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Media Manipulation to Enhance *In Vitro* Pitcher Formation in *Nepenthes mirabilis* (Lour.) Druce for Microfloriculture Development

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Abstract

Microfloriculture refers to souvenirs and Living Room Ornaments made from the *in vitro* cultured ornamental plants, which are innovative, unique, environmentally friendly, and can be produced across the year. *In vitro* pitcher-forming *Nepenthes mirabilis* will increase the attractiveness and market value of microfloriculture products. Two experiments have been carried out to stimulate *in vitro* pitcher formation in *N. mirabilis* using a completely randomized design (CRD) of a two-factorial treatment pattern with three replicates. The first experiment tested different Murashige and Skoog (MS) media formulations (full-strength, 3/4MS, 1/2MS, and 1/4MS) and phytagel concentrations (0, 2.5, 3.5, and 4.5 g l^{-1}). The same media formulations and 6-Benzylaminopurine (BAP) concentrations (0, 0.5, 1.0, and 1.5 µM) were tested in the second experiment. The results showed that the explants cultured on 1/4MS media produced the greatest number of shoots (3.83-8.25) and leaves (26.17-26.75), the earliest pitcher formation (27.75±2.06 days), the highest number of pitchers (6.50±2.65), and pitcher-forming leaves (23.30±5.10%). Liquid media enhanced leaves (28.33±4.97) and pitchers (6.42±3.36) formation as well as pitcher-forming leaves percentage (21.37±8.01%). A 1/4MS supplemented with 0.5 µM BAP maximized pitcher-forming leaves (21.08±7.78%) and pitcher number (5.67±2.52). Adding 1.5 µM BAP on 1/4MS media produced the highest number of leaves (38.67±5.69). Full-strength MS produced the lowest number of shoots, leaves, pitchers, and pitcher-forming leaves. This study developed an affordable and effective method to improve *in vitro* pitcher development in *N. mirabilis*, highlighting how nutrient levels and BAP concentration influence pitcher formation, which aids in optimizing culture techniques for ornamental carnivorous plants.

Keywords: BAP; media formulation; microfloriculture; *Nepenthes mirabilis*; pitcher formation

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INTRODUCTION

Microfloriculture® is a registered trademark of souvenirs and Living Room Ornaments (LivROnt) derived from the *in vitro* culture of ornamental plants (Restianto et al., 2024). Several microfloriculture products have been developed using *Anthurium plowmanii* Croat, *Aglaonema* sp. cv. Ruby, *Aglaonema* sp. cv. Kochin, and *Nepenthes mirabilis* (Lour.) Druce. These are new, environmentally friendly, mass-produced throughout the year, and reasonably priced (Suliyanto et al., 2022). There is a potential to develop *N. mirabilis* as a microfloriculture

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product because of the ability to form pitchers more rapidly than other *Nepenthes* species (Putri et al., 2024). *N. mirabilis* with significant morphological diversity, including distinct pitcher formation across developmental stages, is considered a species of both scientific interest and conservation concern, particularly regarding persistent habitat loss (Gilbert et al., 2020).

Enhancing the micropropagation methods can promote sustainable agriculture in developing countries by producing sustainable products and conserving biodiversity (Sapaeing et al., 2020; Rahayu and Banowati, 2022). *N. mirabilis* is in high demand as a commercial ornamental plant due to the rapid formation of pitchers with attractive shapes and colors (Handayani and Hadiah, 2019; Tarigan et al., 2023). This plant has cylindrical green pitchers with an oval-shaped bottom and an hourglass-like top (Hidayat et al., 2018), as well as red-dotted lips (Wardana, 2023). The pitcher wall has a paper-like structure with long red hairs on the inner compartment (Tarigan et al., 2023). *In vitro* pitcher-forming *N. mirabilis* will become an attractive microfloriculture product because of the uniqueness and higher market value.

Nepenthes, the sole genus of the *Nepenthaceae* family (Novitasari and Isnaini, 2021), is extensively used in traditional medicine for the anti-osteoporotic, antibacterial, and antioxidant characteristics (Liu et al., 2021; Rahman-Soad et al., 2021; Angadam et al., 2022). *N. mirabilis* (Lour.) Rafarin, has been specifically used to treat hepatitis, gastric ulcers, diarrhea, diabetes, and high blood pressure (Ye et al., 2021). *Nepenthes* is a carnivorous plant that lives in poor environments containing low nitrogen, phosphorus, and potassium. The plant adapts to these poor conditions by forming a pouch/pitcher to trap insects and other small animals, which are subsequently digested as a source of organic nutrients (Cross et al., 2022; Mithöfer, 2022). Pitcher formation in *Nephentes* is majorly driven by low nutrients, particularly low nitrogen (Handayani, 2021; Agrawal et al., 2022).

In vitro pitcher formation may be stimulated by reducing nutrient availability and limiting the access of explants to the nutrient medium. Murashige and Skoog (MS-1962) is the most commonly used media with a high salt concentration (Park, 2021; Biswas et al., 2024). Reducing the macronutrients in MS media stimulates pitcher formation in *Nepenthes* (Isnaini and Novitasari, 2023; Dkhar et al., 2024). Furthermore, the concentration of the solidifier in the media determines the media properties. Extremely soft media cause hyperhydration and the extremely hard type can inhibit nutrient and water absorption by the explants (Polivanova and Bedarev, 2022; Avila-Victor et al., 2023). Phytagel is a solidifier derived from bacterial fermentation that contains water-soluble anionic polysaccharides as well as a high degree of purity, promoting more effective mineral absorption and better gel formation (Sánchez-Gutiérrez et al., 2023).

The presence of plant growth regulators (PGRs), such as cytokinins, is another factor affecting the growth and differentiation of *Nepenthes* explants during *in vitro* culture (Miguel et al., 2020). Cytokinins can accelerate cell cycle and differentiation as well as induce lateral shoot formation. 6-Benzylaminopurine (BAP) is among the most commonly used cytokinins (Wu et al., 2021; Rahayu and Banowati, 2022) which plays a vital role in stimulating cell division, shoot formation, and leaf initiation (Hussain et al., 2021), as well as promoting pitcher formation and organogenesis in *Nepenthes* (Bhattacharjee et al., 2024). *In vitro* pitcher formation remains a challenge because the required culture conditions are different from those used for micropropagation purposes. Therefore, the balance conditions between pitcher formation and shoot growth optimization should be formulated.

