

## Effects of cytokinin–auxin combinations on *in vitro* propagation of *Nepenthes mirabilis* (Lour.) Druce

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DOI: <https://doi.org/10.36077/kjas/2026/v18i2.23210>

Received date: 5/2/2026

Accepted date: 1/5/2026

### Abstract

The pitcher plants are rare species of the *Nepenthes* genus, known for their numerous bioactivities and widespread use in folk medicine. Due to overexploitation, the number of *Nepenthes mirabilis* in the wild is rapidly declining, while efficient *in vitro* propagation protocols for this species is limited. This investigation was conducted to evaluate the effects of different concentrations of 6-benzyladenine (BA) and indole-3-butyric acid (IBA) combined with 1-naphthaleneacetic acid (NAA) on the growth and development of *N. mirabilis* (Lour.) Druce under *in vitro* conditions. The results showed that *N. mirabilis* shoots cultured on B5 media supplemented with 2 mg.L<sup>-1</sup> BA and 0.5 mg.L<sup>-1</sup> NAA exhibited the highest shoot multiplication, with 15.67 ± 2.50 new leaves and 10.17 ± 1.90 new shoots/explant after 12 weeks of culture. For rooting, B5 media containing 1-3 mg.L<sup>-1</sup> IBA and 0.5 mg.L<sup>-1</sup> NAA significantly increased root number, with the highest value of 14.83 ± 1.94 roots/explant after 12 weeks of culture. This indicated that appropriate concentrations of plant growth regulators should be applied for micropropagation of *N. mirabilis* in support of conservation and large-scale cultivation.

**Keywords:** *Nepenthes mirabilis*, *in vitro* culture, shoot multiplication, plant growth regulators



## Introduction

*Nepenthes* is a genus of tropical pitcher plants that belongs to the Nepenthaceae family. In nutrient-poor environments, *Nepenthes* species have developed pitcher-shaped structures derived from leaf blades that function as specialized organs for capturing, digesting, and absorbing nutrients from insects (1, 2, and 3). They frequently grow in wet environments like swamps, streams, and small canals (4).

The carnivorous *Nepenthes mirabilis* (Lour.) Druce is a member of the *Nepenthes* and is found worldwide, from southern China to northern Australia and Southeast Asia (3). Due to its attractive shape, *N. mirabilis* is widely used as an ornamental plant (5). Besides, in some regions, this plant is also used to treat jaundice, hepatitis, stomach pain, diarrhea, diabetes, and high blood pressure (6). Currently, these species are being overexploited to serve humanity's interests, and it has become difficult to find them in the natural environment (7). For this reason, many methods have been developed to conserve this species, including *in vitro* techniques, which are among the most popular methods for rapid multiplication of plant shoots. However, information about the effect of cytokinin–auxin combinations on *in vitro* propagation of *N. mirabilis* is limited.

Plant tissue culture enables efficient plant propagation under controlled conditions and has been widely used to produce healthy plantlets, especially in medicinal and rare species (8, 9 and 10). In which plant growth regulators (PGRs) are often added to the culture media to stimulate plant growth and development. Many

reports mention that PGRs can promote growth, cell differentiation and organ formation (11). The interaction between cytokinin and auxin is crucial for controlling organogenesis and regeneration efficiency *in vitro* (11 and 12). Among commonly used PGRs, 6-Benzyladenine (BA) significantly impacts shoot growth and development, while Indole-3-butyric acid (IBA) effectively stimulates rooting of *in vitro* shoots (13, 14, and 15). Naphthaleneacetic acid (NAA) has been shown to improve shoot development and shoot length (15). Therefore, NAA is often used in combination with IBA, BA, and other auxins and cytokinins to increase the efficiency of plant propagation.

This study aims to evaluate the effect of BA and IBA concentrations, in combination with NAA, on the micropropagation of *N. mirabilis* (Lour.) Druce. The results are expected to provide further scientific evidence on how the appropriate combination of cytokinin and auxin enhances shoot and root formation in this species, supporting its conservation and propagation efforts.

