BIODIVERSITAS
Volume 24, Number 7, July 2023
Pages: 4168-4174

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d240756

Shoot regeneration in *Nepenthes mirabilis* as affected by flurprimidol and GA3 application

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Manuscript received: 26 November 2022. Revision accepted: 28 June 2023.

Abstract. *Dwiati M, Widodo P, Susanto AH. 2023. Shoot regeneration in* Nepenthes mirabilis *as affected by flurprimidol and GA3 application. Biodiversitas 24: 4168-4174. Nepenthes mirabilis* (Lour.) Druce. is a pitcher plant species at a higher risk of extinction mainly due to overexploitation. Previous investigations have shown that *in vitro* micropropagation can be used as an approach for *ex-situ* conservation of *N. mirabilis*. Therefore, this study aimed to assess the new shoot formation of *N. mirabilis* under *in vitro* conditions stimulated with the application of flurprimidol and GA3. The three concentrations of flurprimidol and GA3 used as treatments were arranged factorially in a Randomized Complete Block Design (RCBD), with internodes 2, 3, 4, and 5 serving as blocks. Based on the results, F1G0 (flurprimidol of 1 mgL^{-1} without GA3) had the highest shoot formation, which was not accompanied by sufficiently high contents of chlorophyll, leading to improper shoot development. Despite the smaller number of newly formed shoots in F2G2 (flurprimidol of 2 mgL^{-1} and GA3 of 2 mgL^{-1}), significantly higher chlorophyll contents were observed, enabling better development of shoot. Leaf length and shoot diameter also showed similar results under F2G2, indicating that higher concentrations of exogenous GA3 were necessary for better shoot development. This indicated that appropriate concentrations of both plant growth regulators should be applied for micropropagation of *N. mirabilis* in support of *ex-situ* conservation.

Keywords: Flurprimidol, GA3, micropropagation, *Nepenthes mirabilis*

INTRODUCTION

Nepenthes, also known as pitcher plant, is uniquely carnivorous due to the presence of a sac-like structure at the leaf tip, which is used for trapping insects. This genus consists of 114 species distributed worldwide from East Madagascar to New Caledonia, with over 60 endemic species found in Indonesia (Setiawan et al. 2018). Several species of this plant have been intensively traded and subjected to booming demand from 2007 to 2011, leading to overexploitation in their natural habitats occurred and causing a high risk of extinction. *Nepenthes* is now categorized as a protected plant based on some regulations such as Indonesian Law Number 5 Year 1990 on Conservation of Biological Resources and Their Ecosystems, Indonesian Government Regulation Number 7 Year 1999 on Preservation of Plant and Animal Species, and Indonesian Government Regulation Number 8 Year 1999 on Use of Wild Plants and Animals. All *Nepenthes* species are classified as rare according to the International Union for Conservation of Nature (IUCN) and World Conservation Monitoring Centre (WCMC). They are also listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) (Hernawati et al. 2022).

One of *Nepenthes* species, namely *N. mirabilis* (Lour.) Druce., which originates from East Kalimantan, is currently at risk of extinction (Handayani and Hadiah 2019). Therefore, to aid the preservation of this species, *ex-situ* conservation through *in vitro* propagation outside its natural habitat is required to obtain a large-scale generation of vegetatively propagated plantlets.

The propagation of *Nepenthes* species by using stem cutting without *in vitro* culture is relatively difficult to carry out. For example, only two shoots resulted from stem cutting in the propagation of *N. gymnamphora* without *in vitro* culture (Maulidah 2022). Wahdani (2022) also obtained only two shoots when using stem cutting in the *N. adrianii* propagation. Therefore, a more efficient procedure using a micro-cutting approach as explant materials in *in vitro* cultures is needed to obtain a high number of propagated plants. This should be then exposed to a condition where shoot formation is stimulated by increasing synthesis of endogenous cytokinin (Vylíčilová et al. 2020).

