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Oryzalin-induced polyploidy in *Vanda limbata* (Blume): Phenotypic assessment

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ABSTRACT

Background: *Vanda limbata* is a natural orchid species found on Java Island, commonly known as *V. limbata* 'Jawa.' Enhancing plant vigor is essential to increase its potential as an ornamental plant, with one promising approach being induced polyploidy using chemical mutagens such as oryzalin.

Objective: This study aimed to evaluate the effect of oryzalin on inducing polyploidy in *V. limbata* cultured *in vitro*.

Method: Oryzalin was applied at concentrations of 0, 25, 50, 75, and 100 µM, with five replications for each treatment, resulting in 25 experimental units. Several morpho-physiological and anatomical traits were measured as indicators of polyploidy.

Results: The results demonstrated that oryzalin at 100 µM was the most effective concentration for inducing polyploidy in *V. limbata* cultured *in vitro*. This was particularly evident in traits such as reduced leaf length, increased leaf width, enhanced adventitious shoot formation, and enlarged stomatal width.

Conclusion: Oryzalin, when applied at appropriate concentrations, can be effectively used to induce polyploidy in *V. limbate*.

Keywords: in vitro culture, oryzalin, polyploidization, *Vanda limbata*

Introduction

The genus *Vanda* is highly sought after as an ornamental plant due to its significant economic value. Approximately 60 species of *Vanda* are widely distributed throughout Indonesia, with nearly half of them found on Java Island. One species endemic to Java is *Vanda limbata*, which is sometimes referred to as *V. limbata* 'Jawa' [1]. This species is an epiphytic orchid characterized by monopodial growth, strap-shaped leaves, the absence of pseudobulbs, and both aerial and attached root types. The flowers are relatively small, predominantly brown with reddish spots and a purple labellum, and emit a cinnamon-like fragrance [2]. The diverse flower colors and patterns of *V. limbata* make it a popular choice for use as either potted or cut ornamental flowers. Additionally, *V. limbata* exhibits high adaptability to arid conditions and thrives in lowland areas [3].

Genetic improvement of orchids can be achieved through conventional hybridization, but this method is often considered inefficient due to the lengthy process involved. Challenges such as low hybrid formation rates and high sterility in hybrids further complicate this approach [4]. An alternative strategy is mutagen-induced polyploidy, which uses chemical mutagens to generate plant mutants. These mutants can then be selectively cultivated to produce plants with desirable traits [5].

Oryzalin, a chemical mutagen, is commonly used to induce polyploidy. It interferes with mitotic cell division by inhibiting spindle fiber formation, preventing the separation of chromosomes and leading to an increase in chromosome numbers [6]. Polyploid plants often exhibit superior morphological and anatomical traits compared to their diploid counterparts [7].

Inducing polyploidy through chemical mutagens can be performed using *in vitro* culture techniques, which require aseptic conditions and can utilize various plant tissues as explants, such as seeds, shoots, leaves, stems, or roots. Compared to conventional breeding methods, *in vitro* techniques offer a higher likelihood of success in producing high-quality polyploid plants within a shorter time frame. However, the success of this approach largely depends on the plant's ability to regulate molecular, cellular, and physiological processes during regeneration [8].

This study aims to investigate the effects of oryzalin application in inducing polyploidy in *V. limbata* cultured *in vitro*. The assessment focuses on phenotypic changes in morphological, physiological, and anatomical characteristics. To date, no studies have been reported on the genetic improvement of this natural orchid species through mutation breeding. Therefore, this research will provide valuable insights for orchid cultivators, supporting the development of *V. limbata* as an ornamental plant with high economic potential.

Methods

Study location and design

The study was conducted in the Laboratory of Plant Physiology, Faculty of Biology, Universitas Jenderal Soedirman, from December 2021 to May 2022. A Completely Randomized Design (CRD) was employed, with five oryzalin concentrations (0, 25, 50, 75, and 100 µM) as treatments. Each treatment consisted of five replications, resulting in a total of 25 experimental units. Data were collected on morpho-physiological traits, including plant height, leaf number, leaf size, and the number of adventitious shoots, as well as on anatomical traits, such as stomatal number, stomatal size, and stomatal index. Environmental parameters of the *in vitro* culture, including temperature, humidity, and light intensity, were also recorded.

Plant material

Vanda limbata protocorms were used as the plant material. These were obtained through *in vitro* seed culture, where six-month-old seeds were aseptically cultured. Protocorms were produced after one month of incubation on solid Murashige and Skoog (MS) medium.