Two experiments have been conducted to stimulate the *in vitro* formation of *N. mirabilis* pitchers. The first experiment examined the interaction effect between media formulation and phytagel concentration on *in vitro* pitcher formation. The second experiment investigated the effect of the interaction between the same media formulations and BAP concentrations on *in vitro* pitcher formation. *N. mirabilis* forming pitchers *in vitro* will be used in the development of microfloriculture products which should survive for > 3 months and be shock-resistant during distribution. This study is expected to produce a medium that can stimulate pitcher formation, provide nutrients to support explant growth for > 3 months, and not be damaged during distribution. Additionally, there is an expectation to increase the availability of *N. mirabilis* pouched seedlings to support microfloriculture development and conservation efforts as well as explore the possibility of producing chemical products from the pitchers. The success of the *in vitro* propagation and pitcher formation of *N. mirabilis* will support sustainable agriculture

in developing countries by conserving natural resources and contributing to earning through microfloriculture development.

MATERIALS AND METHOD

Experimental design

This study aimed to stimulate the *in vitro* formation of *N. mirabilis* pitchers based on two experiments arranged in a completely randomized design (CRD) of a two-factorial treatment pattern with three replicates. The first experiment was carried out to test the effect of the interaction between media formulation (full-strength, 3/4MS, 1/2MS, and 1/4MS) and phytagel concentrations (0, 2.5, 3.5, and 4.5 g l^{-1}). The second determined the interaction effect between media formulations including full-strength MS, 3/4MS, 1/2MS, and 1/4MS and BAP concentrations of 0, 0.5, 1.0, and 1.5 µM.

Explant preparation

The plant materials used were *N. mirabilis* shoot, a Plant *In vitro* Culture Laboratory culture collection, Faculty of Biology, Universitas Jenderal Soedirman. The initial explants were obtained from germinating embryos, which the microshoots were then induced on MS media (MS, Phytotech M519) supplemented with 20 g l^{-1} sucrose and 15 µM BAP (Sigma-Aldrich B3408) and solidified with 0.25% phytagel (Sigma-Aldrich-P8169), then incubated under continuous light at 24 °C. The microshoots were maintained on MS media (MS, Phytotech M519) supplemented with 20 g l^{-1} sucrose and 5 µM BAP (Sigma-Aldrich B3408) and solidified with 0.8% agar (SNP plant) before being incubated under continuous light at 24 °C. The explants used in this study were apical microshoots collected from the microshoot culture stock by excising at 1.5 cm in length with five leaflets and no pitchers or roots.

Plant *in vitro* culture procedures

Preculture

Apical microshoots were planted aseptically onto sterile media (MS0) consisting of an MS medium supplemented with 3% sucrose and solidified with 0.8% agar. Two microshoots were inoculated into each bottle and then grown for 12 days under continuous light at 24 °C to achieve a similar growth state.

Treatment application

The basic medium used was MS medium supplemented with 2% sucrose. In the first experiment, 16 treatment combinations of media formulations (full-strength, 3/4MS, 1/2MS, and 1/4MS) and phytagel concentrations (0, 2.5, 3.5, and 4.5 g l^{-1}) at 50 ml $bottle^{-1}$ were prepared with three replications. To avoid explants drowning on the media with 0 g l^{-1} phytagel (liquid media), a filter paper bridge was used, and 0 g l^{-1} phytagel supported with a filter paper bridge was subsequently referred to as liquid media. In the second experiment, another 16 treatment combinations of media formulations (full-strength, 3/4MS, 1/2MS, and 1/4MS) and BAP concentrations (0, 0.5, 1.0, and 1.5 µM) supplemented with 0.7% agar were prepared. Each treatment medium was prepared for three replicates at 50 ml $bottle^{-1}$. All media pH was measured and set to 5.8 before sterilization, then sterilization was performed in an autoclave at 0.15 MPa and 121 °C for 20 minutes. Each of the explants precultured at the 12-day stage was aseptically planted onto the individual treatment medium and sealed tightly. Cultures were incubated at 24 °C with continuous tube luminescent light for 16 weeks and closely monitored throughout the culture period.

Data collection

The same sets of parameters were measured in the two experiments, which included the number of shoots and leaves, pitcher formation time, the number of pitchers, and the percentage of pitcher-forming leaves. The number of shoots, leaves, pitchers, and pitcher formation time were counted when the size was > 2 mm. Pitcher formation was observed and recorded at three-day intervals. Shoots, leaves, and pitchers were counted at week 16. Shoot and leave data presented were the increased numbers, of which the data were deducted from an initial number of shoots (1) and leaves (5). The data were recorded, and the explants were photographed using an iPhone XR, while the pitcher image was taken using HAYEAR Simul-focal 7X-45X Zoom Stereo HDMI 14MP Industry Binocular Microscope Head Camera 144 LED Light 0.5X-2X Auxiliary Objective Lens.

Data analysis

Analysis of variance (ANOVA) was conducted for the obtained data using DSAASTAT VER 1.514 software to generate results at 95% and 99% significance levels. The treatment averages were compared using Duncan's multiple range test (DMRT) at a 95% confidence level.

RESULTS AND DISCUSSION

Effect of media formulation and phytagel concentration

The ANOVA results (Table 1) showed that *N. mirabilis* explant growth and the pitcher formation at 16 weeks after planting were controlled by media formulation and phytagel concentration, but no interaction between these factors was observed. Media formulations significantly influenced all parameters measured ($p < 0.05$), while the phytagel concentrations only significantly influenced the number of leaves and pitchers, as well as the percentage of pitcher-forming leaves ($p < 0.05$). These results showed that media formulation and phytagel concentration independently determined explant growth and pitcher formation in *N. mirabilis*. The results of media formulations significantly controlling all parameters measured were in accordance with Oberschelp and Gonçalves (2018), Iswara et al. (2019), and Shrivastav et al. (2020). Iswara et al. (2019) stated that the growth media provides all the necessary nutrients to meet the needs of the plants to grow and develop. Furthermore, Oberschelp and Gonçalves (2018) and Shrivastav et al. (2020) reported that the growth media play an essential role in providing the macronutrients, micronutrients, carbon sources, and vitamins required by the plants to carry out metabolic processes as well as other physiological activities.