Flurprimidol is a retardant plant growth regulator that can increase endogenous cytokinin synthesis, making it applicable to stimulate shoot formation in several plant species. It is the most recent plant growth regulator introduced to ornamental plant growers in commercial nurseries in the US. Flurprimidol belongs to the pyrimidine chemical class that has a nitrogen-containing heterocycle compound. This growth regulator inhibits enzymes involved in the gibberellin biosynthetic pathway, which catalyze the oxidation of ent-kaurene to ent-kaurenoic acid, a gibberellin precursor (Neware 2019). According to previous investigations, the application of 200 mgL^{-1} flurprimidol induced the formation of new shoots from rhizomes or creeping root segments in *Solidago altissima* and *Cayratia japonica*. However, the newly formed shoot

was suffering from some obstacles in the elongation processes (Ito and Ito 2021) due to flurprimidol mode of action, which inhibited gibberellin biosynthesis, thereby terminating cell elongation (Salachna and Zawadzińska 2017; Desta and Amare 2021). Since shoots are formed as a result of differentiation occurring in the dark, it is necessary to recover the newly formed shoot for cell elongation by applying gibberellin (GA3) under light conditions. Konstas and Kintzios (2003) showed that flurprimidol concentrations ranging between 1 and 2 mgL^{-1} successfully stimulated micropropagation of cucumber.

The newly formed shoot that has completely passed through cell elongation due to flurprimidol and GA3 application can be cut and separated to produce novel plant individuals. However, there is no previous report on the combination of flurprimidol and GA3 application in *Nepenthes* species. Therefore, this study aims to investigate the effect of flurprimidol and GA3 application on the growth and development of *N. mirabilis* under *in vitro* conditions. The presence of relatively long internodes in *N. mirabilis* makes it easy to perform micro-cutting in this pitcher plant species. The results are expected to provide an ideal micropropagation model for this endangered species to support its conservation.

MATERIALS AND METHODS

Plant material and treatments

This study was carried out in the Laboratory of Plant Physiology, the Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, from April to October 2022. The experiment was conducted using a Randomized Complete Block Design (RCBD), with two factors, namely flurprimidol and GA3, each at three concentrations (0, 1, and 2 mgL^{-1}). Subsequently, four internode positions served as blocks, with the first internode removed to prevent apical dominance on the micro-cuttings used as explants. The micro-cuttings obtained from internodes 2, 3, 4, and 5 were used as blocks I, II, III, and IV respectively, representing the individual origin of micro-cutting or explant. In total, there were 36 experimental units consisting of F0G0, F0G1, F0G2, F1G0, F1G1, F1G2, F2G0, F2G1, F2G2 treatments, each replicated within a particular block.

Media preparation

The MS medium was prepared and adjusted to a pH of 5.8 by adding HCl 1 N and/or NaOH 1N as required. This was followed by the addition of active charcoal of 1 gL^{-1} and sucrose of 30 mgL^{-1} to the media. The media was heated up to a boil, 8 gL^{-1} agars were added, and sterilized in an autoclave at 0,15MPa and 121ºC for 20 minutes. When the media was cooling down to approximately 60ºC, flurprimidol corresponding to the respective treatment was added using microsyrinx of 0.45 µm in diameter. Meanwhile, some other MS medium was added with GA3 based on each treatment before sterilization.

Nepenthes mirabilis explant preparation and culture establishment

Explants were prepared aseptically by cutting the internodes as previously explained. One leaf was included in each internode to assist in explant photosynthesis. After preparation, the internodes or explants were planted on an MS medium containing flurprimidol with corresponding concentrations, and the explants were incubated for 3 weeks in dark conditions. When shoot primordials appeared, the explants were incubated under 8/16 h light/dark conditions, followed by a 16/8 h light/dark cycle, and finally, under continuous light conditions. Subsequently, the explants were subcultured on MS medium containing GA3 of the respective concentrations. After 85 days of culture initiation, the explants were re-subcultured on an MS medium without any plant growth regulator.

Chlorophyll extraction and quantification

Leaves were weighed to 0.02 g, crushed, and dissolved using 4 mL acetone, filtered, and inserted into a test tube for quantification in a uv-vis spectrophotometer of 646 and 663 nm wavelengths. The formula were as follows: chlorophyll *a* = (12.21 x A_{663})-(2.81 x A_{646}); chlorophyll *b* = (20.13 x A_{646})-(5.03 x A_{663}); total chlorophyll = (17.3 x A_{646})-(7.18 x A_{663}) (Miazek and Ledakowicz 2013).

Data analysis

Shoot number, height, and diameter, with leaf number, length, and width, as well as chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents of the shoot, were analyzed using ANOVA. Further analysis was carried out using the Tukey test at a 0.05% level of confidence when a significant effect of treatments or blocks was observed.

