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Modification of Media Formulation and Agar Concentration to Improve Pitcher Plant (*Nepenthes mirabilis* (Lour.) Druce) Micropropagation for Conservation and Microfloriculture Development

Andes Khofifah Putri[1], Rendie Prasetyo[1], Elly Proklamasingsih[1], Paul A. Davison[2] and Sugiyono[1*]

[1]Department of Botany, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia; [2]School of Biosciences, The University of Sheffield, Sheffield, United Kingdom

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Abstract

The pitcher plant (*Nepenthes mirabilis* (Lour.) Druce) is a unique plant listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II and is protected in Indonesia. Conventional propagation of *N. mirabilis* is difficult and takes a longer time. Therefore, *in vitro* culture method is proposed. This study aimed to determine the best and most economical media formulation and agar concentration for *N. mirabilis* micropropagation. This research has been carried out experimentally using a completely randomized two-factor factorial design. The first factor was the media formulation (full-strength Murashige and Skoog (MS), half-strength MS, half-strength MS + AB mix, and AB mix) and the second factor was agar concentrations (6, 8, and 10 g l^{-1}). Twelve treatment combinations were obtained and repeated 5 times to produce 60 experimental units. The explants were apical microshoots (1.5 cm long with 5 leaflets). The cultures were incubated at 24 °C under continuous light for 16 weeks. The parameters measured included shoot emergence time, number of shoots, number of leaves, and shoot length. The data were analyzed using variance analysis followed by Duncan's multiple range test at a 95% confidence level. The results showed that half-strength MS medium resulted in the highest number of shoots and leaves and the longest shoot length, whereas adding 8 g l^{-1} agar resulted in the fastest shoot emergence time. Half-strength MS medium solidified with 8 g l^{-1} agar could produce many *N. mirabilis* (Lour.) Druce microshoots to support both conservation and microfloriculture development.

Keywords: AB mix; agar; *in vitro*; microshoots; MS

INTRODUCTION

The pitcher plant (*Nepenthes*, a member of the *Nepenthaceae* family) is an ornamental plant with a pitcher of unique form, size, and color (Wardhani, 2019). The genus *Nepenthes* has also been utilized in folk medicine for a long time in India and Southeast Asia countries. They are used to treat leprosy, cholera, night blindness, gastrointestinal discomfort, dysentery, stomachache, and bed-wetting (Sanusi et al., 2017). In addition, *Nepenthes mirabilis* (Lour.) Rafarin has been used as a folk medicine in the treatment of jaundice, hepatitis, gastric ulcers, ureteral stones, diarrhea, diabetes, and high blood pressure (Thao et al., 2016). *Nepenthes mirabilis* (Lour.) Druce is a species of *Nepenthes* well known for producing pitchers of varying colors and shapes (Amanda et al., 2019). According to the International Union for Conservation of

* **Corresponding author**: sugiyono@unsoed.ac.id; gieks_sugiyono@hotmail.com

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Nature (IUCN) Red List, *N. mirabilis* is included in the Least Concern (LC) category but is listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II (Clarke, 2014) and the Indonesian Government protects *N. mirabilis* plant by granting conservation status based on Law No. 5/1990, and Government Regulation No. 7/1999, and No. 8/1999 (Government Regulation, 1999; Handayani et al., 2005; Pranata et al., 2020). The *N. mirabilis* population in its natural habitat is declining due to over-exploitation, poor conservation efforts, the incidence of forest fires, and changes in forest function (Suwardi and Navia, 2015). As a result, conservation efforts need to be carried out using good propagation techniques to provide high-quality seed (Suwardi and Navia, 2015).

The commercial attractiveness of *N. mirabilis* and the availability high-quality seeds will support the development of an eco-friendly, creative economy product called microfloriculture. Microfloriculture is a registered trademark of *in vitro* culture-based ornamental plants for the gift market as souvenirs and Living Room Ornaments (LivROnt) (Restianto et al., 2024). Microfloriculture products offer several benefits, such as (1) being novel and distinctive, (2) eco-friendly, (3) able to be mass-produced year-round, and (4) being reasonably priced when compared to non-green souvenirs (Suliyanto et al., 2022).

Propagation of *Nepenthes* through seeds is limited by the difficulty in obtaining seeds and the length of seed germination time. *N. mirabilis* is a monoecious plant, meaning that each plant has either a male or female flower, which results in difficulty obtaining seeds from natural crosses (Isnaini et al., 2021). In addition, *N. mirabilis* seeds are difficult to plant due to low germination rate and long germination time, and the seedlings take up to a year to grow. Propagation via cuttings, on the other hand, is hampered by the time it takes to prepare the parental plant, the limited number of cuttings obtained, and the difficulty in stimulating shoot and root growth so that the number of offspring produced is small (Siregar, 2020). Therefore, *Nepenthes* propagation via *in vitro* culture could be an alternative due to the ability to produce large numbers of seedlings/ progenies in a relatively shorter time, making it more efficient for large-scale plant propagation (Messyana et al., 2023).

