mL L^{-1} tomato juice, 50 mL L^{-1} carrot juice, or 50 mL L^{-1} bean sprout extract. Additionally, half-strength MS (with half-strength macro elements and full-strength microelements) was modified with 150 mL L^{-1} coconut water (CWH) and combined with the same organic compound treatments. All media were further enriched with 5 mL L^{-1} vitamin stock (niacin, pyridoxine, thiamine, and glycine), 30 g L^{-1} sucrose, and solidified with 0.38% gellan gum.

A total of eight treatment combinations were tested, each replicated into two experimental units, resulting in 24 observational units per treatment. The number of PLB formed was recorded every four weeks for 24 weeks using the same microscope and software described in the germination experiment. The average number of PLB per explant was determined by dividing the total number of PLB by the total number of explants for each treatment. Additionally, the percentage of explants producing PLB was calculated by dividing the number of induced explants by the total number of explants per treatment.

Shoot regeneration using temporary immersion system (TIS) bioreactor

PLB clumps with diameters of approximately 0.7-0.9 cm, obtained from PLB induction cultures, were used as explants. Six explants were planted into a sterilized RITA® TIS bioreactor containing 150 mL of regeneration media (Figure 3M). The regeneration media consisted of half-strength MS medium supplemented with 20 g L^{-1} sucrose, 5 mL L^{-1} vitamin stock (including niacin, pyridoxine, thiamine, and glycine), 30 g L^{-1} banana powder, 100 mL L^{-1} CW, 0.5 g L^{-1} activated charcoal, and 2 mL L^{-1} Plant Preservative Mixture (PPM). The TIS bioreactor was set to two immersion periods, including two minutes of immersion every two and four hours. As a comparison, single explants were cultured on solid media in 90 × 20 mm Petri dishes and in 100 mL Erlenmeyer flasks containing liquid media. Each culture unit contained 25 mL of regeneration media, with liquid cultures agitated on a shaker at 100 rpm. Explants in the TIS bioreactor and liquid cultures were subcultured into fresh media every four weeks. The experiment consisted of four treatments replicated across three experimental units, resulting in 18 observational units for the TIS treatments and three units each for solid and liquid cultures. Observations of PLB and shoot numbers were conducted after 16 weeks.

Culture condition

All experiments were performed under aseptic conditions in a LAFC. Cultures were incubated at 25 ± 3 °C with a light intensity of 3000 lux using white light. For germination experiments, cultures were maintained in complete darkness for the first eight weeks before transitioning to illuminated conditions.

Experimental design and statistical analysis

The experiments followed a completely randomized design, and a statistical analysis was performed using SPSS 27. Significant differences in seed germination and development treatments were determined using analysis of variance (ANOVA) with Duncan's multiple range test (DMRT). For PLB induction, variables were analyzed using the Kruskal-Wallis test, and significant differences were further evaluated with the Mann-Whitney post-hoc test. The PLB induction rate was analyzed using Pearson's chi-squared test to compare treatment effects. Variables related to shoot regeneration were analyzed similarly to those in the germination experiments, and all statistical analysis was conducted at a 95% confidence level.

Genetic stability analysis of shoots and regenerants derived from micropropagation

To assess the genetic stability of shoots and regenerants, leaf samples from 19 regenerant plantlets and the parent plant (sourced from the Bogor Botanical Garden) were collected. The leaves were dried overnight in a sealed container with silica gel and then weighed (approximately 20-50 mg) for DNA extraction. DNA was extracted using liquid nitrogen in combination with the Quick-DNA™ Plant/Seed Miniprep Kit (Zymo Research) and the Plant Genomic DNA Extraction Miniprep System (Viogene).

Extracted DNA was analyzed using ISSR markers with five primers, consisted of UBC-811 (G(Ag)7AC), UBC-818 (C(AC)7Ag), UBC-825 (A(CA)7CT), UBC-827 (A(CA)7Cg), and UBC-857 (A(CA)7Cyg). The PCR reaction mixture consisted of 3 µL master mix, 0.5 µL primer, 0.5 µL DNA, and 2 µL nuclease-free water, resulting in a total volume of 6 µL. The PCR process began with an initial denaturation at 94 °C for five minutes, followed by 35 cycles of denaturation at 94 °C for 45 seconds, annealing at 52-54 °C (depending on the primer) for 45 seconds, and extension at 72 °C for 45 seconds. A final extension was performed at 72 °C for 10 minutes.