RESULTS AND DISCUSSION

Shoot regeneration

Control treatment (F0G0) resulted in only one relatively long shoot from internode 2 (block I), as illustrated in Figure 1. The sufficient growth of the newly formed shoot was due to the absence of apical dominance effect related to the significantly decreasing auxin concentration at the stem tip obtained by trimming the stem tip or removing the first internode. Only one newly formed shoot was also observed from internode 3, 4, and 5, respectively, under F0G0 control treatment, as presented in Figure 2.

Other treatment combinations of flurprimidol and GA3 showed various shoot numbers of *N. mirabilis* ranging from two to six. As illustrated in Figure 2, F1G0 yielded the highest shoot number, with a value of 4.0±1.826. This was mainly achieved in internode 5, although the other internodes also exhibited high shoot formation values, meaning that the explants with flurprimidol 1 mgL^{-1} can be sufficiently stimulated to pass through new shoot formation and growth in the absence of GA3 application. The dormancy of the newly formed shoot due to flurprimidol application lasted merely within two or three weeks. After the shoot of still white color was formed during the dark

period and the explants were gradually exposed to light, GA3 began to be naturally synthesized. Despite the very low content, the endogenous GA3 or gibberellin sufficiently stimulated the growth of new shoot showing some newly formed leaves. There was no inhibition on the elongation of the newly formed shoot observed.

N. mirabilis explants treated with F1G0 tended to be more effective in new shoot formation as the internodes were farther beyond the stem tip. As shown in Figure 3, the more distant internode from which the explant originated, the more new shoot were produced. Despite a relatively small amount, auxin content in internode 5 was still capable of producing new shoots. Meanwhile, cytokinin synthesis induced by flurprimidol application in each internode led to the formation of a new shoot.

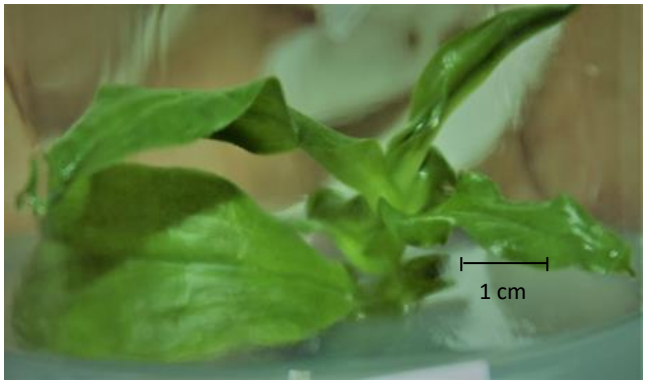

Figure 1. *Nepenthes mirabilis* explant of internode 2 under control treatment (F0G0) 85 days after explant planting

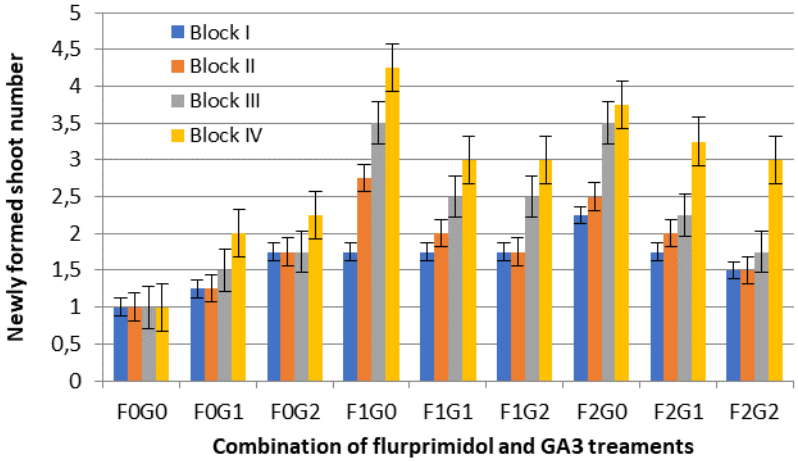

Figure 2. The number of newly formed shoots from each internode (block) under treatment combinations of flurprimidol and GA3 at 60 days after explant planting