The effect of media formulations on shoot growth and pitcher formation of *N. mirabilis* at 16 weeks after planting is presented in Table 2. The poorest medium (1/4MS) produced the greatest average number of shoots (3.83±0.88), leaves (26.75±5.32), and pitchers (6.50±2.65) per explant, earliest pitcher formation (27.75±2.06 days), as well as the largest percentage of pitcher-forming leaves (23.30±5.10%). These were significantly higher than the values found on richer MS media formulations. Explants cultured on full-strength MS medium had the fewest number of shoots (2.17±0.43), leaves (18.83±3.45), and pitchers (2.00±0.72) per explant, as well as the slowest pitcher formation (48.50±1.73 days), and lowest percentage of pitcher-forming leaves (10.16±1.98%). The poorer the media, the better the explant growth and pitcher formation. *N. mirabilis* shoot and leaf appearances on several MS media formulations at 16 weeks after planting are presented in Figure 1, showing that explants grew better in reduced nutrient content.

The results confirmed that pitcher formation in *Nephentes* was mainly driven by low content of nutrients, specifically nitrogen (Handayani,

Table 1. ANOVA results for the effect of media formulations and phytagel concentrations on shoot growth and pitcher formation of *N. mirabilis* at 16 weeks after planting

Source of variance (*p*-value)	Number of shoots[1)]	Number of leaves	Pitcher formation	Number of pitchers[1)]	Percentage of leaves forming pitcher[2)]
Media formulations	0.027	0.000	0.000	0.000	0.000
Phytagel concentrations	0.199	0.000	0.262	0.000	0.027
Media formulation x Phytagel concentrations	0.396	0.162	0.187	0.779	0.973

Note: [1)]Results of √(X+0.5) transformation; [2)]Results of arcus sinus transformation; *p*-value < 0.01 shows highly significant difference at 1% significance level. *P*-value < 0.05 shows a significant difference at a 5% significance level

Table 2. The effect of media formulations on shoot growth and pitcher formation of *N. mirabilis* at 16 weeks after planting. The values shown are averages with n = 12

Treatment	Number of shoots[1)]	Number of leaves	Pitcher formation time (days)	Number of pitchers[1)]	Pitcher-forming leaves[2)] (%)
Full-strength	2.17±0.43^{b}	18.83±3.45^{b}	48.50±1.73^{a}	2.00±0.72^{c}	10.16±1.98^{d}
3/4MS	3.00±0.86^{ab}	20.08±3.86^{b}	40.33±5.27^{b}	3.00±0.90^{bc}	14.50±2.30^{c}
2/4MS	2.17±0.88^{b}	20.08±7.70^{b}	32.75±2.50^{c}	4.42±2.53^{b}	20.71±3.90^{b}
1/4MS	3.83±0.88^{a}	26.75±5.32^{a}	27.75±2.06^{d}	6.50±2.65^{a}	23.30±5.10^{a}

Note: [1)]Results of √(X+0.5) transformation; [2)]Results of arcus sinus transformation; a, b, c—Averages marked with different letters in the same column are statistically different at DMRT 95%

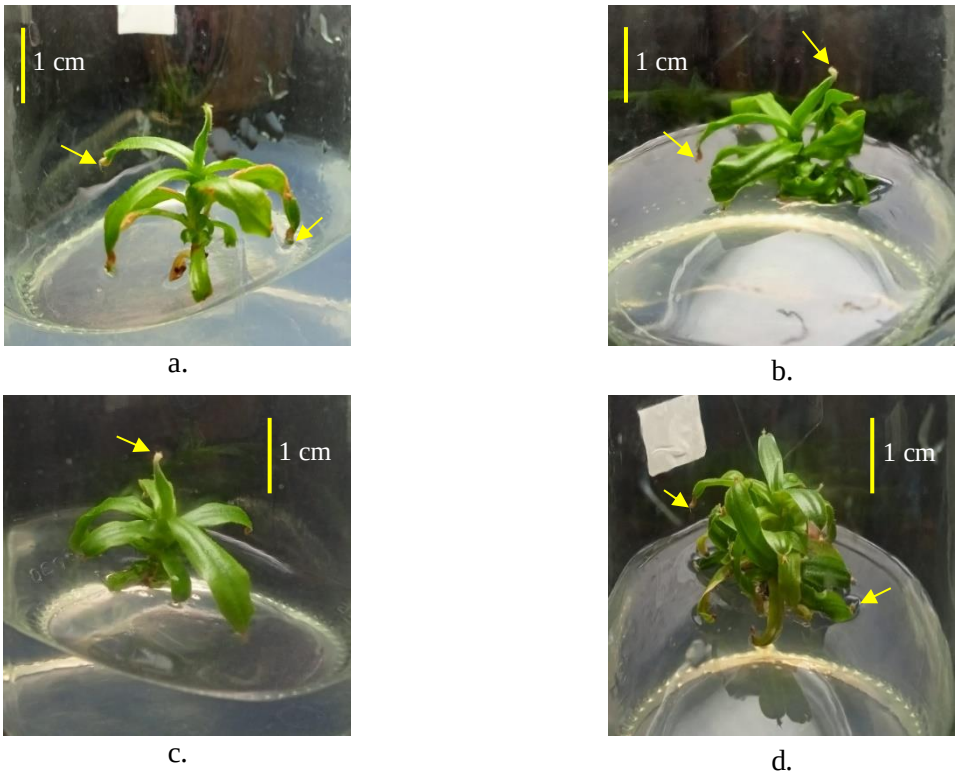


Figure 1. *N. mirabilis* shoot and leaf appearances on several MS media formulations at 16 weeks after planting: (a) Full-strength; (b) 3/4MS; (c) 1/2MS; (d) 1/4MS
Notes: The yellow arrow shows the pitcher

2021), attributed to reducing nutrient availability or limiting the access of explants to the medium. Siregar (2018) reported that media modifications produced different growth responses in *Nepenthes* explants. Optimal type and concentration of nutrients are generally needed for the development and differentiation of plant tissue (Hameg et al., 2020; Arteta et al., 2022). However, Capó-Bauçà et al. (2020) and Givnish and Shiba (2022) found that *Nepenthes* growth was facilitated by limiting factors. *Nepenthes* grow well in nutrient-poor environments, with low essential nutrient content such as nitrogen, phosphorus, and potassium, as well as high soil acidity levels (Dkhar et al., 2024). This plant adapts morphologically and physiologically to suit nutritional demands by forming pitchers because of the poor nutrient availability in the growing habitat (Hernawati et al., 2022; Mansur et al., 2024). Pitchers in *Nepenthes* originate from the modifications of leaf tips (Schwallier et al., 2020), which are intended to trap and dissolve insects to produce organic compounds as a source of nutrients.