## Material and Methods

### Plant material

*N. mirabilis* plants (6 months old) were provided by the tissue culture laboratory of Thu Dau Mot University (Figure 1) and maintained under stable *in vitro* conditions. Before the experiment, the plant materials were preliminarily checked for growth status. The explants were taken from healthy, growing shoots that showed no signs of contamination or physiological abnormalities. They were then used directly for shoot multiplication. The use of



uniform, pre-established *in vitro* materials helped minimize contamination risk, ensuring the repeatability and reliability of the results.



**Figure 1. *N. mirabilis* samples at 6 months of age**

### ***In vitro* establishment**

The explants were removed from the previously established *in vitro* shoot cultures, trimmed with scissors and a scalpel into uniformly sized segments, approximately 2 cm long. These explants were cultured in B5 media containing standard macro- and micronutrients and vitamins according to the original formulation, supplemented with 30 g.L<sup>-1</sup> sucrose, 7.5 g.L<sup>-1</sup> agar and PGRs.

Each treatment was initially established in three culture bottles; however, contaminated bottles were excluded from the analysis. The final data were calculated from two uncontaminated bottles (replicates) per treatment, with three

explants per bottles (six explants/treatment).

After inoculating the explants into the media, they were placed in the plant tissue culture room, where the temperature is controlled at 25 ± 2°C with a 16-hour light cycle at an intensity of 1500 lux.

### **Effect of PGR ratios on the growth and development of *in vitro* shoots**

**Effect of BA and NAA ratios:** Explants were cultured on B5 media, with BA at 1, 2, 3, and 4 mg.L<sup>-1</sup>, 0.5 mg.L<sup>-1</sup> NAA, 30 g.L<sup>-1</sup> sucrose, and 7.5 g.L<sup>-1</sup> agar. The control sample had no added PGRs. The number of shoots and leaves per explant was recorded every 4 weeks. The selected concentrations of BA and NAA were based on previous studies (3 and 5).

**Effect of IBA and NAA ratios:** Stem segments (approximately 2 cm in length) obtained from experiment 1 were cultured on B5 media supplemented with IBA at concentrations of 1, 2, 3, and 4 mg.L<sup>-1</sup>, with NAA 0.5 mg.L<sup>-1</sup>, sucrose 30 g.L<sup>-1</sup>, and agar 7.5 g.L<sup>-1</sup>. The control sample had no added PGRs. The number of shoots and leaves per explant was recorded every 4 weeks, while the number of roots per explant was recorded after 12 weeks of culture. The selected IBA concentrations, in combination with NAA, were chosen to evaluate their rooting efficiency.

### **Statistical analysis**

Data were analyzed using ANOVA based on individual explants (n = 6 per treatment) using Minitab 16. Further analysis was carried out using Tukey's test at p < 0.05 when a significant treatment effect was observed.

### **Results and Discussion**

### Effect of BA and NAA ratios on the growth and development of *N. mirabilis* *in vitro*

In the experiments, *N. mirabilis* samples were cultured in B5 media, with BA and NAA added at different concentrations. Every 4 weeks of cultivation, the number of shoots and leaves for each treatment was recorded (Table 1, 2 and Figure 2).

During the first 8 weeks of culture, the number of shoots was almost unchanged between treatments (Table 2). In contrast, the number of new leaves increased progressively, with the highest number in the treatment with a mixture ratio of 4 mg.L<sup>-1</sup> BA and 0.5 mg.L<sup>-1</sup> NAA, reaching 6.50 ± 1.05 leaves (Table 1).

In the 12<sup>th</sup> week, the treatment supplemented with 2 mg.L<sup>-1</sup> BA and 0.5 mg.L<sup>-1</sup> NAA again showed a statistically significant difference compared to other groups in both leaf and shoot numbers. In this ratio, both leaves and shoots showed superiority, with the average number of leaves reaching 15.67 ± 2.50 (Table 1).