The success of plant *in vitro* culture is controlled by several factors, including the growth medium and solidifying agent. Murashige and Skoog (MS) medium is commonly used in plant *in vitro* culture due to its high nutritional content (Desyana and Isda, 2020; Lengkong et al., 2023). Alternatively, AB mix® is a compound fertilizer typically used in hydroponic systems that may have the potential to be used as an MS substitute since it is nutrient-dense and cheaper (Hidayanti and Kartika, 2019; Arianandа et al., 2020; Pratiwi et al., 2023). The gelling agent is also an essential factor in plant *in vitro* culture. Most plant *in vitro* culture media are in the solid state, which require a solidifying agent. Various solidifying agents used in plant *in vitro* culture include agar, bacto agar, agarose, gelzan, gellan gum, gelrite, and phytagel (George et al., 2008). A correct solidifying agent concentration is important. When media is too soft, it can lead to a hyperhydration. In contrast, nutrient and water absorption will be inhibited when media is too hard (Gangopadhyay et al., 2009).

Agar is often used in plant *in vitro* culture because it is relatively cheap and easy to obtain, has high stability and clarity, and is resistant to metabolism during culture (Priyadarshan, 2019). This study aimed to determine the best and most economical media formulation and agar concentration for the propagation of *N. mirabilis* microshoots inside *in vitro* culture. This research is expected to accelerate and increase the production of *N. mirabilis* plantlets to support the conservation and mass production of microfloriculture products.

MATERIALS AND METHOD

Plant material

The plant material used was *N. mirabilis* microshoots, a culture collection of Plant *In Vitro* Culture Laboratory Faculty of Biology Universitas Jenderal Soedirman. The culture was maintained on media consisting of MS basic medium (Phytotech M519) supplemented with 20 g l^{-1} sucrose and 5 µM BAP (Sigma-Aldrich B3408) and solidified with 8% agar (Swallow®). The explants were prepared by cutting the apical microshoot to 1.5 cm long and consisting of 5 leaflets. The microshoot explants were cultured on MS media without any additional growth regulator and incubated at 24 ℃ under continuous

light for 12 days to obtain explants at the same growth phase.

Microshoot multiplication

The study was conducted experimentally at the Plant *In Vitro* Culture Laboratory, Faculty of Biology Universitas Jenderal Soedirman, using a completely randomized two-factor factorial design. The first factor was the media formulation consisting of full-strength MS, half-strength MS, and half-strength MS supplemented with AB mix and AB mix. The second factor was agar concentration consisting of three levels: 6, 8, and 10 g l^{-1}. All treatment combinations were replicated 5 times, resulting in 60 experimental units. Microshoots were planted onto the treatment medium, 1 microshoot per bottle, and were then incubated for 16 weeks at 24 °C under continuous light. The variable observed was the growth of the *N. mirabilis* microshoot, as measured by shoot emergence time, number of shoots, number of leaves, and shoot length. The shoot emergence was recorded when the shoots were 2 mm long. The observation was carried out at three-day intervals. The number of shoots and leaves formed were counted after 16 weeks of culture. Shoot length measurements were taken at 16 weeks by placing the explants on sterile millimeter blocks, and then the difference between the initial and final lengths of the shoot was calculated. The data obtained were then used for each parameter's Relative Growth Rate (RGR) calculations. The RGR was calculated according to Hunt (1990) using the Equation 1.

$$RGR = \frac{(\ln H_2 - \ln H_1)}{(t_2 - t_1)} \quad (1)$$

Where H is parameter measured at respective time and t is time at two time intervals, t_1 and t_2.

Data analysis

The data obtained were analyzed with Analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at a 95% confidence level using DSAASTAT VER 1.514 software.

RESULTS AND DISCUSSION

The ANOVA results on the effect of media formulation and agar concentration on *N. mirabilis* (Lour.) microshoot growth at 16 weeks after planting (Table 1) showed that the media formulation significantly affected almost all parameters measured except shoot emergence time. This condition follows Poothong et al. (2018), who state that media modification is necessary to obtain optimal *in vitro* culture growth needed by each plant. In contrast, the agar concentration only significantly influenced the shoot emergence time. These results are similar to reports by Podwyszynska and Olszewski (1995) and Khumaida et al. (2012). Podwyszynska and Olszewski (1995) showed that the gelling agent did not significantly affect the multiplication rate of rose, cordyline, and homalomena. In contrast Khumaida et al. (2012) reported that media type did not significantly affect *in vitro* shoot induction of *Anthurium plowmanii*. In addition, the interaction between media formulation and agar concentration had no significant influence on any observed parameters.

