Adventitious shoot growth in *Grammatophylum scriptum* var. Leopard Blume under colchicine and oryzalin applications

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Abstract. Grammatophylum scriptum var. Leopard Blume is of great demand due to its uniqueness flowers showing tiger-like patterns in color. This can still be developed further to make it the main element of a cut flower arrangement. Polyploidization offers the potentials of producing better plant vigour and performance in comparison to its diploid counterparts. This study aims to investigate the effectiveness of colchicine and oryzalin in inducing polyploidization in G. scriptum var. Leopard and to determine the most optimum concentration of the substances in producing desired polyploid G. scriptum var. Leopard. An experiment method was arranged in a Completely Randomized Design employing eight treatments consisting of four colchicine concentrations and four oryzalin concentrations. Some morpho-anatomical characters were used as the parameters to examine. Plants under both colchicine and oryzalin showed fresh green in colour. Even sturdier and larger size were observed in the oryzalin-treated plants. Stomata and epidermal cells enlarged leading to a decrease in stomata number and density. It can be concluded that oryzalin of 75 μM was found to be the most optimum treatment as it produced plants of wide and sturdy leaves supported by numerous roots and higher plant habitus.

1 Introduction

Orchids are highly valued ornamental flowers, either used as potted plants or cut flower arrangements. One of the orchid cultivars renowned for their cut flower appeal is *Grammatophylum scriptum* var. Leopard Blume. This cultivar is so-called because of possessing a green base adorned with brown blotches reminiscent of a leopard's coat. In addition, a robust and upright growth habit exists [1]. Its distribution extends from the Fiji and Solomon Islands through Papua New Guinea and Indonesia to the Philippines. In Indonesia, it is naturally found on the islands of Sumatra, Kalimantan, Sulawesi, Maluku and Papua [2].

Apart from the main characteristics, *G. scriptum* var. Leopard stands out for its impressive size and C3 photosynthetic pathway. The inflorescences can reach up to 50 cm in height and

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each bears 30 to 50 flowers. An individual flower has a diameter of about 3 – 4 cm. Remarkably, *G. scriptum* var. Leopard boasts exceptional longevity, remaining fresh for up to a month even after being cut from the stems. This makes *G. scriptum* var. Leopard a popular choice for centerpiece arrangements in vases. The flowering season extends over a considerable period, namely from January to August [3].

The flowers of diploid *G. scriptum* var. Leopard have typically a diameter of 4 cm. This can be developed further to create a more visually impactful arrangement of blooms within a single inflorescence. With the increasing flower size, it is also desirable to enlarge the bulbs, ensuring the capacity of adequate support on the larger inflorescence and maintaining a harmonious balance with the overall size of the inflorescence. Furthermore, sharpening pigmentation of the flower patterns should also be made. These all can be achieved by creating polyploid *G. scriptum* var. Leopard, which is expectedly of more vigorous specimens with larger flowers and more vibrant colours in comparison to its diploid counterparts. One approach to induce polyploidy in *G. scriptum* var. Leopard is by means of applying some chemical mutagens, such as oryzalin and colchicine [4].

Both oryzalin and colchicine are antimitotic agents with a similar mode of action in inducing polyploidy, which is by effectively inhibiting the polymerization of tubulin proteins into microtubules. These are essential structures for spindle formation and chromosome segregation during cell division. Consequently, cells treated with oryzalin and or colchicine remain arrested in metaphase with duplicated chromosomes congregated at the equatorial plane. As a result, the primary cell wall fails to form, leading to a doubling of the chromosome number within the cell [[4,5].

Although polyploid plants often exhibit unique characteristics that enhance their ornamental value, sterility is a common drawback associated with polyploidy, impeding the propagation of these desirable traits through conventional seed-based methods. This limitation leads to alternative propagation strategies, such as vegetative propagation, to ensure the continued cultivation of these valuable specimens. Therefore, the application of oryzalin and colchicine to induce polyploidy remains a valuable tool for enhancing the ornamental qualities of high-value plants like *G. scriptum* var. Leopard despite the challenges in sterility.

Induced polyploidy in *Rhynchostylis gigantea* var rubrum Sagarik using colchicine has been successfully demonstrated [6]. On the other hands, relatively unexplored applications of oryzalin in orchids exist, even though those in other plant species have been proven effective in inducing polyploidy. Successfully induced polyploidy using oryzalin in *Cymbidium* and *Phalaenopsis* has been reported. However, it was noted that *Cymbidium* exhibited greater resistance to polyploidization in comparison to *Phalaenopsis* [4]. Regardless the limited study on oryzalin-induced polyploidy in orchids, it is necessary to develop optimized oryzalin treatment protocols in *G. scriptum* var. Leopard.