Leaves were well developed, dark green, and had thick, wide leaf blades. On average, each explant formed 10.17 ± 1.90 new shoots (Table 2). The remaining combined ratios for the number of new leaves ranged from 5.50 ± 1.38 to 8.17 ± 0.98, corresponding to the number of shoots from 6.00 ± 0.63 to 8.00 ± 2.07. These findings suggest that such concentration ratios were less favorable for shoot multiplication, as the number of new leaves formed was positively correlated with the number of shoots formed on the original explant. When only BA was present in the media, shoot and leaf formation in the explants was significantly reduced compared to the control. Conversely, when NAA was added to the media along with BA, the cultured shoots tended to increase considerably in the number of shoots and leaves. In addition, when BA was added at concentrations that are too high or too low, this process was significantly inhibited. This indicates that the concentrations of cytokinin and auxin in the media directly affect the shoot-forming process.

**Table 1. Effect of BA and NAA on new leaf number in *in vitro* shoot propagation of *N. mirabilis***

Growth regulator (mg.L <sup>-1</sup> )		New leaves/explant		
BA	NAA	4 weeks	8 weeks	12 weeks
Control		3.67 ± 0.82 <sup>a</sup>	4.83 ± 0.75 <sup>ab</sup>	9.50 ± 3.08 <sup>b</sup>
1	0	3.17 ± 1.17 <sup>a</sup>	4.83 ± 0.75 <sup>ab</sup>	6.00 ± 1.79 <sup>bc</sup>
2	0	3.17 ± 0.41 <sup>a</sup>	5.67 ± 1.21 <sup>ab</sup>	7.00 ± 2.45 <sup>bc</sup>
3	0	4.00 ± 1.10 <sup>a</sup>	5.50 ± 1.22 <sup>ab</sup>	5.50 ± 1.38 <sup>c</sup>
4	0	3.33 ± 0.52 <sup>a</sup>	5.33 ± 0.82 <sup>ab</sup>	6.00 ± 0.84 <sup>bc</sup>
1	0.5	3.50 ± 1.05 <sup>a</sup>	4.67 ± 0.82 <sup>ab</sup>	6.33 ± 1.21 <sup>bc</sup>
2	0.5	3.00 ± 0.89 <sup>a</sup>	5.83 ± 1.47 <sup>ab</sup>	15.67 ± 2.50 <sup>a</sup>
3	0.5	3.00 ± 1.26 <sup>a</sup>	4.17 ± 0.98 <sup>b</sup>	6.33 ± 1.03 <sup>bc</sup>
4	0.5	4.00 ± 0.89 <sup>a</sup>	6.50 ± 1.05 <sup>a</sup>	8.17 ± 0.98 <sup>bc</sup>

Values in the same column with different letters indicate statistically significant differences (*p*-value < 0.05)



These results showed that supplementation with BA, along with NAA, was necessary for rapid shoot induction in *N. mirabilis* stem samples. Supplementing with NAA helps to stabilize the plant's shoot formation process. Similarly, previous studies reported that moderate concentrations of BA in combination with NAA significantly enhance shoot regeneration. For example, BA (2-3 mg/L) combined with NAA helped in the highest number of shoots in *Curcuma longa* L. (16). However, at higher cytokinin concentrations in combination with auxin, shoot induction was inhibited, likely due to the suppression of meristematic cell division and poor interaction with the