Preparation of oryzalin stock solution

A stock solution of oryzalin was prepared at a concentration of 1,000 µM in a total volume of 100 mL. This was achieved by dissolving 0.034 g of oryzalin powder in 2 mL of dimethyl sulfoxide (DMSO) and diluting with distilled water (aquadest) to a final volume of 100 mL. The solution was thoroughly homogenized and stored in a refrigerator. Working solutions of oryzalin for the treatments were prepared by diluting the stock solution to the respective concentrations (0, 25, 50, 75, and 100 µM).

Preparation of media and oryzalin application

Liquid MS medium was prepared in a total volume of 2,000 mL, containing 40 g of sucrose and 8.66 g of MS powder. The pH was adjusted to 5.83 using 1 N NaOH and 1 N HCl. The medium was then heated to boiling and sterilized in an autoclave at 0.15 MPa and 121°C for 15 minutes. Once cooled to approximately 60°C, the medium was divided into five flasks, with each flask containing 400 mL of liquid medium. Oryzalin of the respective concentrations was added to the flasks using a microsyringe, except for the control treatment, which received no oryzalin. For post-treatment protocorm adaptation, additional liquid MS media without oryzalin were also prepared. Each treatment flask contained 40 mL of liquid medium.

Solid MS medium was prepared in a total volume of 3,000 mL, containing 30 g of sucrose, 6.5 g of MS powder, and 12 g of agar. The pH was adjusted to 5.83 using 1 N NaOH and 1 N HCl, and the medium was sterilized using the same procedure as for the liquid medium. No oryzalin was added to the solid medium. Additional solid MS media containing growth regulators were prepared by enriching the medium with 5% coconut water, 3 mg L^{-1} benzylaminopurine (BAP), and 1 mg L^{-1}

Table 1. Morpho-physiological characters of *Vanda limbata* 'Jawa' plantlets under oryzalin treatments

Oryzalin concentration (μM)	Average plantlet height (cm)	Average leaf length (cm)	Average leaf width (cm)	Average number of adventitiuous shoots
0	28.71[b]	24.53[b]	4.77[b]	0.00[a]
25	19.76[ab]	15.32[a]	6.26[a]	0.00[a]
50	19.55[a]	15.11[a]	6.37[a]	1.20[ab]
75	18.57[a]	12.15[a]	6.39[a]	1.20[ab]
100	15.66[a]	11.00[a]	6.47[a]	1.40[b]

Numbers followed by the same letters reveal no significant difference at Tukey test level of 0.05.

kinetin. Solid media without growth regulators were used to acclimate the protocorms from liquid to solid conditions, while the growth regulator-enriched media were used to stimulate normal growth.

Immersion of protocorms into liquid media

Protocorms were immersed in liquid MS media containing oryzalin at the respective concentrations. The flasks were continuously agitated at 100 rpm using an orbital shaker for seven days. After the immersion period, the protocorms were washed three times with sterile distilled water and transferred into fresh liquid MS media without oryzalin. These media were also agitated at the same speed and duration to eliminate residual oryzalin and allow the protocorms to adapt before being transferred to solid media.

Protocorm subculture into growth media

Protocorms were first subcultured onto solid MS media without growth regulators for one month to adapt from liquid to solid media conditions. Following this adaptation period, the protocorms were transferred to solid MS media enriched with growth regulators (5% coconut water, 3 mg L^{-1} BAP, and 1 mg L^{-1} kinetin) to stimulate normal growth. Cultures were placed on racks under continuous illumination from tube luminescent (TL) lamps at 26°C for two months. After one month of growth, the resulting plantlets were transferred into fresh solid media. At the end of the experimental period, the morpho-physiological and anatomical traits of the plantlets were examined over the course of one week.

Data analysis

Quantitative data were analyzed using Analysis of Variance (ANOVA) at 95% and 99% confidence levels. When significant differences among treatments were detected, Tukey's test was performed as a post hoc analysis. Qualitative data, such as stomatal shape, were analyzed descriptively.

Results

Morpho-physiological characters

The results of ANOVA indicate that oryzalin has a significant effect on the morpho-physiological traits of *Vanda limbata* 'Jawa' plantlets. Specifically, higher concentrations of oryzalin result in shorter plantlets, as plantlet height decreases with increasing oryzalin concentrations. A similar trend is observed for leaf length, where higher oryzalin concentrations lead to shorter leaves. Conversely, oryzalin has the opposite effect on leaf width, which increases with higher concentrations. In addition, oryzalin significantly increases the number of adventitious shoots (Table 1).