Pitcher formation was most rapidly produced by *N. mirabilis* cultured on 1/4MS (27.75±2.06 days after planting), corresponding with a previous study (Prawestri et al., 2024) that found the fastest time for pitcher appearance in 1/4MS media. The formation of pitchers in *N. mirabilis* is believed to be related to the increase in the number of leaves and the conditions, such as more pitchers appearing in good leaves. Prawestri et al. (2024) reported that increasing the number of leaves increased the number of pitchers formed. Media with low nutrient content is considered similar to the natural habitat of *N. mirabilis*, thereby stimulating *Nepenthes* to form pitchers. Dkhar et al. (2024) reported that shoots cultivated in full-strength MS media did not produce pitchers, and the formation started during the removal of nitrogen from full-strength MS. Isnaini and Novitasari (2023) showed that *N. ampullaria* and *N. rafflesiana* produced excellent pitchers in media with reduced nutrients, such as the 1/4MS and 1/8MS. The used treatment media mimic the natural habitat of *N. mirabilis*, which is a poor nutrient environment, specifically with low nitrogen content.

Table 3 shows the effect of phytagel concentrations on explant growth and pitcher formation of *N. mirabilis*. Based on the results, explants cultured in liquid media supported with a filter paper bridge produced the greatest number of leaves (28.33±4.97), pitchers (6.42±3.36), and percentage of pitcher-forming

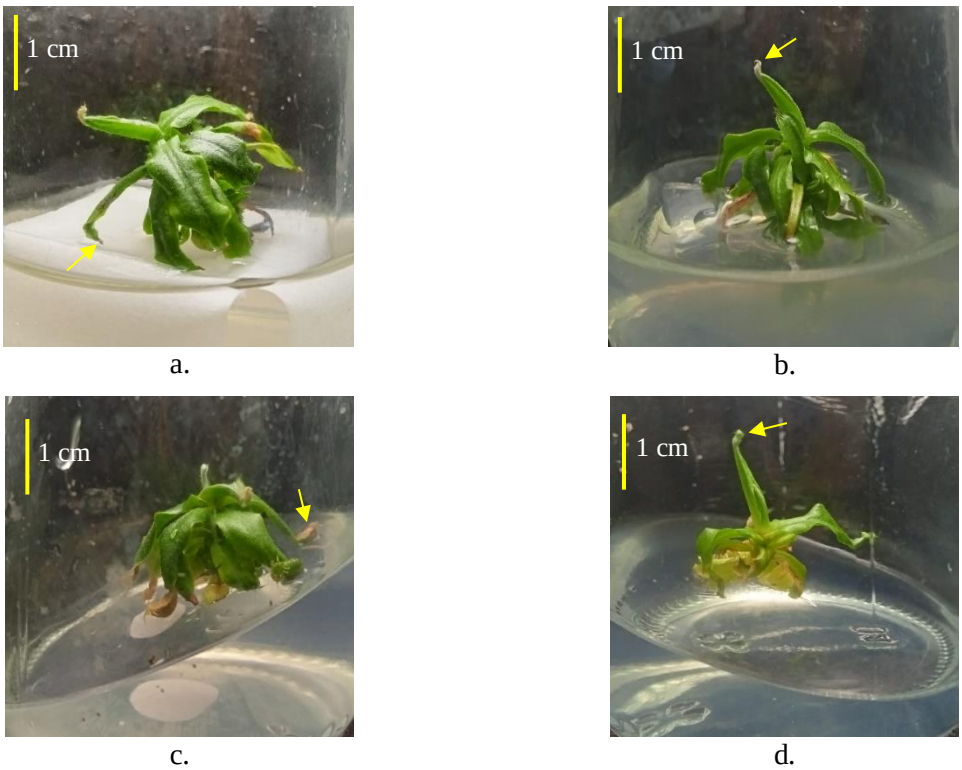
leaves (21.37±8.01) per explant, which were significantly different from those observed in higher phytagel concentrations. Higher concentrations of phytagel (denser media) would lead to a lower number of leaves and pitchers formed, as well as a lower percentage of pitcher-forming leaves. The media solidified with 4.5 g l^{-1} phytagel produced the fewest shoots (17.17±3.38 shoots per explant). According to Figure 2, *N. mirabilis* shoots grown on liquid media supported with a filter paper bridge (0 g l^{-1} phytagel) produced the best shoots, leaves, and pitchers (Figure 2a).

Explants with the best growth in liquid media supported by a filter paper bridge were attributed to the media's physical property which facilitated better nutrient absorption. Meanwhile, counterparts grown on the hardest and most solid media (4.5 g l^{-1} phytagel) showed the smallest number of leaves (17.17±3.38) per explant which could be attributed to reduced nutrient availability and absorption originating from the increased media density, leading to growth inhibition. Explants grown in liquid media absorb nutrients more effectively because of direct contact with the media and there are no physical barriers present in solid media (Nirmal et al., 2023; Nongdam et al., 2023; Zheleznichenko et al., 2023). According to Pasternak and Steinmacher (2024) and Park (2021), the growth in liquid media is more rapid because explants can detect and absorb nutrients better. Nska et al. (2024) and Sun et al. (2025) stated that using liquid culture conditions would accelerate the pace of explant

Table 3. The effect of phytagel concentrations on shoot growth and pitcher formation of *N. mirabilis* at 16 weeks after planting. The values shown are averages with n = 12

Treatment (g l^{-1})	Number of leaves	Number of pitchers[1)]	Pitcher-forming leaves[2)] (%)
0.0	28.33±4.97[a]	6.42±3.36[a]	21.37±8.01[a]
2.5	21.17±4.30[b]	3.75±1.79[b]	16.65±5.51[b]
3.5	19.08±4.02[bc]	2.75±1.13[b]	14.06±3.73[c]
4.5	17.17±3.38[ac]	3.00±1.70[b]	16.59±6.83[b]

Note: [1)]Results of √(X+0.5) transformation; [2)]Results of arcus sinus transformation; a, b, c—Averages marked with different letters in the same column are statistically different at DMRT 95%



a. b. c. d.