Gel electrophoresis was conducted using a 1.5% agarose gel prepared in 1x TAE buffer. To each well, 2 µL loading dye, 100 bp DNA ladder, and 0.5 µL of amplified samples were added. Electrophoresis was carried out at 100 V for 70–80 minutes. The separated amplicons were visualized under UV light using a Gel Doc Imager (BioRad). Band scoring was performed with Gel Analyzer software. The resulting similarity matrix was subjected to Unweighted Pair-Group Method Using Arithmetic Average (UPGMA) cluster analysis to construct a dendrogram using NTSYS 2.1 (Numerical Taxonomy and Multivariate Analysis System).

Results

Effect of supplementation media with different organic compounds on seed germination

Following a 16-week observation period, the germination rates of *Vanda celebica* seeds stored for five years ranged from 56% to 73%, as detailed in Figure 1. The early stage of germination was observed after 4 weeks of culture (Figure 3C). There was no significant variation in germination rates across different media types. However, lower rates were observed on media supplemented with potato puree combined with yeast extract, bean sprout extract, and media added with NAA, BAP, and yeast extract.

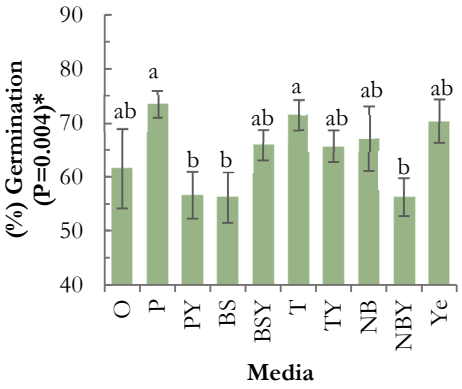

Figure 1. Total germination (stages 1-5) of *Vanda celebica* seeds stored for five years on basic media Knudson C (KC) with different organic compounds after 16 weeks of culture
Mean values (n=3) with standard error are shown in each bar.
The bars with the same letters are not significantly different ($p<0.05$) according to Duncan's Test
Abbreviations: O = KCA; P = KCA added with 40 g L^{-1} potato puree; PY = KCA added with 40 g L^{-1} potato puree and 0.2 g L^{-1} yeast extract; BS = KCA added with 40 mL L^{-1} extract bean sprout; BSY = KCA added with 40 mL L^{-1} extract bean sprout and 0.2 g L^{-1} yeast extract; T = KCA added with 40 mL L^{-1} tomato juice; TY = KCA added with 40 mL L^{-1} tomato juice and 0.2 g L^{-1} yeast extract; NB = KCA added with 0.1 g L^{-1} NAA and 3 g L^{-1} BAP; NBY = KCA added with 0.1 g L^{-1} NAA, 3 g L^{-1} BAP, and 0.2 g L^{-1} yeast extract; Ye = KCA added with 0.2 g L^{-1} yeast extract

Seeds germinated on media supplemented with potato puree progressed to stages 4 and 5, showing seedling development of 9% and 3%, respectively (Figures 2 and 3E). The addition of potato puree combined with yeast extract and tomato juice resulted in seedling development, but only reached stage 4, with a significantly lower success rate of 0.3% and 0.003%, respectively. The development of seeds on media with yeast extract and bean sprout extract was observed to occur at a slow rate, reaching stage 2 after 16 weeks. Additionally, the seeds on the media supplemented with yeast extract exhibited browning at stage 1 (Figure 3D).