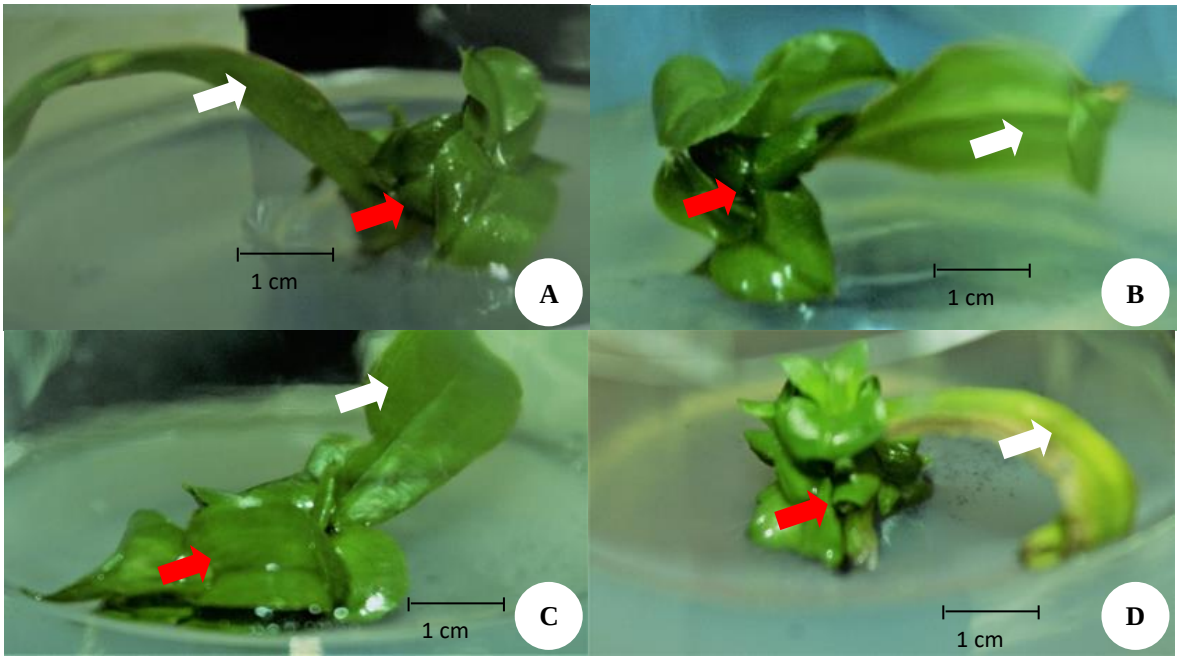

Figure 3. Newly shoot formation in *Nepenthes mirabilis* under flurprimidol of 1 mgL^{-1} without GA3 at 85 days after explant planting. A. internode 2; B. internode 3; C. internode 4; D. internode 5; white arrows = original leaf; red arrows = newly formed shoots

In most plant species, growth began with the increasing level of gibberellin, which stimulated cell elongation, and the oppositely decreasing level of abscisic acid (ABA). ABA is a plant growth regulator with inhibitory effects, causing seed and bud dormancy. Although it was formerly assumed to be responsible for natural leaf drop, a recent study has proven that ABA played a significant part in seed and bud dormancy by inhibiting growth (Dahiru 2018).

Regeneration of explants to produce adventitious shoots was influenced by both internal and external factors, namely plant genotype, plant growth regulator, duration of the dark period, and the physiological status of mother plants. Duration of the dark period, followed by light exposure, was the key factor for the successful formation of adventitious shoots. Light stimulated explants in various biological functions, gene expression, development, and circadian clocks, while molecular and cellular changes occurred during the dark period, resulting in higher competence of regeneration. Auxin degradation occurred more rapidly under light exposure than in dark conditions. Generally, explants incubated in the dark can delay the degradation of both endogenous and exogenous plant growth regulators, while these substances can directly stimulate explant *in vitro* regeneration. Explants incubated under dark treatment exhibited an increasing number of regenerated shoots in comparison to those without dark treatment. This is because some cells grow and differentiate in dark conditions (Jin et al. 2020; Subban et al. 2021).