Figure 2. *N. mirabilis* shoot and leaf appearances on several phytagel concentrations at 16 weeks after planting: (a) 0; (b) 2.5; (c) 3.5; (d) 4.5 g l^{-1}

Note: The yellow arrow shows the pitcher

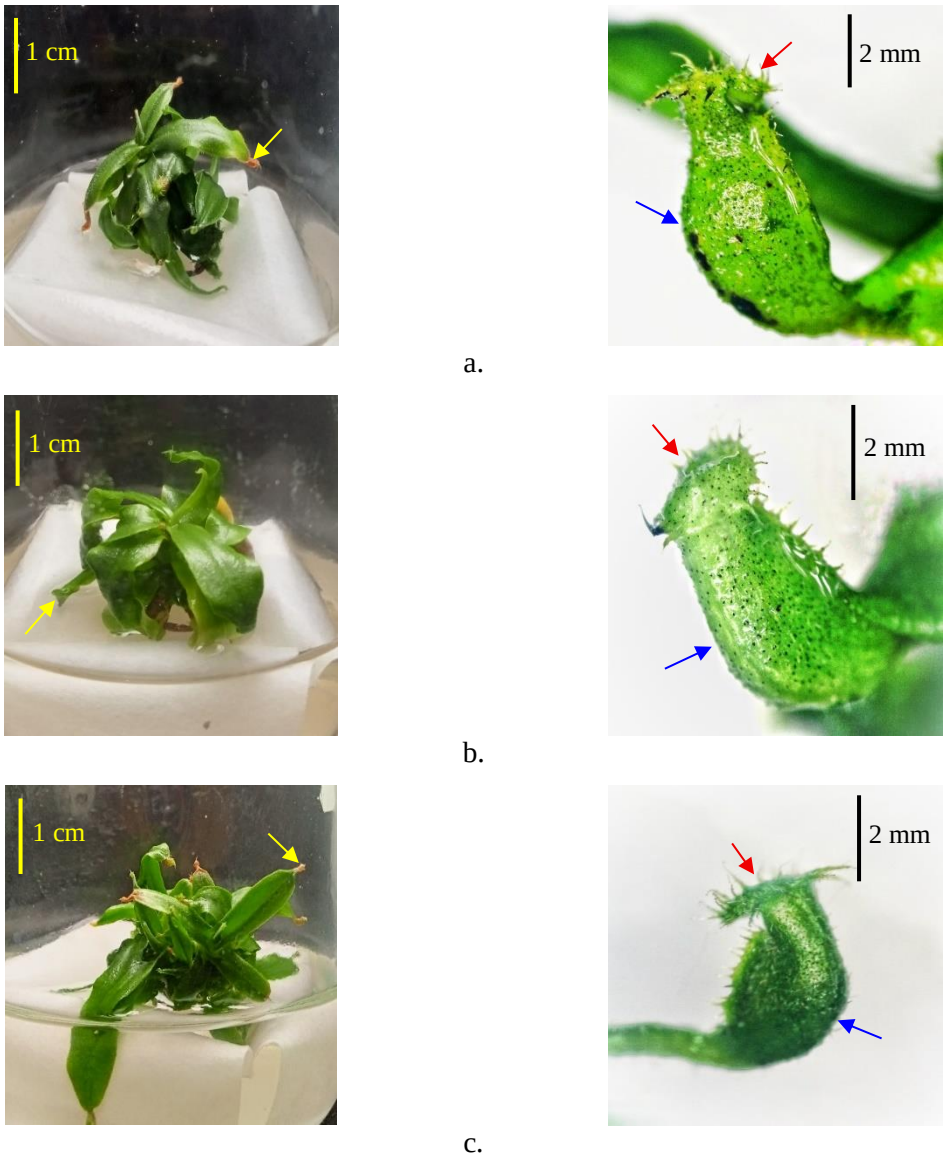


Figure 3. *N. mirabilis* shoot, leaf, and pitcher appearances on liquid 1/4MS at 16 weeks after planting: (a) replication 1; (b) replication 2; (c) replication 3
Note: Arrows colors of blue, red, and yellow show the pitcher body, lid, and pitcher, respectively

cell proliferation, leading to more rapid organ development. The success of *in vitro* culture is influenced by the density of the media used (Sarmah et al., 2017). The density is crucial in modulating the growth rate and differentiation of explants due to the influence on osmolarity and oxygen availability, which are essential for proper *in vitro* development (Polivanova and Bedarev, 2022; Pasternak and Steinmacher, 2024). Although there is no significant interaction between media formulation and phytagel concentration, explants grown on liquid 1/4MS (Figure 3) produced good shoots, leaves, and a perfect pitcher structure, with peristome and lid.

Effect of media formulation and BAP concentration

ANOVA results (Table 4) showed that the interaction between media formulation and BAP concentration produced a highly significant effect on *N. mirabilis* number of leaves and pitchers formed ($p < 0.01$) and significantly controlled the percentage of pitcher-forming leaves ($p < 0.05$). The success of seedling propagation during *in vitro* cultures depends on the medium used, which influences plant development (An et al., 2021; Nhut et al., 2022). In the propagation of *N. mirabilis*, using media with a low nutrient content is sufficient for growth. According to Bhattacharjee et al. (2024), medium modification

by reducing nutrient content and adding cytokinin at suitable quantities promotes *N. khasiana* Hook.f. micropropagation. Marković et al. (2023) and Pasternak and Steinmacher (2024) reported that the availability of nutrients in the media as well as the interaction with added exogenous cytokinin led to enhanced explant development *in vitro* culture.