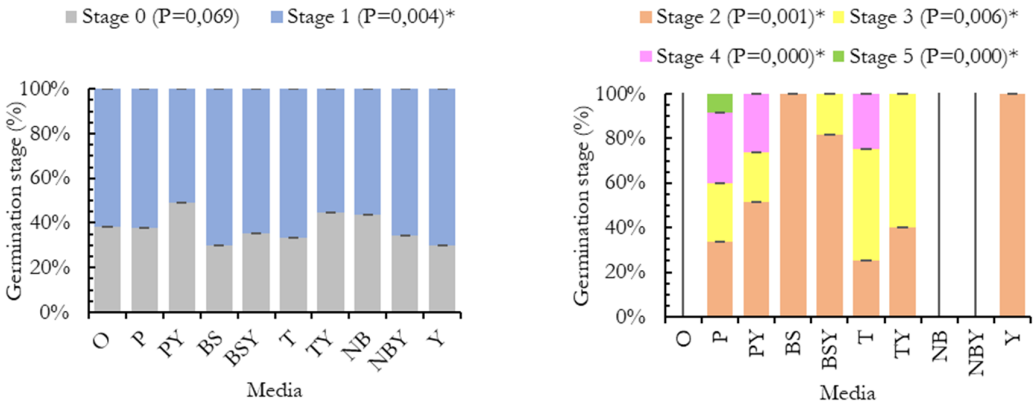

Figure 2. Seedling development (stage 0-5) of five years stored seeds of *Vanda celebica* on basic media KC with various organic additives after 16 weeks of culture
Mean values (n=3) with standard error are shown in each bar. Abbreviations: O = KCA; P = KCA added with 40 g L^{-1} potato puree; PY = KCA added with 40 g L^{-1} potato puree and 0.2 g L^{-1} yeast extract; BS = KCA added with 40 mL L^{-1} extract bean sprout; BSY = KCA added with 40 mL L^{-1} extract bean sprout and 0.2 g L^{-1} yeast extract; T = KCA added with 40 mL L^{-1} tomato juice; TY = KCA added with 40 mL L^{-1} tomato juice and 0.2 g L^{-1} yeast extract; NB = KCA added with 0.1 g L^{-1} NAA and 3 g L^{-1} BAP; NBY = KCA added with 0.1 g L^{-1} NAA, 3 g L^{-1} BAP, and 0.2 g L^{-1} yeast extract; Ye = KCA added with 0.2 g L^{-1} yeast extract