To eliminate the apical dominance effect, the first internode was trimmed in this study. This was consistent with the report of Smith et al. (2014), where the removal of terminal buds will stimulate water uptake and cause redistribution of carbohydrate reserves to the injured parts for healing the wounds and promoting new shoot formation. Similarly, flurprimidol, which is translocated through the xylem will also be uptaken, making it available for regulating gibberellin throughout the whole plant. After trimming, *N. mirabilis* is expected to carry out a reaction of the pentose phosphate cycle. The trimming will be followed by recovery processes involving the synthesis of three substances, namely (1) hemicellulose, that form microfibrils along with cellulose and can be used as a wound covering, (2) pentose, the component of the nucleic acid being responsible for encoding protein synthesis and (3) erythrose will form phenolic substances to protect the wounds from bacterial and fungi infection.

When trimming was performed immediately after flurprimidol application, regrowth occurred before flurprimidol cellularly reached the xylem and arrived at apical buds. This resulted in the growth of newly formed shoots without any influence of flurprimidol. However, when trimming was performed very long after flurprimidol application, an elimination shoot containing flurprimidol may occur. This can cause a decrease in the concentration of flurprimidol in regulating the shoot growth of the applied plant (Smith et al. 2014).

The results on shoot growth of *N. mirabilis* revealed that every internode had the potential of shoot formation. The growth of these newly formed shoot was highly affected by auxin content in the stem tip possessing the highest content of auxin. By trimming the stem tip and removing the first leaf, the auxin content in the second internode will be higher compared to the third internode. Similarly, Koike et al. (2018) stated that auxin in the form of IAA contents decreased with the distance of internodes from the apical region in *Carapichea ipecacuanha*.

The growth shoot in internode 5 will be affected by a lower concentration of auxin in the region. Dwiyani et al. (2010) demonstrated that explants from the stem base of an orchid species, *Vanda tricolor* var. Suavis resulted in more plantlets in comparison to those from the stem tip. Wang et al. (2021) also reported that the bottom internode showed a higher number of actively dividing cells than those in the upper internode of *Phyllostachys edulis*. According to Ribalta et al. (2014), flurprimidol application reduced internode length *in vitro* and increased shoot number in each internode. Demir and Çelikel (2019) stated that flurprimidol is not very mobile in tissues and is active in low concentrations.

Auxin degradation occurred more slowly in the dark compared to light conditions (Jin et al. 2020). In this study, it was assumed that endogenous auxin was still sufficiently available in internodes 2, 3, or 5. Flurprimidol is a retardant compound with the same mode of action as paclobutrazol, which is a gibberellin synthesis inhibitor. This inhibition caused changes in the metabolic patterns of the terpenoid pathway, leading to increased biosynthesis of ABA, chlorophyll, and cytokinin biosynthesis (Soumya et al. 2017) and inhibiting explant growth. Cytokinin is a hormone that plays a role in cell division. When gibberellin biosynthesis is inhibited, cell division still occurs but not cell elongation. Similarly, the growth of stem, leaves, and internode will be inhibited, but lateral growth will be stimulated, causing shorter and more compacted stems (Desta and Amare 2021). Cytokinin affected cell division, new shoot formation, auxin transport, internode elongation, and leaf growth (Dahiru 2018).

Shoot development

Flurprimidol application will increase endogenous cytokinin and prevent cytokinin degradation. Meanwhile, the increase in the level of cytokinin will stimulate chlorophyll synthesis and reduce senescence (Zhu et al. 2004; Desta and Amare 2021). Flurprimidol will also promote chloroplast differentiation and delay chlorophyll degradation (Fletcher et al. 2000). With higher chlorophyll contents, light energy capture in photosystem I and II will increase. Since chlorophyll *a* and chlorophyll *b* are the centers of reaction in photosystem I and II respectively, the electrons captured by photosystem I will be used to reduce NAD into $NADH_2$, while electrons accepted by photosystem II will be used to form ATP. Carbon reaction in the photosynthesis process will also be stimulated and the photosynthates in the form of hexoses will increase in number. Therefore, the more photosynthates available for the formation of adventitious shoots, the newer shoot can be obtained.

The highest chlorophyll contents of *N. mirabilis* had values of 1.390 ± 0.030 μg g^{-1}FW for chlorophyll *a*, 1.811 ± 0.039 μg g^{-1}FW for chlorophyll *b*, and 1.125 ± 0.034 μg

g^{-1}FW for total chlorophyll, due to treatment with F2G2. The increment of 38.68%, 45.20%, and 31.58% over those in control was observed for chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents, respectively, as shown in Table 1. This indicated that the higher levels of both flurprimidol and GA3 will increase chlorophyll contents. According to Salachna et al. (2014) flurprimidol of 30 mgL^{-1} applied to *Ornithogalum saundersiae* through soil drench *ex vitro* increased the relative content of chlorophyll by 24.61% compared to the content in the control plant. Furthermore, Wróblewska (2013) stated that the longer chlorophyll perseveres in fresh and optimum conditions, the more photosynthates will be produced.