Plant responses to media formulation and compactor concentration can differ due to differences in nutrient content and media density. According to Inaya et al. (2021), nutrients are essential chemical elements that plants need in balanced amounts to carry out various metabolic processes and physiological activities that support growth and differentiation. Furthermore, Avila-

Table 1. Summary of ANOVA results on the effect of media formulation and agar concentration on *N. mirabilis* (Lour.) microshoot growth at 16 weeks after planting

Source of variance	Parameters						
	Shoot emergence time	Number of shoots	RGR-number of shoot	Number of leaves	RGR number of leaves	Shoot height	RGR-shoot height
p-media formulation	0.0726	0.0000**	0.0000**	0.0000**	0.0000**	0.0000**	0.0000**
p-agar concentration	0.0382*	0.4648	0.2905	0.7344	0.6527	0.9670	0.9491
p-media x agar	0.1585	0.6169	0.5549	0.4196	0.4462	0.1515	0.2041

Note: ** and * are statistically different at F test 99% and 95%, respectively

Victor et al. (2023) showed that solidifying agents play a role in determining the density of the media, with the concentration level influencing both water availability and nutrient uptake.

Looking at the *N. mirabilis* microshoot emergence time, DMRT results (Figure 1) on the effect of agar concentration on the average of *N. mirabilis* microshoot emergence time at 16 weeks after planting showed that microshoots cultivated on media solidified with 8 g l^{-1} agar produced the fastest shoot emergence, with an average emergence time of 27.90 ± 1.73 days after planting. Figure 1 also shows that agar concentrations lower or above 8 g l^{-1} delayed shoot emergence. This result is slightly different from that reported by Li (2020), in which adding 6 and 8 g l^{-1} agar to media can result in faster shoot growth because the lower agar concentration allows explants to make better contact with the media, increasing nutrient penetration into plant tissue and increasing the diffusion rate. Muzika et al. (2024) stated that gelling agents support explants and impact their development and differentiation. Gelling agents such as agar change culture humidity, the availability of water, dissolved media components, and the nutrient diffusion process, all of which influence explant growth.

The highest agar concentration, 10 g l^{-1}, showed the longest shoot emergence time. These

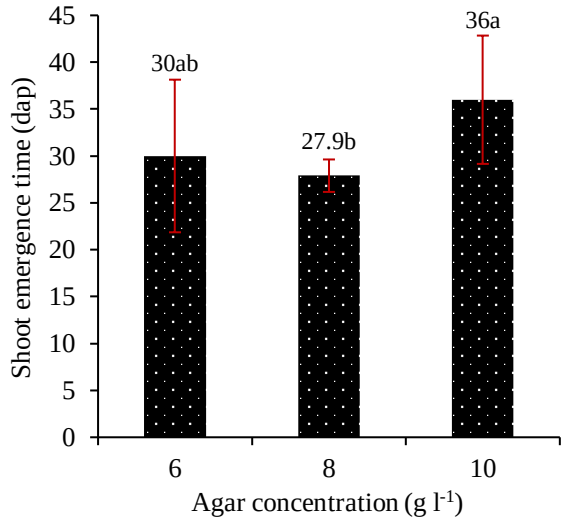

Figure 1. The effect of agar concentration on the average *N. mirabilis* microshoot emergence time (n = 20)

Note: a, b —Means marked with different letters in the same line are statistically different at DMRT 95%

results indicate that increasing agar concentration inhibits explant growth or differentiation. According to Priyadarshan (2019), high medium viscosity reduces water availability and makes it harder for explants to touch the media, which decreases nutrient absorption. In addition to restricted nutrient absorption, excessive media viscosity leads to a lack of water availability, which might hinder explant development due to the explants experiencing water stress (Kosmiatin et al., 2014).

One of the most important parameters of *in vitro* plant propagation is shoot development. DMRT results (Figures 2 and 3) showed the average number of shoots and the average RGR number of shoots under different media formulations. The data in Figure 2 showed that *N. mirabilis* microshoots grown on half-strength MS media resulted in the highest average number of shoots (10.13 shoots $explant^{-1}$). In contrast, the lowest number of shoots was observed on the explants grown on AB mix media, with an average number of shoots formed of 5.67 shoots $explant^{-1}$. RGR analysis also showed consistent data in which the highest RGR was observed in *N. mirabilis* grown on half-strength MS media with an RGR of 0.14 shoots per week, and the lowest RGR was shown by explants grown on AB mix media with an RGR of 0.11 shoots per week. Microshoots appearance in different media formulations is shown in Figure 4. Figure 4 shows that microshoots grown on half-strength MS media produced significantly more shoots (Figure 4C) compared to *N. mirabilis* microshoots grown on full-strength MS media (Figure 4B) and half-strength MS + AB mix (Figure 4D) or AB mix (Figure 4E).