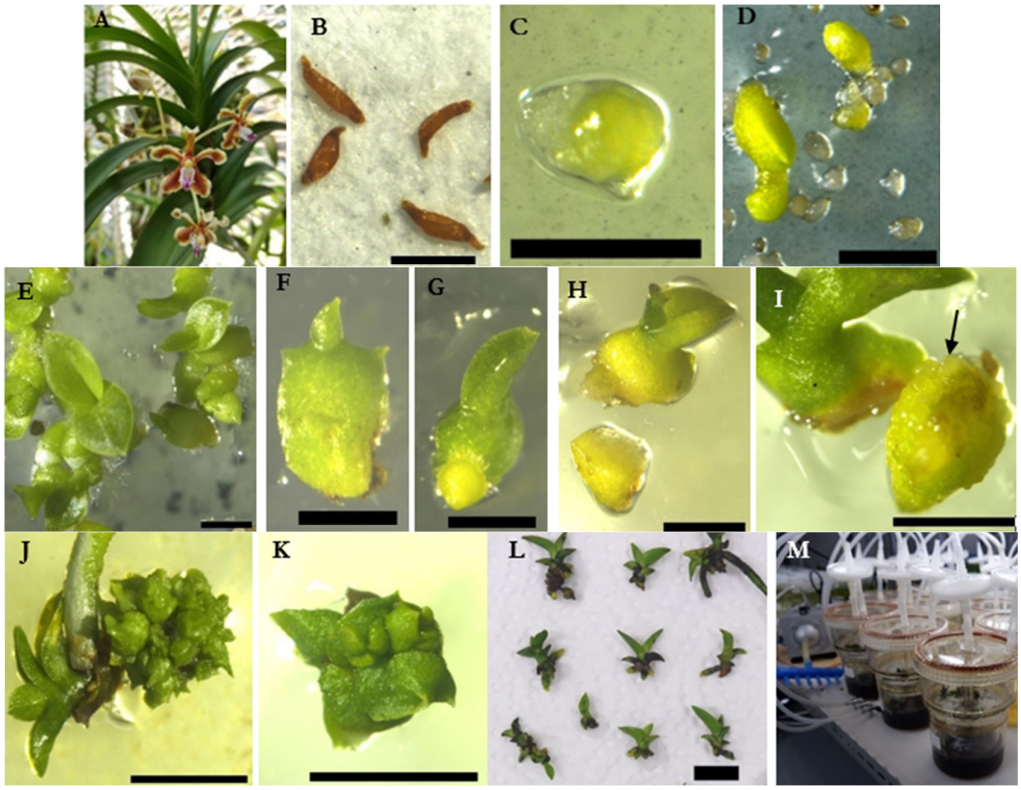

Figure 3. Micropropagation protocol of *V. celebica* from germination of stored seeds to regeneration of PLB using TIS; (A) Inflorescence of *V. celebica*; (B) *V. celebica* seeds stored for five years; (C) Germination of seeds on media KCA with potato puree after four weeks; (D) Seeds germinated on media KC with yeast extract exhibited browning after 16 weeks; (E) Stage 4 and 5 of germination on media KC with 40 g L^{-1} potato puree after 16 weeks; (F) Initial stage of PLB induction using whole protocorm stage 3 and; (G) stage 4, as well as; (H) Divided protocorm; (I) Globular stage initiation of divided protocorm on media ½ MS with 40 mL L^{-1} tomato juice after six weeks and; (J) PLB regeneration after 16 weeks; (K) PLB induction of whole protocorm stage 3 on media MS with 40 mL L^{-1} tomato juice after 16 weeks; (L) Shoot regeneration after 16 weeks; (M) PLB regeneration in TIS bioreactor
Scale bar: B = 300 µm; C = 1000 µm D-H = 2000 µm; I-K = 5000 µm; L = 2 cm

Effect of different explants, concentration of basal media, and organic compounds on PLB induction

The effect of media on observed variables was assessed at 24 weeks of culture, as outlined in Table 1. The addition of organic compounds to ½-MS media resulted in significantly higher PLB induction compared to full-MS media. Media containing CW and other organic compounds had a higher survival rate of PLB compared to media without these additions. Specifically, the combination of CW and carrot juice in ½-MS media led to the highest explant induction rate of 30.56%. A similar basal media treatment with the addition of CW and tomato juice resulted in a 25% explant induction rate, while media with only CW resulted in a 23.6% induction rate.

Table 1. PLB induction competence on induction media with different concentrations of basic media MS and organic additives after 24 weeks of culture

Media	Number of PLB per explant	PLB initiation times (weeks)	PLB survival rate (%)	Explant produced PLB (%)
CWF	0.94 ± 0.47[b]	14.20 ± 2.69	45.38 ± 22.83[b]	6.94
CWF+Tomato juice	0.58 ± 0.29[b]	14.80 ± 2.22	100.00 ± 0.00[ab]	6.94
CWF+Carrot juice	0.56 ± 0.24[b]	13.13 ± 1.90	100.00 ± 0.00[a]	11.11
CWF+Bean Sprout extract	0.19 ± 0.12[b]	23.00 ± 0.00	100.00 ± 0.00[ab]	4.17
CWH	3.99 ± 1.11[a]	14.35 ± 1.33	79.98 ± 8.51[b]	23.61
CWH+Tomato juice	2.71 ± 0.67[a]	12.33 ± 1.37	87.09 ± 6.19[ab]	25.00
CWH+Carrot juice	2.22 ± 0.53[a]	14.43 ± 1.68	96.54 ± 2.51[a]	30.56
CWH+Bean Sprout extract	2.08 ± 0.65[ab]	15.77 ± 1.73	92.31 ± 7.69[ab]	18.06
P-value	0,000*	0.434	0.014*	<0.001**