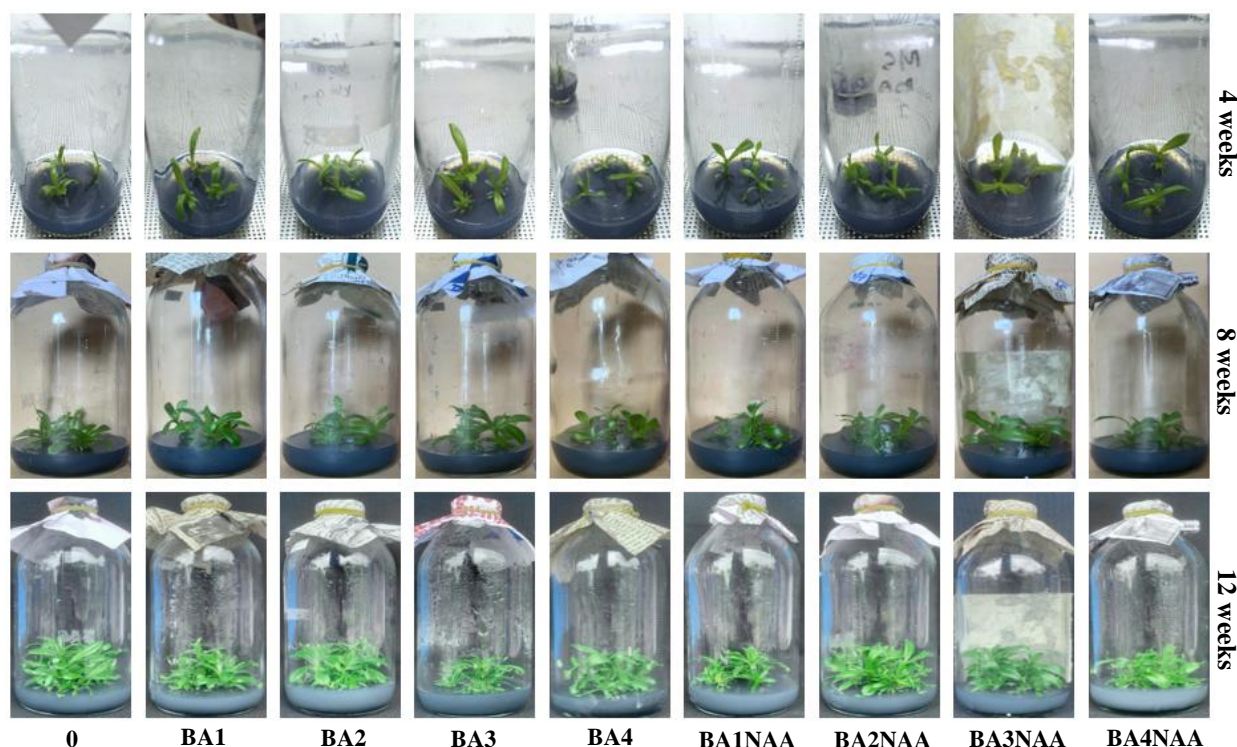
culture medium, resulting in reduced shoot growth (17). By contrast, a report by Yudhanto *et al.* (2015) showed that cytokinin supplementation was unnecessary for *in vitro* shoot multiplication of *N. mirabilis*, as only 1 mg.L<sup>-1</sup> NAA in MS media yielded 5.2 shoots after 10 weeks of culture (18). Nevertheless, this result was lower than when BA and NAA were combined and added to the B5 media in the experiment. Therefore, the selection of plant growth regulator concentrations and appropriate mixing ratios for optimal plant growth and development needs further investigation to find the optimal ratio and concentration for *in vitro* propagation of this species.

**Table 2. Effect of BA and NAA on new shoot number in *in vitro* shoot propagation of *N. mirabilis***

Growth regulator (mg.L <sup>-1</sup> )		New shoots/explant		
BA	NAA	4 weeks	8 weeks	12 weeks
Control		4.00 ± 0.89 <sup>a</sup>	5.83 ± 0.75 <sup>a</sup>	6.83 ± 0.88 <sup>bc</sup>
1	0	3.00 ± 0.63 <sup>a</sup>	4.67 ± 0.52 <sup>a</sup>	6.00 ± 0.88 <sup>c</sup>
2	0	3.00 ± 1.10 <sup>a</sup>	5.17 ± 0.75 <sup>a</sup>	6.33 ± 0.60 <sup>bc</sup>
3	0	3.33 ± 0.52 <sup>a</sup>	5.50 ± 0.84 <sup>a</sup>	6.00 ± 0.63 <sup>c</sup>
4	0	3.83 ± 1.33 <sup>a</sup>	6.00 ± 0.89 <sup>a</sup>	6.50 ± 0.93 <sup>bc</sup>
1	0.5	4.50 ± 0.84 <sup>a</sup>	6.17 ± 0.75 <sup>a</sup>	7.00 ± 0.89 <sup>bc</sup>
2	0.5	3.83 ± 1.60 <sup>a</sup>	6.33 ± 1.51 <sup>a</sup>	10.17 ± 1.90 <sup>a</sup>
3	0.5	3.00 ± 0.98 <sup>a</sup>	5.50 ± 0.84 <sup>a</sup>	8.00 ± 2.07 <sup>b</sup>
4	0.5	5.00 ± 1.26 <sup>a</sup>	6.33 ± 0.82 <sup>a</sup>	7.50 ± 1.17 <sup>bc</sup>

Values in the same column with different letters indicate statistically significant differences ( $p$ -value < 0.05).