Caldeira et al. (2021) reported that carnivorous plants can adapt to low-nutrient conditions by modifying their leaves to form an insect trap. In addition, Rejthar et al. (2014) reported that reducing nutrient concentrations in the media and supplementing with low concentrations of cytokinin is quite effective for the micropropagation of carnivorous plants. Nutrition can also indirectly influence the auxin-cytokinin ratio produced by plants. Kocjan Ačko et al. (2019) and Xia et al. (2021) reported that lower nutrient availability may interfere with meristematic cell development, leading to reduced apical dominance and increased lateral shoot development. Therefore, the large increase in the number of shoots on microshoots grown

in half-strength MS medium might be due to a decrease in the auxin ratio due to meristem formation inhibition caused by a lower nutrient supply to the explants.

According to Poothong et al. (2018), plant *in vitro* culture growth media can be improved by modifying the media to suit the nutrients needed by each plant. Optimizing nutrients in the medium can produce significant changes in shoot morphology, such as increased shoot length and numerous and healthier leaves. MS media is known to have a high mineral salt content and is widely used for various plants, but this does not mean it is optimal for all types of plants. Media with a lower total nutrient content, such as half-strength MS media, can more effectively stimulate cell differentiation in several plant species, including *N. mirabilis.*

Shoot growth can also be related to the osmotic potential of the medium. Based on the availability of ions in the treatment media, full-strength MS and half-strength MS + AB mix media have a higher ion content than half-strength MS. In contrast, AB mix has a lower ion content than the three other media. An increase in the concentration of ions leads to a decrease in the osmotic potential of the medium. The highest number of shoots produced by explants on half-strength MS media is thought to be due to the ion content and osmotic potential of this media was

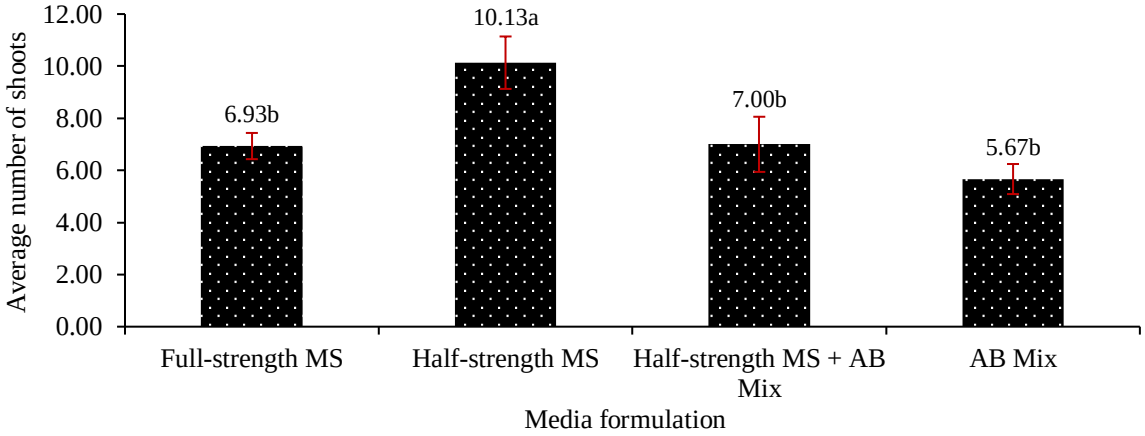

Figure 2. The influence of media formulation on the average number of shoots 16 weeks after planting (n = 15)

Note: a, b —Means marked with different letters in the same line are statistically different at DMRT 95%