Table 5 shows the effect of the interaction between media formulation and BAP concentration. Based on the results, explants cultured on 1/4MS supplemented with 0.5 µM BAP had the highest percentage of pitcher-forming leaves (21.08±7.78%), leading to the largest number of pitchers formed (5.67±2.52) per explant. There was no pitcher formed on full-strength-0 µM BAP, full-strength-0.5 µM BAP, 3/4MS-0.5 µM BAP, 2/4MS-0.5 µM BAP, and 1/4MS-1.5 µM BAP. Moreover, explants cultured on 1/4MS supplemented with 1.5 µM BAP had the highest number of leaves (38.67±5.69) formed without pitchers, significantly different from those observed in other treatments tested. A greater number of leaves leads to a smaller size of leaves produced, and pitchers are formed from good leaves. Appearances of *N. mirabilis* shoots, leaves, and pitchers on several media formulations and BAP concentrations at 16 weeks after planting are presented in Figure 4, which shows perfect pitcher structures including peristome and lid.

The 1/4MS media produced the best response in the formation of *N. mirabilis* leaves, corresponding with a report by Putri et al. (2024) that lower concentrations of MS media stimulated *Nepenthes* growth and leaf formation. The formation of pitchers and leaves in *Nepenthes* is shaped by nutrient-deficient substrates, which ecophysiologically propel the evolution of carnivorous adaptations in response to nutritional

Table 4. ANOVA results for the effect of media formulations and BAP concentrations on shoot growth and pitcher formation of *N. mirabilis* at 16 weeks after planting

Source of variance	Number of shoots[1)]	Number of leaves	Number of pitchers[1)]	Percentage of pitcher-forming leaves[2)]
Media formulations	0.000	0.000	0.002	0.019
BAP concentrations	0.004	0.008	0.012	0.017
Media formulations × BAP concentrations	0.093	0.001	0.000	0.016

Note: [1)]Results of √(X+0.5) transformation; [2)]Results of arcus sinus transformation; *p*-value < 0.01 shows a highly significant difference in probability test with 1% significance level, *p*-value < 0.05 shows a significant difference in the probability test with a 5% significance level

Table 5. The effect of the interaction between MS media formulations and BAP concentrations on shoot growth and pitcher formation of *N. mirabilis* at 16 weeks after planting. The values shown are averages with n = 3

Treatment	Number of leaves	Number of pitchers[1)]	Pitcher-forming leaves[2)] (%)
Full-strength-0 µM BAP	23.33±2.08^{bcd}	0.00±0.00^{d}	0.00±0.00^{d}
Full-strength-0.5 µM BAP	15.67±2.31^{de}	0.00±0.00^{d}	0.00±0.00^{d}
Full-strength-1 µM BAP	17.33±4.51^{cde}	0.67±1.15^{cd}	5.13±8.88^{bcd}
Full-strength-1.5 µM BAP	20.00±3.61^{bcde}	0.67±1.15^{cd}	3.51±6.08^{bcd}
3/4MS-0 µM BAP	11.67±0.58^{e}	1.00±1.73^{cd}	8.33±14.43^{bcd}
3/4MS-0.5 µM BAP	12.33±2.08^{e}	0.00±0.00^{d}	0.00±0.00^{d}
3/4MS-1 µM BAP	13.33±3.51^{e}	1.67±0.58^{bcd}	13.15±6.27^{ab}
3/4MS-1.5 µM BAP	19.67±4.73^{bcde}	0.67±1.15^{cd}	2.67±4.62^{bcd}
2/4MS-0 µM BAP	15.00±6.24^{de}	0.33±0.58^{cd}	1.96±3.39^{cd}
2/4MS-0.5 µM BAP	15.67±5.51^{de}	0.00±0.00^{d}	0.00±0.00^{d}
2/4MS-1 µM BAP	29.00±6.00^{b}	3.00±1.73^{ab}	10.55±5.99^{abc}
2/4MS-1.5 µM BAP	18.67±3.21^{cde}	1.00±1.00^{bcd}	5.56±5.09^{bcd}
1/4MS-0 µM BAP	20.00±8.72^{bcde}	1.33±1.15^{bcd}	8.93±7.78^{abcd}
1/4MS-0.5 µM BAP	27.00±8.72^{bc}	5.67±2.52^{a}	21.08±7.78^{a}
1/4MS-1 µM BAP	19.00±5.57^{cde}	2.00±1.00^{bc}	10.34±4.05^{abc}
1/4MS-1.5 µM BAP	38.67±5.69^{a}	0.00±0.00^{d}	0.00±0.00^{d}

Note: [1)]Results of √(X+0.5) transformation; [2)]Results of arcus sinus transformation; a, b, c, d, e—Averages marked with different letters in the same column are statistically different at DMRT 95%