This study aims to elucidate the effectiveness of colchicine and oryzalin in inducing polyploidization in *G. scriptum* var. Leopard and to determine the most optimum concentration of both substances in producing desired polyploid individuals of the orchid cultivar. The pursuit of polyploid propagation in *G. scriptum* var. Leopard presents both challenges and opportunities. The challenges are particularly with regard to sterility, while the opportunities are related to the potential benefits of increasing ornamental quality. By embracing the challenges and pursuing further study on *G. scriptum* var. Leopard, horticulturists and orchid enthusiasts can unlock the full potential of this remarkable orchid cultivar.

2 Methods

The study was carried out experimentally arranged in a Completely Randomized Design employing eight treatments consisting of four colchicine concentrations, i.e. 0, 250, 500 and 750 µM, and four oryzalin concentrations, i.e. 0, 25, 50 and 75 µM. Each treatment was subjected to three replications giving rise to a total of 24 experimental units.

2.1 Preparation of liquid medium containing colchicine and oryzalin

Colchicine stock solution of 1,000 µM was prepared in 100 mL aquadest and added with 2% DMSO, while that of 100 µM oryzalin was prepared in 200 mL aquadest and added with 2% DMSO. A liquid MS (1962) medium was prepared by dissolving 4.35 g MS powder (Phytotech) in a total volume of 1,000 mL aquadest. As many as 24 liquid MS media of 20 mL were then put into sterile 100 mL Erlenmeyer flasks. The media were sterilized using an autoclave at a pressure of 0.15 MPa and a temperature of 121°C for 20 minutes. Once they had cooled down into approximately 60°C, colchicine or oryzalin, corresponding to the designed treatment, was aseptically added using a microsyringe. This was performed by diluting the colchicine stock solution into the liquid MS medium giving rise to colchicine concentration as the respective treatment. This was also the case with preparation of liquid MS medium containing oryzalin.

2.2 Immersion of protocorms into liquid media

Forty *G. scriptum* var. Leopard protocorms, with their basal regions wounded using a scalpel, were gently placed into each Erlenmeyer flask containing the respective liquid MS medium. The flasks were then aseptically sealed with aluminium foil and placed on a shaker at 100 rpm for seven days.

After seven days, the protocorms were carefully washed three times with sterile aquadest to remove any residual colchicine or oryzalin. Subsequently, they were transferred into a fresh MS liquid medium lacking of colchicine and oryzalin and shaken further at 100 rpm for 14 days.

2.3 Plant regeneration

After 14 days of shaking, the protocorms developed into seedlings. These were then carefully subcultured onto solid MS media enriched with growth regulators, i.e. 5% coconut water, 3 mg/L benzyl adenine (BA) and 1 mg/L kinetin. The seedlings were further grown for eight weeks to resume normal growth following exposure to colchicine and oryzalin. This extended period of growth ensured that the seedlings had fully recovered from the polyploidy induction process and were ready to exhibit their enhanced ornamental potentials [7].

2.4 Subculturing and acclimatization

Every two months, the seedlings were carefully subcultured onto fresh MS medium devoid of growth regulators. This repeated processes enabled the seedlings to gradually adapt to a less nutrient-rich environment, preparing them for ex vitro growth. As the seedlings matured with the increasing size and fully developed leaves, they revealed the ornamental values of G. scriptum var. Leopard.

2.5 Data analiysis

Plant height, leaf number, leaf length and width, root number, longest root length, adventitious shoot number and stomatal number were the growth parameters to examine. All the data were analyzed by using ANOVA to determine the significance of the effects of colchicine and oryzalin on the observed growth parameters. Further analysis of Tukey's Honestly Significant Difference (HSD) test was performed when significant effects of treatments were observed.

3 Results and Discussion

3.1 Protocorm Survival Rate

In general, preliminary observations on protocorm condition indicate that *G. scriptum* var. Leopard individuals applied with oryzalin show better adaptation to the presence of antimitotic agents in comparison to those treated with colchicine. This is evident from the relatively healthier vigour of oryzalin-treated plants with bright green protocorm colour. In contrast, those treated with colchicine exhibit relatively poorer growth and development, some of which even fail to grow due to poor adaptation. Significantly reduced number of protocorms under colchicine of 750 µM with only 75% survival rate was observed, while protocorms treated with 75 µM oryzalin still exhibited 95% survival rate (Table 1).