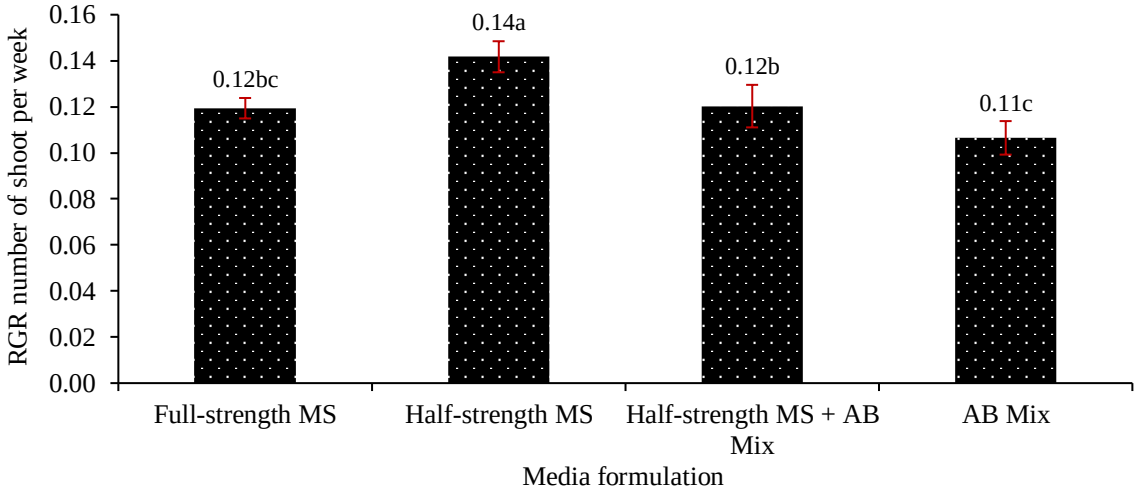

Figure 3. The influence of media formulation on the average RGR number of shoots of *N. mirabilis* at 16 weeks after planting (n = 15)

Note: a, b, c —Means marked with different letters in the same line are statistically different at DMRT 95%

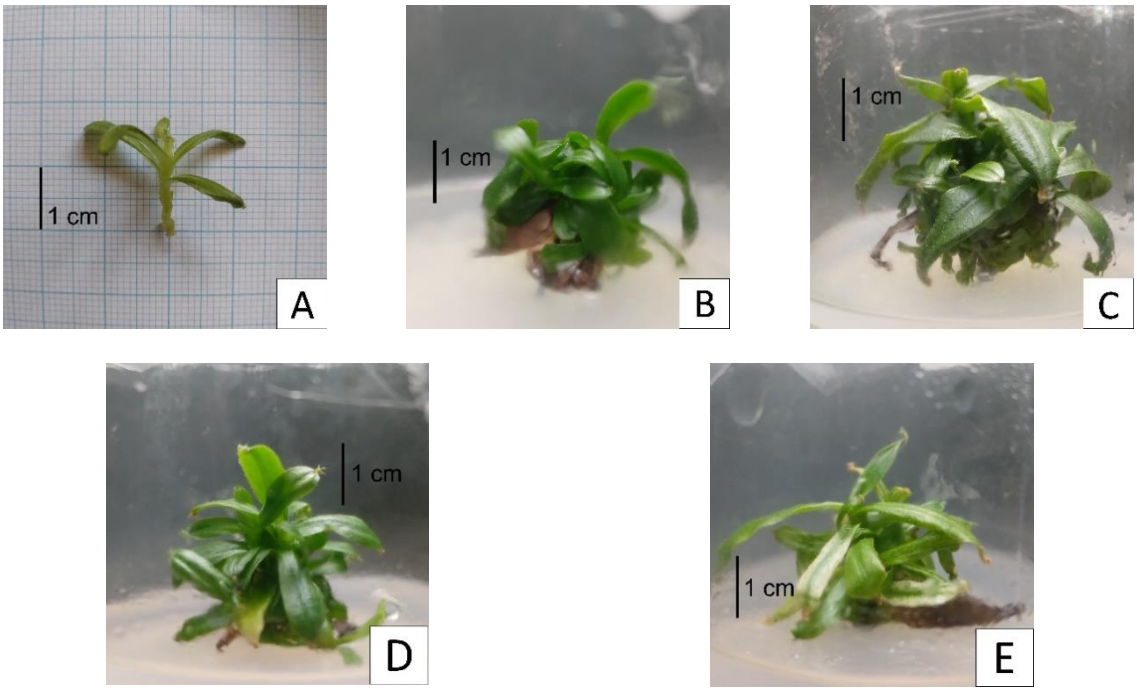

Figure 4. Shoots of *N. mirabilis*: (A) Early shoots at 0 weeks after planting; (B) Shoots grown on full-strength MS; (C) MS half-strength; (D) Half-strength MS + AB Mix; (E) AB Mix at 16 weeks after planting

optimal for the explant's needs. *N. mirabilis* grown on media with a low osmotic potential due to the high content of dissolved ions, such as full-strength MS and half-strength MS + AB mix media, must maintain their turgor pressure by reducing the osmotic potential, which resulted in lower microshoot growth (Novenda and Nugroho, 2016).

An increase in plant height indicates primary growth due to cell division and elongation in the meristematic area (Prasetya et al., 2021). The growth regulator auxin plays a significant role in increasing plant height. In the apical bud, the cells are still actively dividing, so they will regenerate more easily (Alfaris et al., 2020). The apical microshoot (Figure 4A) used in this study has a meristematic area that can produce endogenous auxin to stimulate cell division and elongation (Supriyanto et al., 2022). Cell division and elongation in the microshoot will be manifested as an increase in microshoot height.

The DMRT results on the effect of media formulation on average shoot height (Figure 5) showed that the use of half-strength MS media resulted in the highest average shoot height (31.87 mm). Moreover, DMRT results on the effect of media formulation on the average RGR of shoot height of *N. mirabilis* (Figure 6) also showed that using half-strength MS media resulted in the highest RGR calculation of 0.046 mm week^{-1}. These results showed that in addition to auxin, the nutritional composition can also influence the ability of plant cells to divide and elongate.