Data are means (n=24) with standard error. *significantly different according to Kruskal Wallis's test (p<0.05). **significantly different according to the Chi-square test (p<0.05). Data with the same letters are not significantly different according to Mann-Whitney's test (p<0.05). Abbreviations: CWF; MS full with 150 ml L^{-1} CW; CWH = ½ MS with 150 ml L^{-1} CW

The number of PLB per explant, initiation time, and the percentage of explants that produced PLB were significantly influenced by the explant type (Table 2). The highest number of PLB per explant was observed in whole protocorm stage 3 (Figure 3F, K) and divided protocorm stage 4 (Figures 3H, I, J), with values of 1.54 and 4.54, respectively. Divided protocorm stage 4 and whole protocorm stage 4 showed the shortest PLB initiation time, occuring at 8.32 and 16.50 weeks, respectively. The lowest number of PLB per explant (0.58) was recorded for the whole protocorm stage 4.

Table 2. PLB induction competence on induction media with different explants after 24 weeks of culture

Explant	Number of PLB per explant*	PLB initiation times (weeks)*	PLB survival rate (%)	Explant produced PLB (%)
W-3	1.54 ± 0.53[ab]	22.50 ± 0.86[b]	100.00 ± 0.00	37.50
W-4	0.58 ± 0.47[b]	16.50 ± 0.79[ab]	100.00 ± 0.00	8.33
D	4.54 ± 0.53[a]	8.18 ± 0.66[a]	93.40 ± 4.68	45.83
P-value	0.011*	<0.001*	0.385	0.012**

Data are means (n=24) with standard error. *Significantly different according to Kruskal Wallis's test (p<0.05). **significantly different according to the Chi-square test (p<0.05). Data with the same letters are not significantly different according to Mann-Whitney's test (p<0.05). Abbreviations: W-3 = whole protocorm stage 3; W-4 = whole protocorm stage 4; D = divided protocorm stage 4

Effect of temporary immersion system (TIS) bioreactor on shoot regeneration

The shoot regeneration rate was 64.84% when PLB was immersed in media for two minutes every four hours (Figure 3L). This rate was not significantly different from that observed when PLB was immersed every two hours (Table 3). The lower regeneration rate (24.57%) occurred when PLB was cultured in solid media, while no shoot regeneration was observed in liquid culture. The survival rate of PLB and shoots in solid media was 86.81%, which was not significantly different from the results observed in the TIS-2 and solid culture.

Genetic stability of shoots and regenerants derived from micropropagation

The dendrogram reconstruction showed genetic similarity coefficients between 0.74 and 0.89 for shoots and regenerants obtained from micropropagation, compared with the parent plant (Figure 4). The dendrogram was divided into two groups, with the intersection occurring at a similarity coefficient of 0.76. Group 1 had a higher genetic similarity coefficient (0.80-0.86) with the parent plant, predominantly consisting of shoots

obtained from seed germination. Meanwhile, group 2 showed a slightly lower range (0.77–0.86) and was dominated by regenerants derived from PLB.

Table 3. Shoot regeneration and survival rate in different culture systems after 16 weeks of culture

Culture system	Shoot regeneration of PLB explant (%)	PLB explant and shoot survival rate (%)
TIS-2	37.07 ± 0.16^{ab}	55.13 ± 0.16^{a}
TIS-4	64.84 ± 0.19^{a}	74.26 ± 0.19^{a}
Solid	24.57 ± 0.10^{bc}	86.81 ± 0.10^{a}
Liquid	0.00 ± 0.04^{c}	4.44 ± 0.04^{b}
P-value	0,018*	0,012*

Data are means (n=3) with standard error. Data with same letters are not significantly different. Abbreviations: TIS-2 = TIS with 2 min immersion every 2 h; TIS-4 = TIS with 2 min immersion every 4 h