As the concentration of oryzalin increased, the protocorms turned darker brown and the survival rate decreased (Fig. 1). Similar results in korarima plants (*Aframomum corrorima*) showing considerably lower survival rate, i.e. 37%, after colchicine application in comparison to that of oryzalin-treated plants, i.e. 67%, were reported [7]. Increasing concentrations of either colchicine or oryzalin led to a higher mortality of treated plants. A relatively high dosage of colchicine is needed to inhibit microtubule formation. This substance has a low affinity to microtubules in plants, leading to reduced plant survival due to its sufficiently high toxicity [8].

Table 1. Protocorm survival rate at 3 weeks after colchicine and oryzalin applications

Treatment	Protocorm Colour	Protocorm Survival Rate (%)
colchicine 0 µM	bright green	100
colchicine 250 µM	bright green	100
colchicine 500 µM	yellowish green	80
colchicine 750 µM	brown	75
oryzalin 0 µM	bright green	100
oryzalin 25 µM	bright green	100
oryzalin 50 µM	yellowish green	100
oryzalin 75 µM	brown	95

It is suggested that morphological segregation resulting from colchicine and oryzalin applications is attributed to differences in morphological vigour, colour intensity and leaf size [4]. The number of tetraploid plants obtained from oryzalin treatment was found significantly higher, i.e. 17 individuals, than that from colchicine treatment, i.e. 11 individuals [7].

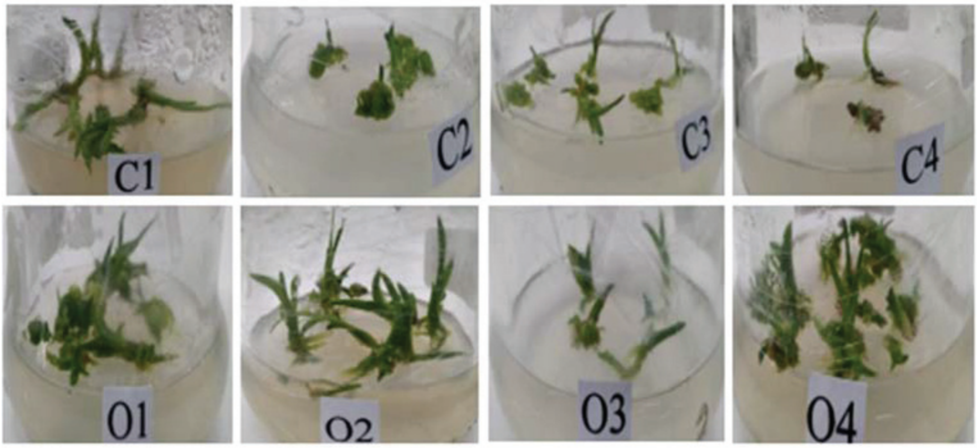

Fig. 1. Colchicine- and oryzalin-treated protocorms at 23rd week after applications (C1: 0 µM colchicine; C2: 250 µM colchicine; C3: 500 µM colchicine; C4: 750 µM colchicine; O1: 0 µM oryzalin; O2: 25 µM oryzalin; O3: 50 µM oryzalin; O4: 75 µM oryzalin)

3.2 Plant Regeneration

Following the addition of 5% coconut water, 3 mg/L BA and 1 mg/L kinetin at the 23rd week after colchicine and oryzalin applications, fresh green colour plants were seen (Fig. 1). This is because the addition of BA and kinetin growth regulators can prolong the lifespan of chlorophyll, resulting in fresh green leaves. It is also noted that colchicine application leads to greener leaves in comparison to control plants, since increased number of chloroplasts in the leaves of tetraploid plants than that of diploid plants [8,9]. Increasing chloroplast number in Viola acuminata plants treated with 10% colchicine was also reported. This significantly enhanced leaf chlorophyll contents, making the leaves appear visually greener [10].

The addition of BA (cytokinin) and kinetin into the culture medium increased the number of new *G. scriptum* var. Leopard shoots (Table 2). Colchicine-treated plants at 750 µM produced 3.33 bulbs, and oryzalin-treated plants at 75 µM produced 3.33 bulbs as well. It is stated that the addition of cytokinin into the culture medium stimulates shoot proliferation by inducing rapid cell division, ultimately leading to the formation of new shoots [11]. The number of shoots in both *Dendrobium nobile* and *Paphiopedilum villosum* was found increasing by the addition of BAP and kinetin as well as coconut water into the treatment medium. Coconut water contains cytokinins, auxins, sugars and amino acids, all of which play active roles in the cell division process. However, high levels of cytokinin in the medium can suppress shoot length but are assumsed to increase the number of axillary buds . It seems likely that the number of shoots is also influenced by the inherent characteristics of the orchid itself. *G. scriptum* orchids have a tendency to clump, especially those being without leaves on their bulbs, which have a tendency to form stolons. Adventitious shoot primordia are formed at specific points on the ends of stolons as shown in Fig. 2.