Morphological changes indicative of polyploidization are evident at an oryzalin concentration of 25 μM. Compared to the control, plantlets treated with 25 μM oryzalin are significantly shorter in height (Figure 1). Additionally, control plantlets exhibit longer leaves and roots, while plantlets treated with oryzalin show more rounded leaves and smaller roots.

At the highest concentration tested (100 μM), oryzalin significantly reduces leaf length, with treated plantlets showing much shorter and wider leaves compared to the control plantlets, which

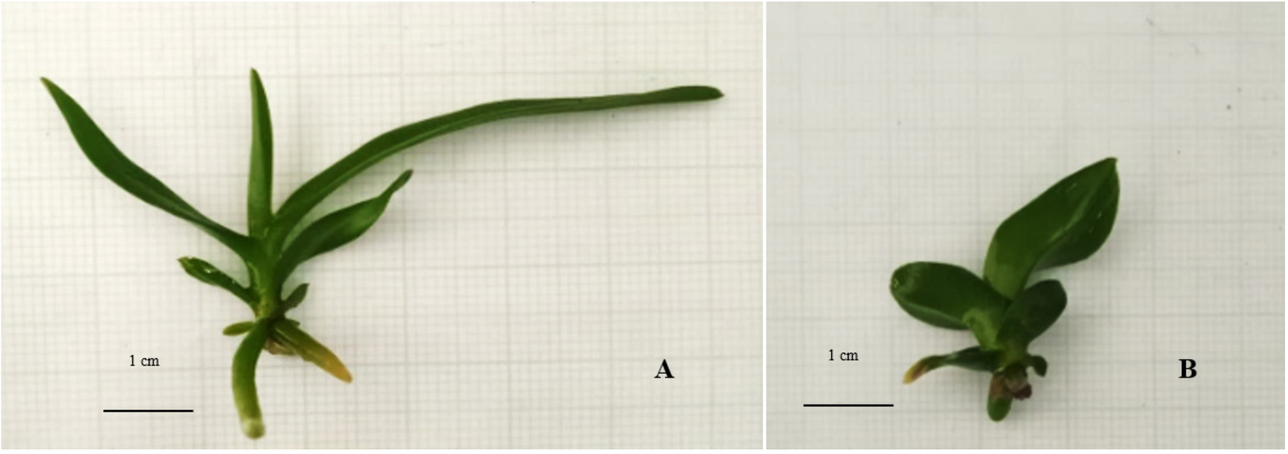

Figure 1. Plantlet height of *Vanda limbata* 'Jawa'. A. Control plantlet, B. Plantlet under oryzalin of 25 µM

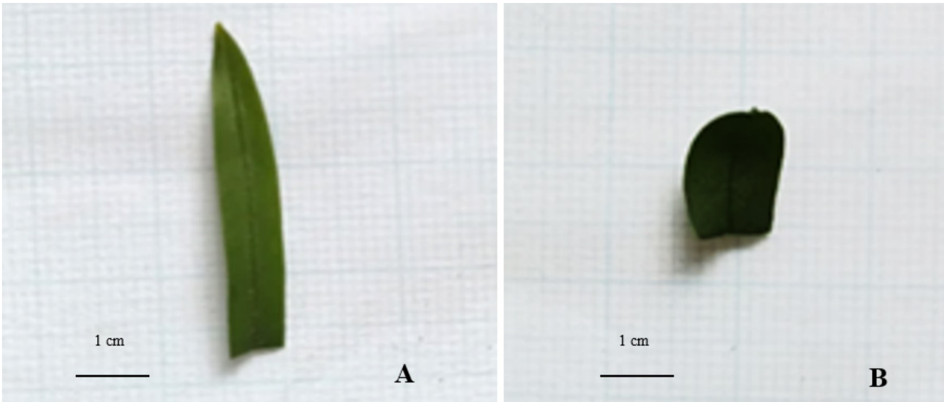

Figure 2. Leaf length of *Vanda limbata* 'Jawa'. A. Control plantlet, B. Plantlet under oryzalin of 100 µM

exhibit longer, narrower leaves (Figure 2). This demonstrates that oryzalin has contrasting effects on the length and width of *V. limbata* 'Jawa' leaves.