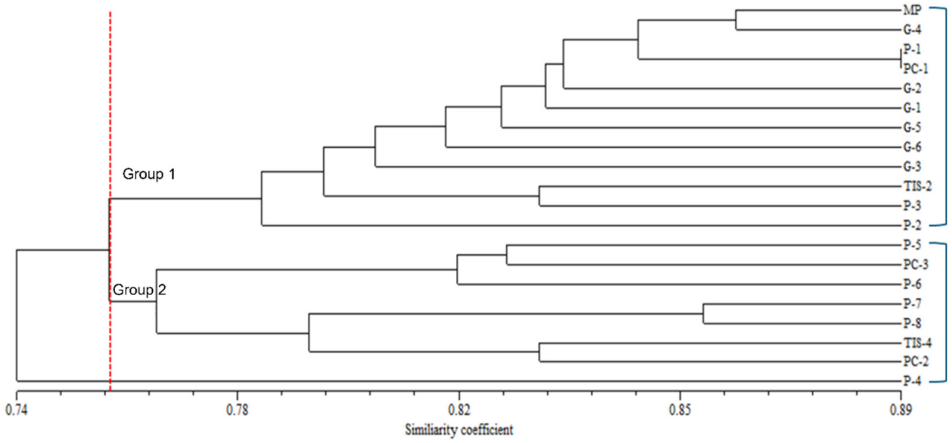

Figure 4. Dendrogram of shoots from each experimental stage compared with parent plant similarity analysis using five ISSR markers

The number following letters indicate repetition, except TIS. Abbreviations: MP = mother plant (parent plant); G = shoots from germination; P= regenerants from PLB induction; PC = regenerants from PLB induction through callus; TIS-2: regenerants from PLB in TIS with 2 min immersion every 2 h; TIS-4 = regenerants from PLB in TIS with 2 min immersion every 4 h

Discussion

Supplementation media with different organic compounds affected seed germination

Germination of *Vanda celebica* seeds showed a significant decline by approximately 50% after being stored for five years at a temperature of -20 °C. This decline was consistent with observations made for *Phalaenopsis amabilis* seeds, which also had a similar reduction in germination after being stored for four years under identical conditions (Puspitaningtyas and Handini, 2021). In contrast, *Cymbidium finlaysonianum* seeds showed a 50% decline in germination after being stored for two years under the same conditions (Puspitaningtyas and Handini, 2020). It was crucial to consider the effects of temperature and relative humidity on seeds storage (Magrini *et al*., 2019). An optimal combination of seeds moisture and a consistently low temperature was essential for maintaining seeds viability over time. The observed reduction in seeds germination during storage was attributed to the gradual aging process of seeds, which was slowed by low temperature and humidity (Ranganathan and Groot, 2023).

The addition of potato puree and tomato juice to the germination media supported seeds development into seedlings. Potatoes were rich in essential vitamins and minerals, particularly vitamins C, B1, B6, and minerals such as K, Fe, and Mg. They also contained amino acids comprising lysine and arginine (Bártová and Bárta, 2009). The optimal root length and quantity in *Vanda roxburgii* were achieved with the addition of 100 mL L^{-1} potato extract (Islam *et al*., 2011). Similar results were observed in *Phalaenopsis* sp., where 150 g L^{-1} potato extract promoted seedling development (Ambarwati et al., 2021). Lycopene and vitamin C in tomatoes function as antioxidants that can mitigate the effects of browning in plant cultures. The addition of tomato juice at a concentration of 100-200 g L^{-1} to *Vanda tricolor* Lindl. var. suavis has been demonstrated to inhibit the oxidation of phenolic compounds secreted from the basal part of the protocorm, thereby supporting the growth and development of the protocorm (Dwiyani *et al*., 2015).

In contrast, the addition of yeast extract to germination media resulted in slow rate of seeds development into seedlings at stage 2, accompanied by browning at stage 1. This is different from what Jawan *et al*. (2010) found, where 2 mg L^{-1} yeast extract made shoots form and grow in *Vanda dearei*. Similarly, David *et al*. (2015) observed that adding 3 mg L^{-1} yeast extract increased germination within two weeks in *Vanda helvola*, even though the subsequent development of sprouts into shoots was not achieved.