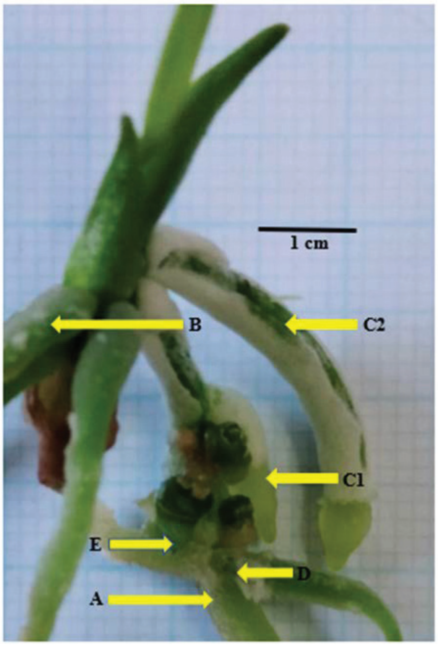

Fig. 2. Adventitious shoot primordia emerging from around *Gramatophyllum scriptum* rooting zone (A: root; B: old root; C1: young root; C2: root injured longitudinally forming scars containing chlorophyll; D: adventitious shoot primordia; E: adventitious shoot emerging from root)

Adventitious shoot primordia were observed in several sites within the rooting zone, particularly due to high concentrations of colchicine, i.e. 500 to 750 µM. Initially, the roots sustained injuries because of the high concentration of colchicine applied. These injuries appeared as longitudinal wounds along the roots. Subsequently, the wounds underwent healing, and even new chlorophyll-containing tissues emerged within the scar tissue, visually appearing as elongated green lines (Fig. 2 C2). These green roots then underwent dedifferentiation into juvenile tissues, giving rise to numerous new shoot primordia (Fig. 2 D). The adventitious shoot primordia subsequently developed into adventitious shoots on the roots (Fig. 2 E). The number of adventitious shoots induced by 500 and 750 µM colchicine and other treatments is summarized in Table 2. Observations were conducted when the adventitious shoots reached a length of over 3 mm. Therefore, the counted adventitious shoot number presented in Table 2 does not include adventitious shoot primordia of smaller than 3 mm as illustrated in Fig. 2 E.

Root number of *G. scriptum* var. Leopard increased with increasing concentrations of colchicine, except for those under 750 µM (Table 2). This is contrary to that reported in *Phalaenopsis amabilis*, where root number was not affected by colchicine application [12]. With respect to longest root length, it was found that colchicine application showed significant effects. Correspondingly, oryzalin significantly increased root number with increasing concentrations of the substance (Table 2).

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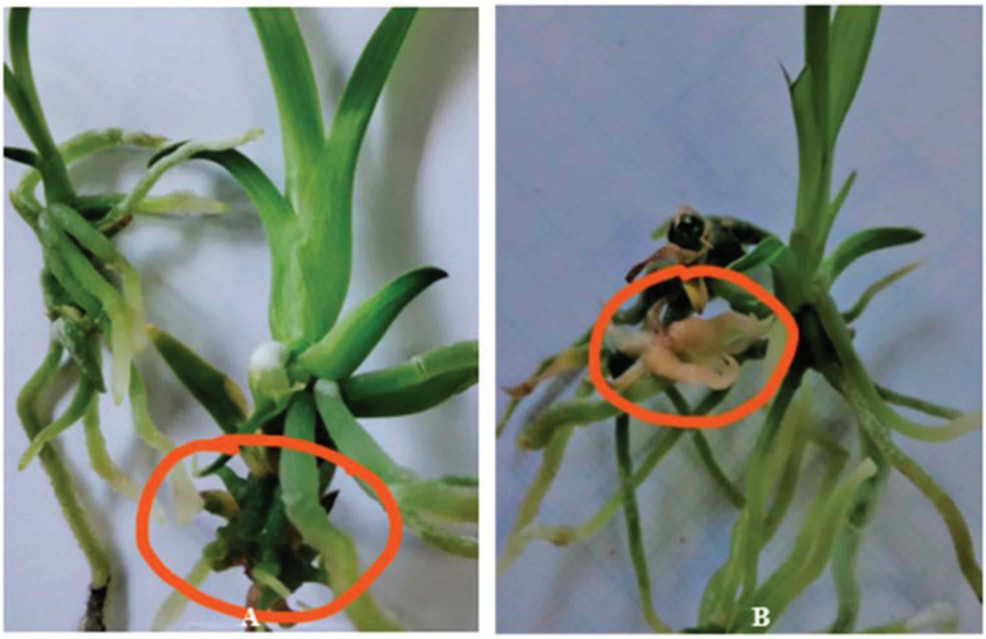