Flurprimidol is translocated through the xylem for micro-cuttings planted as explants in an *in vitro* culture medium to enable appropriate translocation of water, nutrients, and flurprimidol. Therefore, flurprimidol will reach growth spots, which are axillary shoots of the respective micro-cutting. Triazole compounds such as flurprimidol can increase chloroplast differentiation, chlorophyll biosynthesis and inhibit chlorophyll degradation. Moreover, the delay of chlorophyll degradation in the explants will increase its biosynthesis and extend the active phase. This indicated that chlorophyll would be more efficient in increasing the longer photosynthesis process to obtain relatively higher net photosynthates applicable as raw matters for new shoot formation (Desta and Amare 2021).

Table 2 presented the significant effect of flurprimidol and GA3 application on the other parameters of shoot development, except for leaf width, as indicated by the results of the ANOVA.

The highest shoot number was obtained under F0G2 treatment with a length of 27.1 mm, 155 days after culture establishment. In the absence of flurprimidol application, the newly formed shoot elongated with the increasing level of exogenous GA3 applied to explants. Table 2 also showed that shoot height tended to decrease with the increasing level of flurprimidol. Salachna et al. (2014) reported that plant height decreased with the increasing level of flurprimidol application on *O. saundersiae ex vitro*. Application of 30 mgL^{-1} flurprimidol resulted in a decrease in plant height of 40.45% compared to that in the control plant. In this study, treatment with *N. mirabilis*, F1G0 which resulted in the highest new shoot formation, showed the lowest shoot height, namely 10.8 mm, while the control plant (F0G0) had a shoot of 24.1 mm in length. This indicated that F1G0 showed a 55.19% decrease in shoot height compared to in control plant. Flurprimidol of 1 mgL^{-1} significantly reduced shoot height, and in the absence of GA3 application, there was no stimulation of shoot height occurred. The newly formed shoots under F1G0 were short with clustering leaves (Figure 3D).

Table 1. Chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents of *Nepenthes mirabilis* at 155 days after explant planting under flurprimidol and GA3 application

Treatment	Chlorophyll *a* (µg g^{-1}FW)	Chlorophyll *b* (µg g^{-1}FW)	Total chlorophyll (µg g^{-1}FW)
F0G0	1.002 ± 0.068 f	1.247 ± 0.034 n	0.855 ± 0.030 w
F0G1	1.138 ± 0.053 de	1.371 ± 0.065 lm	0.851 ± 0.195 w
F0G2	1.211 ± 0.076 cd	1.467 ± 0.012 l	0.961 ± 0.022 v
F1G0	1.094 ± 0.075 ef	1.267 ± 0.068 mn	0.717 ± 0.059 x
F1G1	1.171 ± 0.085 de	1.311 ± 0.071 mn	0.736 ± 0.055 x
F1G2	1.214 ± 0.072 cd	1.460 ± 0.137 l	0.874 ± 0.134 vw
F2G0	1.282 ± 0.049 bc	1.673 ± 0.057 k	0.907 ± 0.026 vw
F2G1	1.346 ± 0.025 ab	1.766 ± 0.019 k	1.063 ± 0.090 u
F2G2	1.390 ± 0.030 a	1.811 ± 0.039 k	1.125 ± 0.034 u

Note: F: flurprimidol; G: GA3; FW: fresh weight; numbers followed by the same letter show no significant difference at a 0.05% level of confidence

Table 2. Shoot development parameters of *Nepenthes mirabilis* at 155 days after explant planting under flurprimidol and GA3 application