**Figure 2. Growth of *N. mirabilis* on B5 media supplemented with BA and NAA after 12 weeks of culture**

#### Effect of IBA and NAA ratios on the *in vitro* growth and development of *N. mirabilis*

IBA is a plant growth regulator belonging to the auxin group, acting as an auxin precursor by converting to the active auxin (indole-3-acetic acid, IAA) (19). Its primary function is stimulating root initiation (19 and 20). Its effectiveness depends on concentration and plant species

(20 and 21). In plant tissue culture, the combination of IBA and NAA is commonly used to enhance root induction and overall plant development. However, there is limited information regarding the appropriate concentration and ratio of these two plant growth regulators for *N. mirabilis*. Therefore, this experiment was performed to evaluate the impact of combining IBA and NAA on this process.

**Table 3. Effect of IBA and NAA on new shoot number in *in vitro* shoot propagation of *N. mirabilis***

Growth regulator (mg.L <sup>-1</sup> )		New shoots/explant		
IBA	NAA	4 weeks	8 weeks	12 weeks
Control		2.50 ± 1.22 <sup>a</sup>	4.83 ± 1.17 <sup>ab</sup>	11.83 ± 2.32 <sup>b</sup>
1	0	3.17 ± 0.75 <sup>a</sup>	5.50 ± 1.05 <sup>ab</sup>	12.00 ± 2.53 <sup>b</sup>
2	0	2.83 ± 1.83 <sup>a</sup>	4.83 ± 2.32 <sup>ab</sup>	11.17 ± 4.22 <sup>bc</sup>
3	0	1.67 ± 0.82 <sup>a</sup>	6.00 ± 2.19 <sup>a</sup>	23.83 ± 3.60 <sup>a</sup>
4	0	1.33 ± 0.52 <sup>a</sup>	3.00 ± 0.89 <sup>b</sup>	6.50 ± 1.05 <sup>c</sup>
1	0.5	3.17 ± 0.75 <sup>a</sup>	5.67 ± 0.52 <sup>ab</sup>	10.67 ± 3.14 <sup>bc</sup>
2	0.5	2.17 ± 0.98 <sup>a</sup>	5.67 ± 1.37 <sup>ab</sup>	8.00 ± 2.10 <sup>bc</sup>

3	0.5	2.83 ± 0.98 <sup>a</sup>	4.33 ± 1.21 <sup>ab</sup>	8.17 ± 2.14 <sup>bc</sup>
4	0.5	1.83 ± 0.75 <sup>a</sup>	4.83 ± 1.60 <sup>ab</sup>	6.83 ± 2.99 <sup>bc</sup>

Values in the same column with different letters indicate statistically significant differences ( $p$ -value < 0.05)

**Table 4. Effect of IBA and NAA on new leaf number in *in vitro* shoot propagation of *N. mirabilis***

Growth regulator (mg.L <sup>-1</sup> )		New leaves/explant		
IBA	NAA	4 weeks	8 weeks	12 weeks
Control		3.83 ± 1.60 <sup>a</sup>	8.00 ± 2.37 <sup>bc</sup>	33.00 ± 2.37 <sup>ab</sup>
1	0	4.17 ± 1.17 <sup>a</sup>	11.83 ± 2.32 <sup>ab</sup>	34.00 ± 4.98 <sup>ab</sup>
2	0	4.00 ± 2.10 <sup>a</sup>	9.83 ± 2.48 <sup>bc</sup>	29.33 ± 9.05 <sup>bc</sup>
3	0	4.00 ± 1.41 <sup>a</sup>	15.17 ± 3.97 <sup>a</sup>	39.67 ± 3.33 <sup>a</sup>
4	0	2.67 ± 0.52 <sup>a</sup>	6.33 ± 1.21 <sup>c</sup>	15.67 ± 1.63 <sup>c</sup>
1	0.5	4.00 ± 1.26 <sup>a</sup>	9.83 ± 1.94 <sup>bc</sup>	21.83 ± 7.36 <sup>cde</sup>
2	0.5	3.50 ± 1.05 <sup>a</sup>	7.67 ± 1.63 <sup>bc</sup>	20.33 ± 6.12 <sup>cde</sup>
3	0.5	3.67 ± 1.03 <sup>a</sup>	9.00 ± 3.22 <sup>bc</sup>	26.33 ± 4.68 <sup>bcd</sup>
4	0.5	2.83 ± 0.98 <sup>a</sup>	8.83 ± 2.71 <sup>bc</sup>	18.00 ± 2.97 <sup>de</sup>