Fig. 3. Adventitious shoot growth of *Grammatophylum scriptum* var. Leopard: A)Rooting zone undergoing adventitious shoot growth under oryzalin of 25 µM; B)Pink adventitous shoot formed after oryzalin of 75 µM application

The number of adventitious shoot buds formed was slightly different among the oryzalin concentrations used. Dedifferentiation of root tissues, such as those under high colchicine treatments (500 and 750 µM), was also observed in oryzalin-treated roots. However, the number of buds in these areas was not as high, and they were characterized by smaller bumps rather than prominent new adventitious shoot buds. Roots were also smoother and more slender in oryzalin-treated plants with sufficiently high adventitious shoot number (Fig. 3A). Interestingly, some of the adventitious shoot buds formed in *G. scriptum* var. Leopard treated with 75 µM oryzalin were pink in colour (Fig. 3B). These buds appeared to be mutants, and their frequency was relatively low, only occurring in *G. scriptum* var. Leopard treated with 75 µM oryzalin. These pink buds exhibited pigment abnormalities and required special care for further development. The lack of pigment, particularly chlorophylls a and b, which are responsible for the green and light green colours of plants, respectively, impaired the capture of light energy and the electron transport reactions in Photosystems I and II. Consequently, the production of NADPH and ATP from the light reactions was limited, resulting in poor growth of the pink buds. These buds likely required different light conditions in comparison to normal *G. scriptum* var. Leopard buds, as they were mutated in pigment formation. Preliminary experiments have shown that certain treatments with boron, calcium, and high sucrose can slightly improve the growth of these pink *G. scriptum* plants. In addition, these pink *G. scriptum* var. Leopard may require irradiation with wavelengths different from those used for normal plants to optimize the function of their pigments in capturing available light energy. Therefore, specialized lamps may be necessary to provide the appropriate light conditions for the pigments in these *G. scriptum* var. Leopard seedlings. Due to the pigment abnormalities in the leaves of the pink *G. scriptum* plants, the production of NADPH2 and ATP was severely limited, leading to reduced hexose production from the carbon reactions. Consequently, special care is required to maintain the survival of these pink *G. scriptum* plants.

The application of both colchicine and oryzalin significantly reduced leaf number of *G. scriptum* var. Leopard. However, leaf length tended to be positively influenced by colchicine but tended to be negatively impacted by increasing concentrations of oryzalin. Interestingly, *G. scriptum* var Leopard treated with 75 µM oryzalin exhibited widest leaves, but those treated with 750 µM colchicine showed narrowest leaves. This suggests that a specific dosage of oryzalin can promote leaf width in *G. scriptum* var. Leopard.

The application of 75 µM oryzalin resulted in a suppression of several physiological parameters, including plant height, leaf width and root number. However, it was observed that adventitious shoot number increased. This growth suppression appeared to induce the formation of more adventitious shoots. Another report demonstrated that the highest oryzalin concentration (289 µM) was able to induce tetraploidy in Hebe 'Oratia Beauty' plants by 45.7% . Hence, further studies should be performed to investigate the chromosome number of *G. scriptum* var. Leopard exhibiting increased growth in comparison to the control plants, to determine if chromosomal duplication has occurred.

Tetraploid *G. scriptum* var. Leopard generally exhibit a more compact stature in comparison to their diploid counterparts. This corresponds to the findings that a decrease in node number and internodal length in Hebe 'Oratia Beauty' plants with increasing oryzalin concentrations was observed, resulting in a more compact appearance of tetraploid individuals [5].

Variations in leaf shape among tetraploid genotypes, despite their homogeneous genetic background, have been documented in other plant species, such as *Pyrus communis* [13] and *Dendrobium officinale* [14,15]. Meanwhile, variations in floral traits are attributed to chromosomal duplication and gene redundancy, rather than structural variations or gene mutations. It is suggested that such phenotypic variations arise from heterozygosity [16].