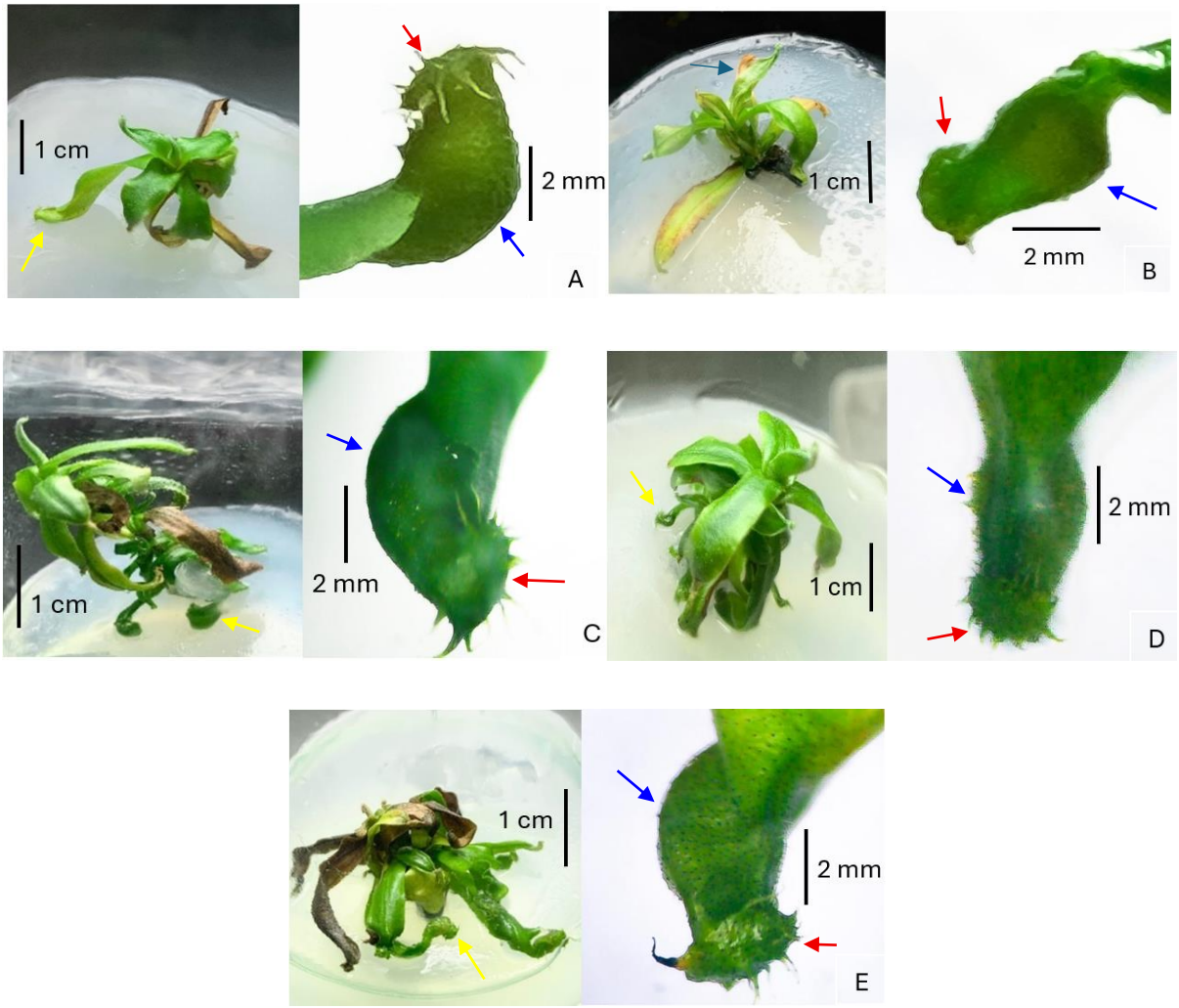


Figure 4. *N. mirabilis* shoot, leaf, and pitcher appearances on several combinations of media formulation and BAP concentration at 16 weeks after planting: (a) 1/4MS-0 µM BAP; (b) 3/4MS-1.0 µM BAP; (c) 1/4MS-1.0 µM BAP; (d) 2/4MS-1.0 µM BAP; (e) 1/4MS-1.0 µM BAP

Note: Arrows colors of blue, red, and yellow show the pitcher body, lid, and pitcher, respectively

scarcity in the native environments (Pavlovič et al., 2016; Adamec et al., 2021). Meinaswati et al. (2022) reported that nitrogen concentrations on media ≤ 1/2-Strength MS produced optimal pitcher growth compared to media with higher nitrogen. García-Pérez et al. (2020) stated that *in vitro* culture media can be modified by adjusting the nutrients needed in each plant to achieve optimal growth. Adding BAP into the media stimulates pitcher formation by affecting the balance of endogenous growth regulators in plant tissues. According to Rahayu and Banowati (2022), the balance level between medium and growth regulators is capable of promoting growth.

The results in Table 5 showed that the formation of pitchers under adequate nutrient conditions required the presence of BAP in relatively higher concentrations (1 to 1.5 µM). However, adding 1.5 µM BAP in poor nutrient conditions did not produce pitchers. The number of shoots and leaves increased at high BAP concentrations, with the shoots appearing smaller and unable to support pitcher formation. The addition of BAP at high concentrations stimulates excessive vegetative growth, such as shoots or leaves, leading to fewer pitcher formations. According to Miguel et al. (2020), BAP can promote the induction of shoot formation followed by leaf formation. *Nepenthes* explants with leaves showing good growth conditions and perfect development often allow pitchers to form at the tips.

It is suggested that 1/4MS media supplemented with 0.5 µM BAP was the optimal

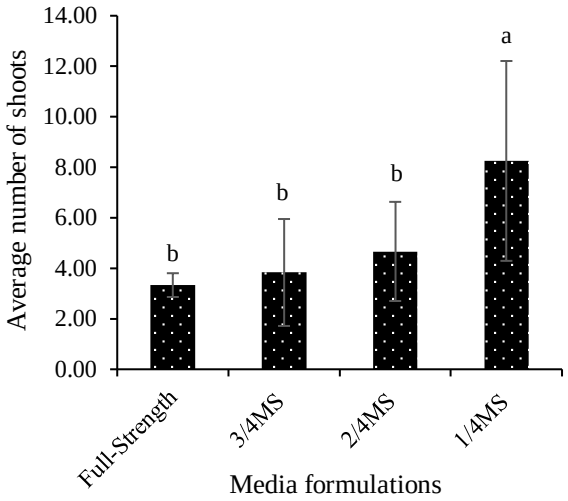


Figure 5. The effect of media formulations on the average number of *N. mirabilis* shoots at 16 weeks after planting. The values shown are averages with n = 12

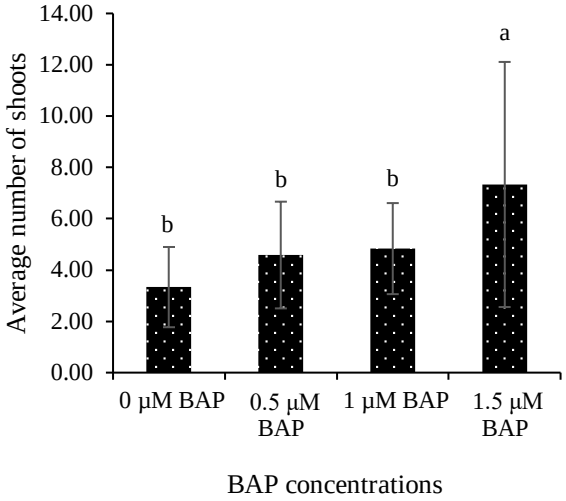


Figure 6. The effect of BAP concentrations on the average number of *N. mirabilis* shoots at 16 weeks after planting. The values shown are averages with n = 12