The application of oryzalin also has a significant visual effect on the formation of adventitious shoots in *V. limbata* 'Jawa' plantlets. The number of adventitious shoots increases with higher concentrations of oryzalin (Figure 3). No adventitious shoots are observed in either the control plantlets or those treated with 25 µM oryzalin. In contrast, plantlets treated with 50 µM, 75 µM, and 100 µM oryzalin produce one, two, and three adventitious shoots, respectively.

Anatomical characters

The anatomical analysis of foliar stomata provides further evidence of polyploidization in *V. limbata* 'Jawa' plantlets under oryzalin treatments. ANOVA reveals that oryzalin has no significant effect on stomatal number. However, notable changes in stomatal size are observed, as higher concentrations of oryzalin significantly increase stomatal width (Table 2, Figure 4).

Table 2. Stomatal size of *Vanda limbata* 'Jawa' plantlets under oryzalin treatments

Oryzalin Concentration (µM)	Average stomatal width (mm)
0	47.83[a]
25	48.85[ab]
50	49.38[ab]
75	49.91[ab]
100	55.00[b]

Numbers followed by the same letters reveal no significant difference at Tukey test level of 0.05.

Oryzalin also has a significant effect on stomatal shape. Plantlets treated with the highest concentration (100 µM) exhibit more rounded stomata compared to the elliptical stomata observed in the control plantlets (Figure 5).

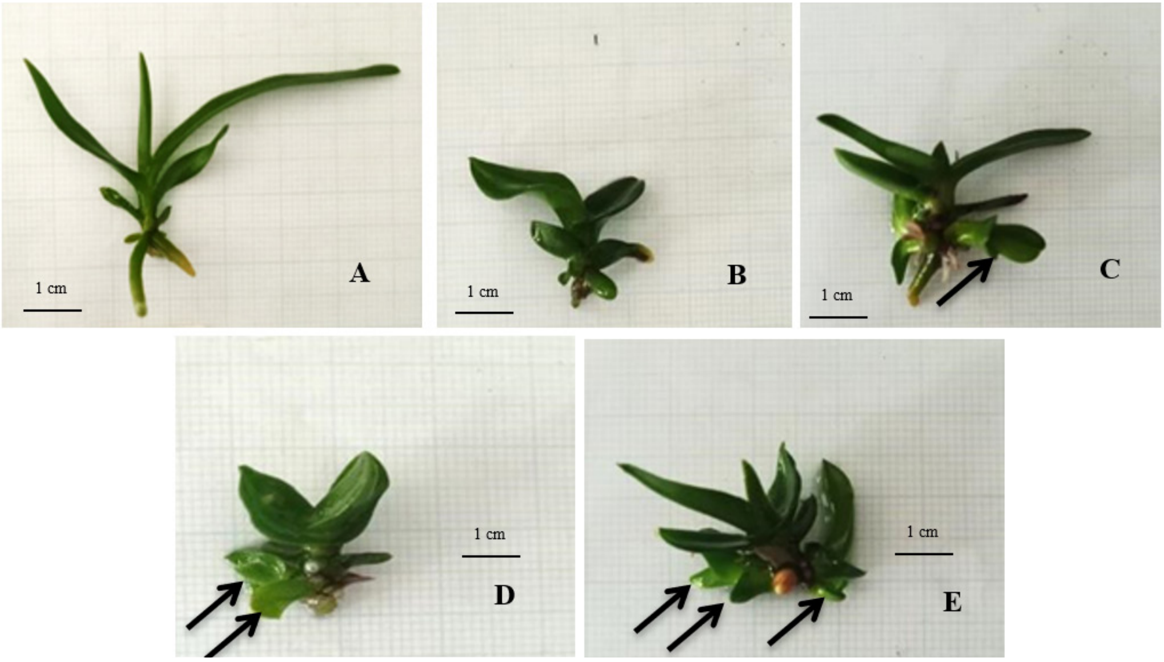

Figure 3. Adventitious shoot formation in *Vanda limbata* 'Jawa' plantlets. A. Control plantlet, B. Plantlet under oryzalin of 25 µM, C. Plantlet under oryzalin of 50 µM, D. Plantlet under oryzalin of 75 µM, E. Plantlet under oryzalin of 100 µM