Table 2. Some morpho-anatomical characters of *Grammatophylum scriptum* var. Leopard under colchicine and oryzalin applications

Treatment	Leaf Number	Leaf Length (mm)	Leaf Width (mm)
colchicine 0 µM	4.00 de	17.93 c	2.57 c
colchicine 250 µM	3.33 ef	19.27 c	1.50 d
colchicine 500 µM	2.00 g	26.43 a	1.53 d
colchicine 750 µM	3.00 f	22.57 b	1.37 d
oryzalin 0 µM	6.00 a	25.33 a	3.70 b
oryzalin 25 µM	4.67 cd	21.77 b	2.67 c
oryzalin 50 µM	5.67 ab	17.70 c	2.97 c
oryzalin 75 µM	5.00 bc	22.70 b	4.27 a

Note: numbers followed by the same letters in the same column show no significant difference at Tukey test of 5%

Table 2. Some morpho-anatomical characters of *Grammatophylum scriptum* var. Leopard under colchicine and oryzalin applications *(continuation)*

Treatment	Roots Number	Longest Root Length (mm)	Plant Height (mm)	Adventitious Shoot Number	Stomatal Number
colchicine 0 µM	2.33 c	51.33 f	24.33 d	1.33 a	4.67 a
colchicine 250 µM	3.00 c	65.67 ef	29.47 c	2.00 a	1.67 de
colchicine 500 µM	5.67 b	91.67 c	37.13 a	2.67 a	0.33 f
colchicine 750 µM	2.67 c	70.33 de	29.07 c	3.33 a	2.00 cd
oryzalin 0 µM	3.33 c	86.33 cd	26.13 d	1.00 a	4.00 b
oryzalin 25 µM	5.67 b	113.67 ab	32.20 b	2.00 a	2.33 c
oryzalin 50 µM	6.00 b	102.00 bc	35.50 b	2.00 a	1.33 e
oryzalin 75 µM	14.67 a	127.00 a	37.07 a	3.33 a	2.33 c

Note: numbers followed by the same letters in the same column show no significant difference at Tukey test of 5%

Observations of stomatal number revealed a decreasing trend with increasing concentrations of both colchicine and oryzalin. Similar findings were reported in *Vanda lombokensis* J.J. Sm. and in *Paphiopedilum villosum* treated with colchicine. These studies demonstrated that colchicine application leads to a reduction in stomatal number and density in comparison to control plants. This phenomenon is attributed to the enlargement of stomata and epidermal cells, resulting in a lower overall stomatal density[14,15]. It is supported that colchicine application can induce larger stomata in comparison to diploid counterparts, while the number of stomata decreases with increasing colchicine concentration [5,9]. Consistent with these findings, the present study also showed a reduced stomatal number in *G. scriptum* var. Leopard treated with both colchicine and oryzalin, with the effect becoming more pronounced at higher concentrations (Table 2). This observation may be partly attributed to the culture conditions, particularly the adequate supply of sucrose (30 g/L), which may have reduced the need for *G. scriptum* var. Leopard to engage in vigorous photosynthesis. Under these conditions, the treated plants may have existed in a heterotrophic state, leading to slower rates of photosynthesis and respiration.

Upon acclimatization, the number of stomata on new leaves is expected to increase, as stomata play a crucial role in gas exchange (CO2 and O2) when the plant changes into an autotrophic state. Photosynthesis and respiration become essential processes for plant growth and survival. This is particularly important for C3 orchids like *G. scriptum* var Leopard, where the capture of CO2 and O2 by Rubisco occurs alternately. Rubisco's high affinity for CO2 drives photosynthesis, leading to an increase in O2 in the surrounding environment. Consequently, photosynthetic photorespiration increases, potentially reducing net photosynthetic yield. Photosynthates and respiration products will be utilized to meet the growth requirements of *G. scriptum* var. Leopard during the acclimatization period.

4 Conclusion

The *G. scriptum* var Leopard plants treated with oryzalin appear sturdy and large in comparison to those treated with colchicine. This indicates that the application of oryzalin can make the orchid plants strong and compact with more roots. Specifically, oryzalin of 75 μM was found to be the most optimum treatment as it produced plants of wide and sturdy leaves supported by numerous roots and higher plant habitus.

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Murni Dwiati collects the research data and takes responsibility for the whole project.
Agus Hery Susanto analyses the data and writes the manuscript.

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