Note: The averages marked with different letters represent significant differences at 5% DMRT

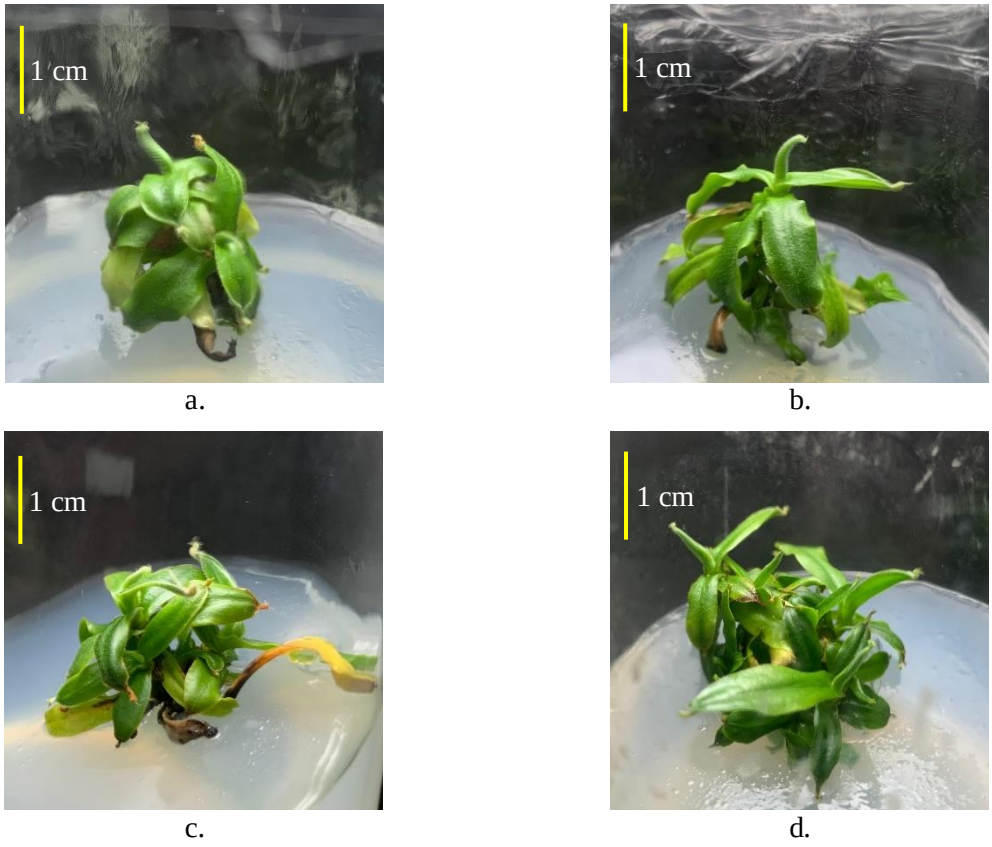


Figure 7. *N. mirabilis* shoot appearances on several BAP concentrations at 16 weeks after planting: (a) 0 ; (b) 0.5; (c) 1.0; (d) 1.5 µM

combination of treatments for pitcher formation in *N. mirabilis*. Low nutrient conditions and BAP concentrations in the media appeared suitable for stimulating pitcher formation, with BAP addition facilitating cell division and differentiation. Low BAP concentrations stimulate optimal pitcher growth and increase the percentage of pitcher-forming leaves. However, higher BAP concentrations can cause uncontrolled shoot growth and suppress the development of other

organs in the explants (Wu et al., 2021; Prasad, 2022).

The ANOVA results (Table 4) also showed that the two independent factors of media formulations and BAP concentrations controlled the number of shoots formed. Explants grown on 1/4MS produced the highest number of shoots (8.25±3.96 shoots per explant), which was significantly different from other media formulations (Figure 5). Poorer media was found to form a greater number of shoots and the lowest number was produced by *N. mirabilis* cultured on full-strength MS (3.33±0.47 shoots per explant), although with no significant difference from 3/4MS and 2/4MS.

BAP addition at 1.5 µM produced the highest average number of shoots (7.33±4.78 shoots per explant), which was significantly different from all applied BAP concentrations (Figure 6). Meanwhile, *N. mirabilis* grown on MS media without BAP produced the lowest number of shoots (3.33±1.56 shoots per explant), showing that adding higher BAP concentration would lead to the formation of more shoots (Figure 7). Asthana et al. (2024) and Ruan and Yi (2022) reported that adding growth regulators such as BAP can increase plant growth by stimulating cytokinesis and organogenesis. Miguel et al. (2020) stated that BAP applied to *in vitro* culture media was very effective in spurring shoot formation compared to conditions without growth regulators, an essential process in plant growth regulation includes cell division, cell elongation, and morphogenesis, followed by shoot formation (Septasari and Mercuriani, 2023). BAP plays a role in cell differentiation, triggers bud cell rupture at the bud eye, and spurs shoot growth and proliferation (Cárdenas-Aquino et al., 2023; Yin et al., 2025).

CONCLUSIONS

The results showed that *N. mirabilis* explants cultured on 1/4MS media produced the most shoots (3.83±0.88), the highest number of leaves (26.75±5.32), and the earliest pitcher formation (27.75±2.06 days), as well as the greatest pitcher count (6.50±2.65) with pitcher-forming leaves (23.30±5.10%). Liquid media enhanced leaf (28.33±4.97) and pitcher formation (6.42±3.36), as well as pitcher-forming leaves percentage (21.37±8.01%). A 1/4MS with 0.5 µM BAP maximized pitcher-forming leaves (21.08±7.78%) and pitcher number (5.67±2.52), while adding 1.5 µM BAP to 1/4MS produced the most leaves (38.67±5.69). More studies on enhancing pitcher color, size, and bioactive compound analysis would benefit microfloriculture development and medicinal investigations.

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