Rejthar et al. (2014) found that increasing nutrients in the media reduced the height of carnivorous plants such as *Drosera intermedia*. Shoots grown on MS medium with lower nutrient concentrations were much taller than shoots grown on full-strength MS medium. According to Fahmi (2022), a lack of nutrition can decrease plant productivity; conversely, excess nutrition can inhibit plant growth and increase economic costs. Pandey et al. (2021) and Ren et al. (2022) stated that nutrient concentrations exceeding the required threshold may reduce plant growth and quality due to nutrient toxicity.

The lowest shoot height was observed on *N. mirabilis* grown on AB mix medium (Figure 5), with an average height of 22.60 mm and an RGR of 0.025 mm week^{-1} (Figure 6). This low shoot elongation might be due to the unavailability of vitamins in the AB mix medium. Srilestari and Suwardi (2019) stated that vitamins such as thiamine (B1) are essential and often used in plant *in vitro* culture to accelerate cell division. Thiamine acts as a coenzyme in cell metabolism

and can increase hormonal activity in plant tissue, encouraging cell division and growth. In addition, myoinositol has also been reported to increase plant height because it plays a role in controlling auxin (Heriansyah et al., 2014), which is also absent in the AB mix medium.

The lower osmotic potential resulting from the high ion content in full-strength MS and half-strength MS + AB mix media can inhibit explant elongation. A lower osmotic potential will inhibit metabolic processes and decrease cell turgor pressure, which ultimately inhibiting cell division. The correct water balance is needed in cell elongation process because the force for cell elongation is caused by turgor pressure (Anugrah et al., 2022). It is reported by Hadiyanti and Mariyono (2019) that a plant water deficit reduces the diameter and height of cats' whiskers (*Orthosiphon aristatus*) due to decreased turgor pressure.

The DMRT results on the effect of media formulation on the average number of leaves of *N. mirabilis* at 16 weeks after planting (Figure 7) showed that the highest average number of leaves was produced by *N. mirabilis* grown on half-strength MS media, which produced 58.333 leaves. In contrast, *N. mirabilis* cultured on AB mix medium produced the lowest number of leaves, averaging 30.067 leaves explant^{-1}. Moreover, DMRT results on the effect of media formulation on the average RGR number of leaves of *N. mirabilis* at 16 weeks after planting (Figure 8) also showed that the highest average of RGR was produced by *N. mirabilis* grown on half-strength MS media, with the highest relative growth rate of 0.151 leaves week^{-1}. The lowest

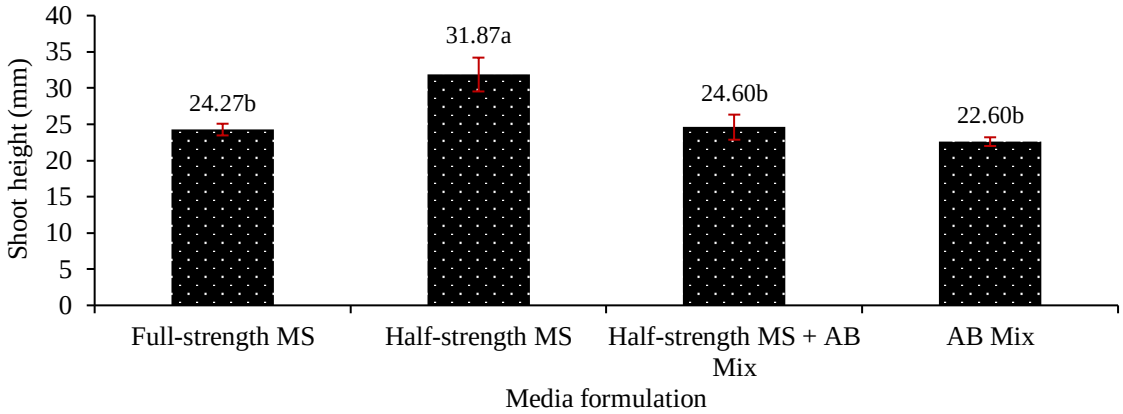

Figure 5. The effect of media formulation on the average shoot height of *N. mirabilis* at 16 weeks after planting (n = 15)

Note: a, b —Means marked with different letters in the same line are statistically different at DMRT 95%