Different explants, concentrations of basal media, and organic compounds affected PLB induction

The reduction of macronutrient concentration and the inclusion of organic compounds in induction media led to a higher PLB response compared to media with the full macronutrient concentration. Changing macronutrients and micronutrients in media was often done to make explants grow and develop better. For *Vanda stangeana*, PLB was made in six weeks using ½ MS media with 0.5 mg L^{-1} NAA and 0.05 mg L^{-1} kinetin (Pebam *et al*., 2016). However, for *Vanda tricolor var. pallida*, it took eight weeks to get PLB from embryogenic callus using basal media with 0.05 mg L^{-1} NAA and 0.01 mg L^{-1} BAP (Hardjo and Savitri, 2017). Full MS media with 1.5 mg L^{-1} BAP worked faster for *Vanda sumatrana*, forming PLB after four weeks (Aini *et al*., 2015). In *Vanda bicolor*, PLB also appeared after six weeks when using 0.6 mg L^{-1} NAA and 0.7 mg L^{-1} BA in the same kind of basal media (Deb *et al*., 2018).

Adding CW and other organic compounds to the media gave better results for surviving PLB than just using CW. Media with 100 mL L^{-1} tomato juice helped reduce browning and improved orchid growth for *Vanda helvola Blume* (David *et al*., 2015). Tomatoes had many nutrients like carbohydrates, vitamins, and minerals. They also had lycopene, an antioxidant, which is good for preventing cell damage (Toor *et al*., 2005). Additionally, lycopene could act as an initiator of embryo development (Setiari *et al*., 2017). Somatic embryo regeneration in *Phalaenopsis amabilis* showed optimal growth in media supplemented with 150 g L^{-1} tomato extract, outperforming other organic compounds (Mose *et al*., 2020). Carrots shared similar properties with tomatoes, though they were higher in vitamin A and riboflavin, while tomatoes contained more vitamin C (Farida and Yenisbar, 2021). In *Phalaenopsis* hybrid 'Pink', the best growth in terms of plant height and leaf length was observed with the addition of 100 mL L^{-1} carrot juice to the culture media (Zahara *et al*., 2016). Supplementing culture media with 150 g L^{-1} of bean sprout extract also promoted the growth of *Phalaenopsis fuscata* (Rahayu *et al*., 2011). Similarly, media supplemented with 150 g L^{-1} of bean sprout extract and 5 mg L^{-1} thiamine produced the best plant growth in *Phalaenopsis* sp. (Zahra *et al*., 2023). Bean sprouts were rich in essential plant vitamins and minerals, such as riboflavin, thiamine, phosphorus, sodium, and manganese, as well as natural auxins and cytokinins, which influenced cell growth and development (Rosdeen *et al*., 2023).

Whole stage 3 protocorm explants and divided stage 4 protocorm explants showed more PLB induction than whole stage 4 protocorm explants. The earlier stage of protocorm explants demonstrated a greater responsiveness to embryogenesis, which can be attributed to the higher proportion of meristematic cells that had not yet differentiated into leaf and root organs, as observed in the later stage of protocorm explants (Figure 3G). A comparable outcome was achieved in young protocorm explants of *Dactylorhiza umbrosa*, which yielded

a greater quantity of PLB (5.37) than old protocorm explants and crown explants (Fatahi *et a*l., 2023). Stage 4 protocorm explants, both whole and divided, showed more rapid initiation times.

Roots in stage 4 whole explants made media absorption better, while stage 3 only had rhizoids, which did not absorb media as efficiently (Jones and Dolan, 2012; Gao *et al*., 2023). Divided stage 4 protocorms with wounds had faster initiation times. Wounding helped cells regenerate because it triggered totipotency and shoot development (Iwase *et al*., 2011, 2017). A similar result was observed in the basal leaf sections of *Vanda tricolor var. pallida*, which was initiated after eight weeks of culture (Hardjo and Savitri, 2017), as well as in the apical, middle, and basal protocorm sections of *Phalaenopsis* sp., initiated after six weeks (Soe *et al*., 2014).