Values in the same column with different letters indicate statistically significant differences ( $p$ -value < 0.05)

The number of shoots, leaves and roots for each treatment was recorded after 12 weeks (Tables 3, 4, 5 and Figures 3, 4). At the 4<sup>th</sup> week of culture, the number of shoots and leaves in the experimental treatments did not differ significantly ( $p > 0.05$ ). Nonetheless, by the 8<sup>th</sup> week and 12<sup>th</sup> week, the differences between the treatments became more apparent. Specifically, in the 12<sup>th</sup> week, the treatment supplemented with IBA at 3 mg.L<sup>-1</sup> resulted in the highest average number of shoots per explant ( $23.83 \pm 3.60$ ), exceeding that of the control (Table 3). Similarly, this sample had the highest number of new leaves, averaging  $39.67 \pm 3.33$  per cultured shoot (Table 4). When combined with NAA, shoot formation in this sample was significantly inhibited. The number of shoots varied only between  $6.83 \pm 2.99$  and  $10.67 \pm 3.14$ , with the number of leaves also decreasing from  $26.33 \pm 4.68$  in the treatment added 3 mg.L<sup>-1</sup> IBA and 0.5 mg.L<sup>-1</sup> NAA to  $18.00 \pm 2.97$  in the

treatment supplemented with 4 mg.L<sup>-1</sup> IBA and 0.5 mg.L<sup>-1</sup> NAA.

Overall, the addition of IBA and NAA had limited effect on the shoot multiplication process of pitcher plants because the primary function of IBA is to stimulate root formation rather than the growth and development of other organs. This result showed that the shoot and leaf development of *N. mirabilis* was not affected by exogenous auxin, depending mainly on endogenous auxin. This was also noted in Setiawan's (2025) study on the same genus pitcher plant (*N. reinwardtiana* Miq.). This data indicated the potential to develop a propagation process for this species without exogenous auxin, significantly reducing costs and simplifying the *in vitro* propagation process (22).

However, when considering the number of roots in the explants, the addition of IBA to the media showed superiority, with samples producing significantly more



roots, mostly higher than in the control. When only IBA was added, root count varied considerably among treatments, with the highest at 1 mg.L<sup>-1</sup> IBA (12.67 ± 3.33 new roots/explant) and the lowest at 2 mg.L<sup>-1</sup> IBA (5.00 ± 1.79 new roots/explant) (Table 5). This result demonstrated that IBA supplementation had a pronounced effect on root induction in *N. mirabilis*. Although when used alone, this process was inconsistent. Rooting was more stable when 0.5 mg.L<sup>-1</sup> NAA was added to the media in all treatments, reaching the highest level in the treatment supplemented with 3 mg.L<sup>-1</sup> IBA and 0.5 mg.L<sup>-1</sup> NAA, with 14.83 ± 1.94 new roots formed (Table 5 and Figure 4). Auxin's physiological role in plants can explain this result. Auxins such as IBA are involved in regulating root formation by increasing cell pressure, stimulating cell division and protein synthesis, thereby promoting cell expansion, elongation, and water uptake (19 and 20). Furthermore, the combination of IBA and NAA can enhance root formation, as IBA promotes root development while NAA helps maintain stable auxin levels and supports root elongation (20 and 23). However, when IBA concentrations increased to 4 mg/L, whether used alone or in combination with NAA, the number of roots formed tended to decrease, possibly due to the toxic effects of excessively high PGR concentrations (23).