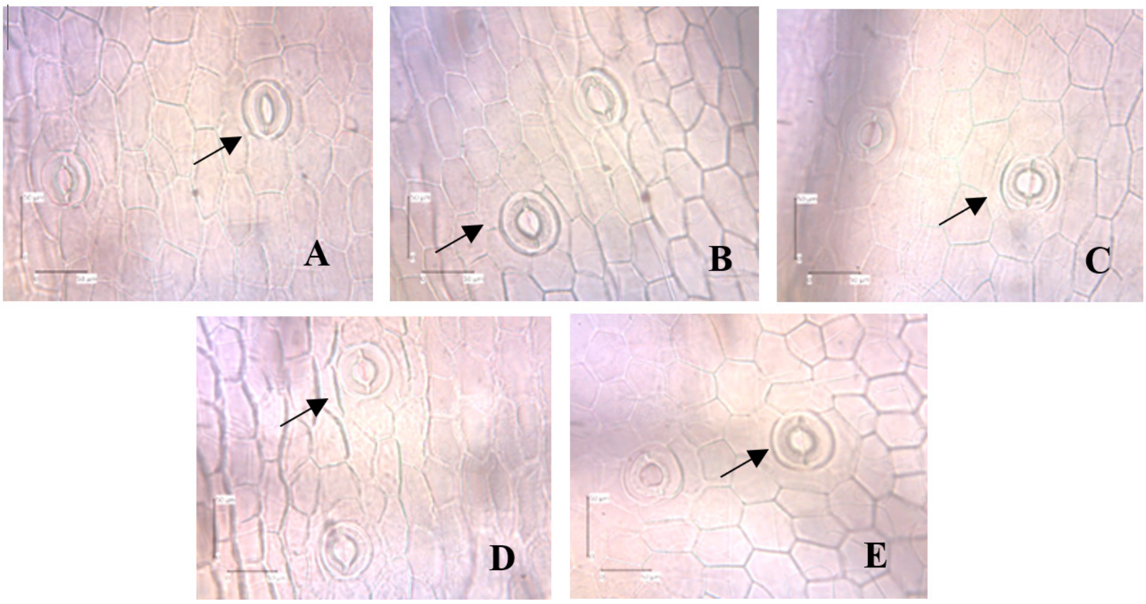

Figure 4. Stomatal size of *Vanda limbata* 'Jawa' plantlets (scale: 50 µm). A. Control plantlet, B. Plantlet under oryzalin of 25 µM, C. Plantlet under oryzalin of 50 µM, D. Plantlet under oryzalin of 75 µM, E. Plantlet under oryzalin of 100 µM

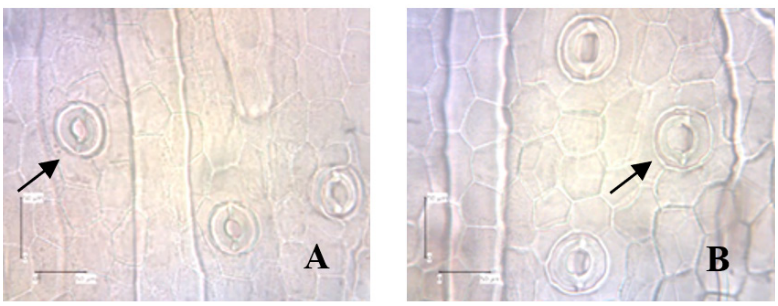

Figure 5. Stomatal shape of *Vanda limbata* 'Jawa' plantlets (scale: 50 µm). A. Control plantlet, B. Plantlet under oryzalin of 100 µM

Discussion

The application of oryzalin significantly influences the morpho-physiological and anatomical characteristics of *Vanda limbata* 'Jawa' plantlets, indicating its potential as an effective agent for inducing polyploidy. The height of *V. limbata* 'Jawa' plantlets is significantly reduced with increasing concentrations of oryzalin, consistent with previous findings. For instance, in oryzalin-induced hexaploid *Populus* species, plantlet height decreased by approximately 50% compared to triploid controls, with hexaploids being induced by oryzalin at 5 mgL^{-1} for 72 hours [9]. Similarly, in *Tectona grandis*, oryzalin at 30 μM reduced plantlet height from 5.06 cm in controls to 2.92 cm in treated plantlets, with comparable results observed using colchicine [10].