Temporary immersion system (TIS) bioreactor affected shoot regeneration

TIS bioreactor has been observed to yield the most optimal results in terms of shoot regeneration when compared to solid and agitated liquid systems. TIS was used for plant growth and produced better shoots. It stopped vitrification because the explants were not always in the media. Air exchange in TIS improved photosynthesis (Aragón *et al*., 2014). For *Dendrobium nobile*, shoots grown in TIS with five minutes of immersion every six hours got higher fresh weight and more shoots than solid media (Zhang *et al*., 2022). Also, *Cattleya walkeriana* did better with three minutes of immersion every 1.5 hours (Moreira *et al*., 2013). *Paphiopedilum rothschildianum callus* became PLB with five-minute immersions every 125 minutes (Masnoddin *et al*., 2018), and *Cattleya forbesii* PLB became shoots with one-minute immersions every four hours (Ekmekçigil *et al*., 2019).

Genetic stability of shoots and regenerants derived from micropropagation

Shoots and regenerants obtained through micropropagation in this study were derived from protocorm explants, which were germinated from seeds collected from artificial crosses of *Vanda* celebica plants from the wild. Genetic stability analysis showed that such shoots and micropropagated regenerants had genetic diversity, with a similarity coefficient ranging from 0.74 to 0.89. These findings are comparable to the genetic diversity of orchids observed in natural populations. Most orchid species in nature reproduced through cross-pollination, which promoted genetic diversity, essential for their survival (Ackerman *et al*., 2023). The genetic diversity of *Vanda* species in the wild, using similar analytical methods, have been reported by (Rindyastuti *et al*., 2015) for *Vanda coerula*, with a similarity coefficient of 0.67-0.96. Shoots obtained from seed germination showed higher genetic similarity to the parent plant, while regenerants derived from PLB induction and shoot regeneration using TIS indicated lower genetic similarity. The longer the propagation stages, the greater the likelihood of genetic changes from the parent plant. Genetic analysis of *Phalaenopsis gigantea* showed a decrease in genetic similarity from 1.00 during initial PLB induction to 0.95 after 16 weeks of subculture, and further to 0.80 after 20 weeks (Samarfard *et al*., 2014). Similarly, in *Dendrobium fimbriatum*, the genetic similarity declined slightly from 1.000 to 0.977, observed between shoots germinated from seeds and those from vegetative multiplication (Tikendra *et al*., 2021).

Conclusions

In conclusion, micropropagation of *Vanda celebica* was conducted with varying concentrations of basal media, explants, organic compounds, culture systems, and an analysis of the genetic stability of the regenerants. Seeds germination, from those stored for five years, ranged between 56% and 73%. The germination rate and the subsequent development of the protocorm into shoots were influenced by the presence of organic compounds. The addition of 40 g L^{-1} potato puree to the germination media resulted in optimal protocorm development. Among the variables tested, media containing ½-MS supplemented with 150 mL L^{-1} CW and 50

mL L^{-1} carrot were the most promising for further use in proliferation, with 2.22 PLB induced per explant, 14.43 weeks required for initiation, a 96.54% survival rate, and a 30.56% explant induction rate. Dividing protocorm stage 4 a considerably optimal outcome, with PLB induction occurring 8.18 weeks after culture, yielding 4.54 PLB per induced explant. The TIS bioreactor system yielded the most optimal results in terms of shoot regeneration, with a shoot regeneration percentage of 64.84% with an immersion time of two minutes every four hours and 37.07% with an immersion time of two minutes every two hours. Moreover, the genetic analysis of shoots and regenerants showed genetic diversity, with similarity coefficients ranging from 0.74 to 0.89. The results demonstrate that the genetic diversity observed within regenerants was comparable to that observed in natural populations, which provides support for conservation of *Vanda celebica*.

Authors' Contributions

Conceptualization: ANL, DRW, MA, DMP; Formal analysis: ANL, SRA; Funding acquisition: DRW; Investigation: ANL, DRW; Methodology: ANL, MA, DRW, DMP, SRA; Project administration: BWH; Resources: DMP, SRA, BWH; Software: AFM; Supervision: MA, DRW, SRA; Validation: DRW, MA, AFM; Visualization: ANL, DRW, MA; Writing - original draft: ANL; Writing - review and editing: ANL, DRW, MA, AFM. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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