**Table 5. Effect of IBA and NAA on new root number in *in vitro* shoot propagation of *N. mirabilis* after 12 weeks**

Growth regulator (mg.L <sup>-1</sup> )	New roots/explant
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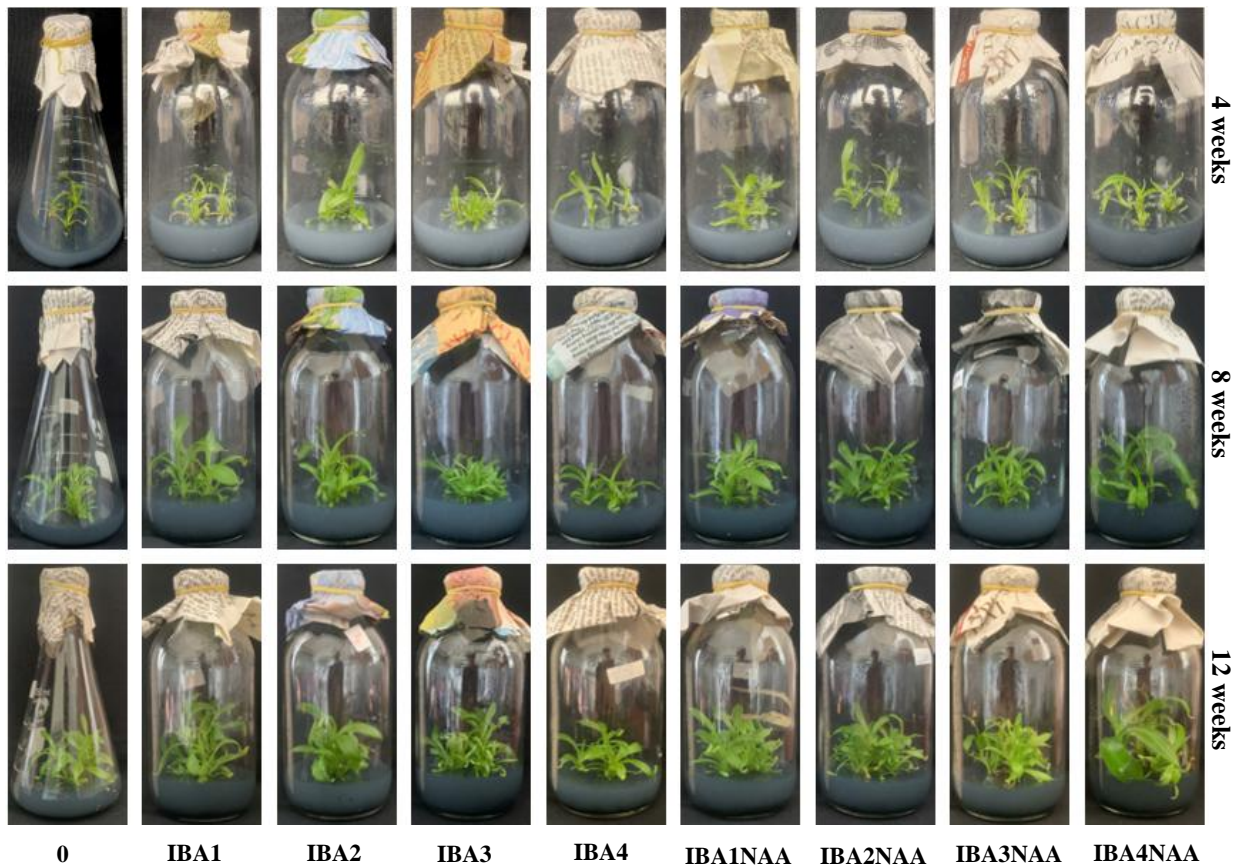
IBA	NAA	
Control		3.00 ± 1.41 <sup>d</sup>
1	0	12.67 ± 3.33 <sup>ab</sup>
2	0	5.00 ± 1.79 <sup>d</sup>
3	0	10.17 ± 1.72 <sup>bc</sup>
4	0	6.83 ± 0.98 <sup>cd</sup>
1	0.5	12.67 ± 3.01 <sup>ab</sup>
2	0.5	12.83 ± 3.06 <sup>ab</sup>
3	0.5	14.83 ± 1.94 <sup>a</sup>
4	0.5	13.67 ± 2.94 <sup>ab</sup>

Values in the same column with different letters indicate statistically significant differences ( $p$ -value < 0.05).