Treatment	New shoot number	Shoot height (mm)	Shoot diameter (mm)	Leaf number	Leaf length (mm)	Leaf width (mm)
F0G0	1.5 ± 0.577 c	24.1 ± 1.159 b	2.8 ± 0.150 f	11.2 ± 0.957 a	19.9 ± 5.876 de	7.5 ± 0.271 bcd
F0G1	2.5 ± 0.577 abc	25.7 ± 0.829 ab	3.4 ± 0.082 de	9.2 ± 2.630 ab	29.9 ± 7.873 b	8.0 ± 2.830 bc
F0G2	3.2 ± 0.957 ab	27.1 ± 1.454 a	3.7 ± 0.171 c	10.5 ± 0.577 ab	38.4 ± 0.775 a	10.6 ± 0.469 a
F1G0	4.0 ± 1.826 a	10.8 ± 0.676 g	3.1 ± 0.129 e	10.0 ± 2.160 ab	16.1 ± 7.018 e	5.8 ± 0.785 d
F1G1	2.0 ± 0.816 bc	11.9 ± 0.510 fg	3.8 ± 0.096 bc	8.5 ± 2.380 b	20.8 ± 3.142 cde	7.0 ± 1.756 cd
F1G2	2.7 ± 0.500 abc	22.2 ± 0.486 c	4.1 ± 0.340 ab	8.5 ± 1.291 b	26.3 ± 2.045 bcd	8.9 ± 0.556 ab
F2G0	3.7 ± 0.500 a	13.5 ± 0.403 f	3.3 ± 0.216 e	9.2 ± 0.500 ab	17.6 ± 0.311 e	7.2 ± 0.685 bcd
F2G1	3.7 ± 1.500 a	17.6 ± 2.484 e	3.6 ± 0.208 cd	8.7 ± 1.258 b	26.8 ± 0.250 bc	8.1 ± 1.144 bc
F2G2	2.7 ± 0.957 abc	19.9 ± 1.333 d	4.4 ± 0.294 a	8.5 ± 0.577 b	40.9 ± 5.392 a	8.6 ± 0.829 bc

Note: F: flurprimidol; G: GA3; numbers followed by the same letter in the respective column show no significance different at a 0.05% level of confidence

The highest leaf width was also obtained in F0G2 and statistically showed a highly significant difference from F1G0, indicating that GA3 effectively overcame the inhibiting effect of flurprimidol in shoot development. Meanwhile, the highest leaf number was shown in the control explant (F0G0) and no significant difference between F0G2 and F1G0 was observed. This indicated that the relatively high leaf number was obtained in F1G0, but it was not accompanied by optimum leaf extension. Exogenous GA3 application was strongly assumed to assist full leaf extension, which was performed 85 days after explant planting during the second subculture period. In F1G0, less proper shoot development regarding leaf length and width was observed, indicating the important role of GA3 application in stimulating further shoot development.

Leaf length showed the highest value at F2G2, namely 40.9 mm at 155 days after explant planting, which was highly different from the values obtained at the other treatments. This indicated that the combination of flurprimidol and GA3 in high-level concentrations will stimulate the leaf elongation process. Meanwhile, F1G0 showed the lowest value of leaf length in comparison to the other treatment combinations. Despite the high shoot formation in F1G0, GA3 application should be made 85 days after explant planting, as endogenous gibberellin can still stimulate leaf elongation and extension after explant planting. However, exogenous GA3 of relatively high concentration is needed to promote further shoot development, especially regarding leaf elongation and extension. The frequency of GA3 application on micro-cuttings should be increased at 85 days after explant planting to provide maximum leaf elongation and extension. Therefore, it is expected that the newly formed shoot of *N. mirabilis* under F1G0 can develop more optimally.

Based on the results, it can be concluded that the appropriate application of flurprimidol and GA3 combination will optimally stimulate shoot growth and development of *N. mirabilis* in an *in vitro* culture. This method can be further developed as a micropropagation technique to support the *ex-situ* conservation of the species.

ACKNOWLEDGEMENTS

The authors are grateful to the Institute for Research and Public Service, Universitas Jenderal Soedirman, Puwokerto, Indonesia, for funding this project through the scheme of *Riset Dasar Unsoed* (RDU) financial year 2022 with the contract number T/1136/UN23.18/PT.01.03/2022. Furthermore, the authors address also high appreciation to Prof. Dr. Sri Wulan Manuhara, Prof. Yafar Vafaee, Ph.D. and Prof. Dr. Enni Suwarsi Rahayu, for thoroughly reviewing this manuscript as well as the anonymous reviewers. The authors are very grateful to Supriyono and Simbar Sulanjari Susanto, for the laboratory assistance and statistical analysis rendered respectively.

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