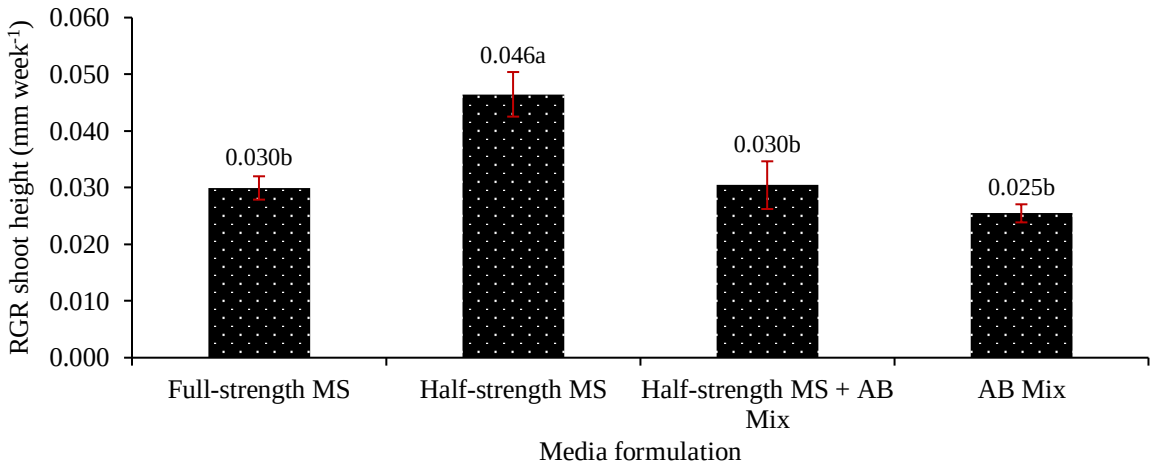

Figure 6. The effect of media formulation on the average RGR shoot height of *N. mirabilis* at 16 weeks after planting (n = 15)

Note: a, b —Means marked with different letters in the same line are statistically different at DMRT 95%

RGR was also produced by *N. mirabilis* cultured on AB mix medium with an average RGR of 0.111 leaves week^{-1}. These results were also consistent with the number of shoots (Figure 2) and shoot height (Figure 5) data, in which the highest number of shoots and shoot height were also shown by explants grown on half-strength MS medium, and conversely, the lowest number of shoots and shoot height were shown by explants cultured on AB mix medium. The number of leaves seems to be correlated with the number of shoots produced and shoot height attained. Amelia et al. (2020) showed that the number of shoots can influence the number of leaves, as an increase in the number of shoots will be followed by an increase in the number of leaves. Shoot height can also affect the number of leaves because increasing plant height may also cause an increase in the number of nodes from which leaves can emerge.

The *N. mirabilis* cultured on half-strength MS also showed longer and broader leaf morphology than *N. mirabilis* grown on other media (Figure 4). According to Hidayat et al. (2020), increasing number of leaves indicates that plant growth has been improved. The appearance of leaves growing well on a half-strength MS medium suggests that the nutrients in this media meet all the explant's

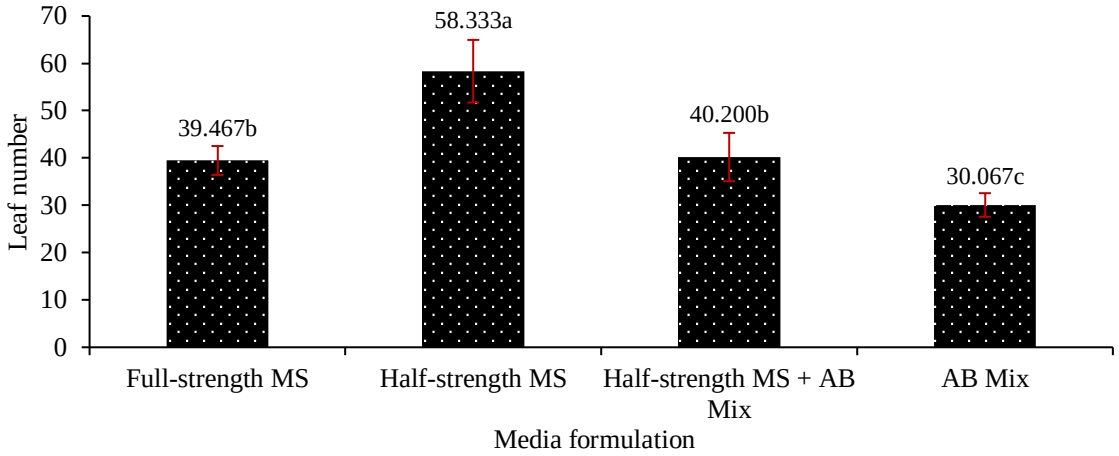

Figure 7. The effect of media formulation on the average number of leaves of *N. mirabilis* at 16 weeks after planting (n = 15)

Note: a, b, c —Means marked with different letters in the same line are statistically different at DMRT 95%