The report by Joshi *et al.* (2022) also noted that an experiment on the same genus (*N. khasiana* Hook. F.) at a concentration of 1 mg.L<sup>-1</sup> IBA resulted in 17.58 ± 0.87 roots per shoot after 3 months of culture on MS media (24). Bhattacharjee *et al.* (2024) also reported root formation in *N. khasiana* Hook. F. with 11.25 ± 1.33 roots produced when NAA was added at a concentration of 3 mg.L<sup>-1</sup>, on shoots cultured on MS ½ media after 28 days of culture. This suggests that adding NAA to the media stabilises and stimulates rooting in cultured shoots (25). In addition, Miguel *et al.* (2020) noted that *N. mirabilis* shoots produced on 1 mg.L<sup>-1</sup> BA media, after transfer to a media added 0.2-1 mg.L<sup>-1</sup> IBA, showed enhanced root formation, with a success rate of 92-100%. Adding 0.5-2 mg.L<sup>-1</sup> IBA to the media promoted strong root development, with a high number of roots (± 11 roots/shoot), with the highest concentration at 2 mg.L<sup>-1</sup> IBA (11.53 ± 1.37 roots/shoot) (2). Furthermore, Ilham *et al.* (2025) also noted that the addition of each NAA individually effectively stimulated root elongation and shortened the time required for root development. Meanwhile, the combination of IBA and NAA stimulated root growth in young oil palm trees (*Elaeis guineensis* Jacq.) (23). The positive effect of

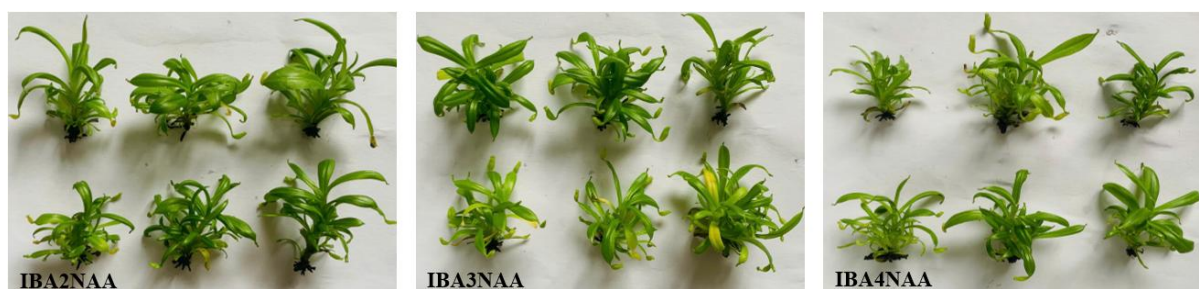


combined IBA and NAA on root induction observed in this study is consistent with general findings reported for *in vitro* cultured plant species.



**Figure 3. Growth of *N. mirabilis* on B5 media supplemented with IBA and NAA after 12 weeks of culture**





**Figure 4. Effect of IBA and NAA on shoot and root propagation from *N. mirabilis* stem explants after 12 weeks of culture.**

## Conclusion

Based on the results, appropriate combinations of BA, IBA, and NAA play important roles in the *in vitro* growth and development of *N. mirabilis* (Lour.) Druce. The combination of BA (2 mg.L<sup>-1</sup>) and NAA (0.5 mg.L<sup>-1</sup>) effectively enhanced shoot and leaf development, while IBA (1-3 mg.L<sup>-1</sup>) combined with NAA (0.5 mg.L<sup>-1</sup>) promoted root formation. These findings provide a useful basis for developing an efficient micropropagation protocol for this species. Further studies are required to improve root length and enhance plantlet adaptability during the transition to the *ex vitro* stage.

## Conflict of Interest

The authors declare that they have no known financial conflicts of interest or personal relationships that could influence the research reported in this paper.

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