Leaf length is also significantly reduced by oryzalin in *V. limbata* 'Jawa,' a pattern that has been reported in other species. For example, in *Allium cepa*, higher concentrations of oryzalin led to shorter leaves, with 25 μM reducing leaf length to 0.6 ± 0.05 cm compared to 3.1 ± 1.3 cm in controls [11]. Similarly, in oryzalin-induced tetraploid *Colocasia esculenta*, the average leaf length of 22.71 ± 2.75 cm was significantly shorter than that of diploid controls (28.92 ± 3.47 cm) [12].

In contrast to plantlet height and leaf length, oryzalin increases the leaf width of *V. limbata* 'Jawa' plantlets, as higher concentrations result in wider leaves. This response is species-dependent, as oryzalin had the opposite effect in *C. esculenta*, where tetraploids exhibited narrower leaves (17.46 ± 2.50 cm) compared to diploid controls (21.50 ± 3.09 cm) [12]. Meanwhile, in *Dendrobium 'Sonia'*, only a slight increase in leaf width was observed, with plantlets treated with 14.14 μM oryzalin showing an average leaf width of 6.72 ± 0.49 mm compared to 6.10 ± 0.37 mm in controls. However, higher concentrations (28.90 μM) were lethal to the plantlets [13]. These findings suggest that oryzalin's effect on leaf width varies among species and is concentration-dependent.

In addition to leaf morphology, oryzalin significantly increases the number of adventitious shoots in *V. limbata* 'Jawa.' This aligns with studies on oryzalin-induced tetraploid *Antirrhinum majus*, where higher shoot numbers were observed in tetraploids compared to diploid controls [14]. However, oryzalin can also exhibit toxicity, as reported in *Vaccinium corymbosum*, where no shoot regeneration was observed. In contrast, colchicine (250 μM) induced the regeneration of 63 shoots in the same species [15].

Oryzalin has no significant effect on the stomata number of *V. limbata* 'Jawa' plantlets, differing from results in other species. For example, *A. cepa* treated with 75 μM oryzalin showed significantly fewer stomata compared to controls [11]. Similarly, tetraploid *C. esculenta* exhibited lower stomata density (139.11 ± 78.42/mm^2) compared to diploid controls (191.51 ± 120.26/mm^2) [12]. Decreased stomata numbers have also been observed in oryzalin-induced polyploids of *T. grandis* [10], *Populus* species [9], *Caladium* species [16], *Vaccinium corymbosum* [15], and *Vitis* species [17].

In contrast, oryzalin significantly increases stomatal size, particularly stomatal width, in *V. limbata* 'Jawa.' This aligns with findings in *Citrullus lanatus*, where tetraploids showed an average stomatal width of 3.46 ± 0.05 μm compared to 2.93 ± 0.05 μm in diploids [18]. Similarly, in *T. grandis*, oryzalin at 30 μM significantly increased stomatal width compared to controls [10]. However, in *Dendrobium 'Sonia'*, no significant differences in stomatal width were observed between polyploid and diploid plants [13].

Plantlets of *V. limbata* 'Jawa' showed good survival rates at eight weeks post-treatment, with only minor symptoms of leaf discoloration (brownish-yellow leaves) likely caused by oryzalin exposure. Similar symptoms were reported in oryzalin- and colchicine-treated *Caladium* 'Red Flash,' where some leaves turned dark yellow [16]. However, oryzalin application has been associated with reduced survival rates in other species, such as *Limonium sinuatum*, where prolonged exposure decreased seedling survival regardless of concentration [19]. Morphological abnormalities, including variation in leaf shape and size, are also commonly observed in oryzalin-treated orchids, making this compound a valuable tool for orchid breeders aiming to produce polyploid individuals with desirable traits [19].

The environmental conditions during *in vitro* culture, including an average temperature of 24°C, humidity of 65%, and light intensity of 1,230 lux, were within the optimal range for plant growth.

Conclusion

The findings of this study demonstrate that oryzalin at a concentration of 100 μM is the most effective treatment for inducing polyploidy in *Vanda limbata* cultured *in vitro*. This is particularly evident in phenotypic traits such as reduced leaf length, increased leaf width, enhanced adventitious shoot formation, and enlarged stomatal width. These results suggest that oryzalin, when applied at appropriate concentrations, can serve as an effective agent for polyploidization in *V. limbata*.

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Declaration of interest

The authors declare that they have no conflicts of interest, financial or otherwise, with any private, public, or academic entity related to the content of this manuscript.

Author contributions

MD conceptualized and designed the experiment and supervised the overall work. WNH was responsible for data collection. AHS prepared the manuscript draft. All authors reviewed and approved the final version of the manuscript for publication.

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