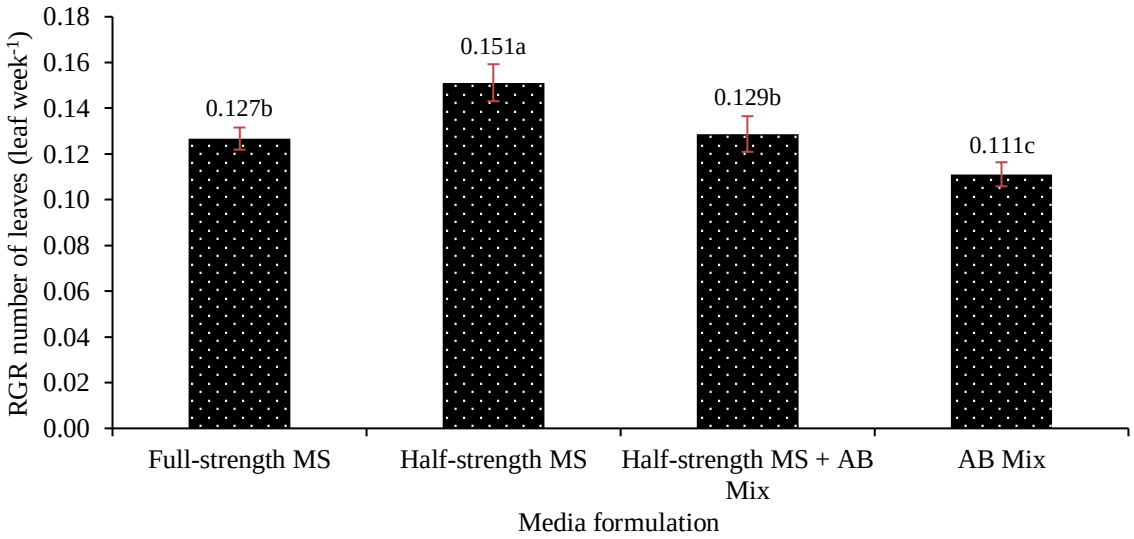

Figure 8. The effect of media formulation on the average number of leaves and RGR number of leaves of *N. mirabilis* at 16 weeks after planting (n = 15)

Note: a, b, c —Means marked with different letters in the same line are statistically different at DMRT 95%

nutritional requirements for forming leaves. The influence of growth media on leaf growth has been reported by Siti-Suhaila and Norwati (2021), where hybrid *Nepenthes* (*N. viking x N. miranda*) grown in half-MS medium produced longer and broader leaves than those grown on full-strength MS medium. Moreover, Siti-Suhaila and Norwati (2021) also reported that good *Nepenthes* leaf growth might be due to adaptation to an environment with a lower nutrient content. A low nutrient content may induce leaf formation, leading to increased pitcher formation to trap insects as a nutritional supplement. According Handayani (2020), the tips of the leaves will form pockets attached to tendrils when the nutrients in the environment are insufficient. The tendrils originate from the mother leaves, and the tendril's tip is modified into a sac to catch and digest insects. The more leaves that are formed, the better the morphology, the greater the number of pitchers that can be produced, and the better the pitcher structure.

The growth of *N. mirabilis* was influenced by the media formulation as measured by the best number of shoots, number of leaves, and shoot height, all of which were consistently better on half-strength MS medium. Half-strength MS media is known to have lower nutrient concentrations than full-strength MS and half-strength MS supplemented with AB mix. These findings indicate that the nutritional content in half-strength MS media is both sufficient and not excessive for *N. mirabilis* to support growth and differentiation. Half-strength MS also has a dissolved ion content that is neither too high nor too low, so the osmotic potential between plants and the media is balanced, reducing the possibility of a decrease in cell turgor pressure that can inhibit explant growth.

The lowest growth of *N. mirabilis*, as seen on AB mix media, could be due to the low ion content of this media and the lack of supplemented vitamins and myoinositol. Although using AB mix did not significantly increase growth, this study shows that *N. mirabilis* explants will still grow in this medium. AB mix medium has previously been put forward by Alfaris et al. (2020) for use in plant *in vitro* culture as an alternative to MS media, as it provides essential nutrients for explant growth and differentiation.

CONCLUSIONS

The increase in the number of shoots, leaves, and shoot height of *N. mirabilis* (Lour.) Druce is influenced by the media formulation used, while the time of shoot emergence is only influenced by the agar concentration used. The half-strength MS media formulation produced the highest number of shoots, leaves, and shoot height of *N. mirabilis* microshoots. At the same time, an agar concentration of 8 g l^{-1} resulted in the fastest shoot emergence time. Half-strength MS medium solidified with 8 g l^{-1} agar could produce many *N. mirabilis* (Lour.) Druce microshoots, which in turn will support both the propagation and conservation of these potential medicinal and ornamental plants. Further research is needed to induce root formation to produce good plantlets and ease acclimatization.

ACKNOWLEDGEMENTS

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