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Research Article

IBA-induced rooting in *Nepenthes reinwardtiana* Miq. cuttings for germplasm conservation

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ABSTRACT

Nepenthes reinwardtiana Miq. is an endemic species distributed in Sumatra and Kalimantan and classified as least concern due to the lack of recent research data on its abundance and distribution. Excessive exploitation, deforestation, the long time required to reach the generative phase, and the dioecious flowering type have caused a population decline. Plant propagation through cuttings can support conservation activities. The success of cuttings is determined by the concentration of plant growth regulators (PGR), making studies on PGR concentration important. This research aimed to determine the best indole-3-butyric acid (IBA) concentration for inducing roots and shoots in N. reinwardtiana. The study was conducted from February to October 2023. The research was arranged based on a completely randomized design with IBA concentration treatments consisting of 5 levels: 0, 5, 10, 15, and 20 mg L^{-1}. The results showed that there was no significant effect of IBA on all variables. However, 5 mg L^{-1} IBA gave the best responses on shoot height increase (10.9 cm), leaf number increase (5.0 leaves), and root length (8.7 cm). These findings suggest that the growth of N. reinwardtiana cuttings may rely heavily on endogenous auxin levels, reducing the observable effects of exogenous IBA application. The non-significant results highlight the potential of this species to thrive without external auxin under certain conditions, which could simplify propagation protocols.

Keywords: biodiversity; conservation; endemic species; extinct; plant growth regulator

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INTRODUCTION

According to the World Rain Forest Report (Butler, 2023), Indonesia ranks 8th in plant biodiversity with a total of 19,232 species. One of Indonesia's endemic flora species is the Pitcher Plant (*Nepenthes* sp.), a carnivorous plant capable of trapping and consuming animals. Currently, there are 123 *Nepenthes* species worldwide, with 64 species (52%) found in Indonesia. The distribution of *Nepenthes* in Indonesia includes 31 species in Sumatra, 3 species in Java, 20 species in Kalimantan, 10 species in Sulawesi, 3 species in Maluku, and 12 species in Papua (IUCN, 2024).

It is important to note that *Nepenthes* sp. is protected by Government Regulation No. 7 of 1999 (Appendix PP No. 7/1999) and the Minister of Environment and Forestry Regulation No. P.92/MENLHK/SETJEN/KUM.1/8/2018 (KLHK, 2018). This is in accordance with the Convention on International Trade in Endangered Species (CITES) regulations, where *Nepenthes rajah* and *Nepenthes khasiana*, which are endangered, are listed in Appendix I (species that are prohibited from international trade in any form), while other *Nepenthes* species are listed in Appendix II (species that are not currently endangered but may become so if trade continues). The extinction of these species can be prevented through strict monitoring of international trade in specimens from their natural habitats, which is only allowed for specific non-commercial purposes with special permits (CITES, 2024).

Among the pitcher plant species, *Nepenthes reinwardtiana* Miq. is an endemic species found in Sumatra and Kalimantan. This species is classified as least concern due to the lack of recent research data on its abundance and distribution (IUCN, 2024). Despite its least concern status, excessive exploitation for trade and habitat destruction/deforestation can lead to a decline in natural populations. Additionally, the long time required to reach the generative phase and the dioecious flowering type also contribute to the population decline of *Nepenthes*.

In situ and ex-situ conservation programs are crucial for preserving *N. reinwardtiana.* Several studies have been conducted to develop propagation methods, including seed germination (Nurchayati, 2023), in vitro culture (Novitasari & Isnaini, 2021; Dwiati et al., 2023), and stem cuttings (Wahdani et al., 2022). Vegetative propagation using cuttings is a simple and rapid solution for conserving *N. reinwardtiana.* The advantage of the cutting method is that the parent plant remains alive and produces new shoots. However, the success of cuttings is highly dependent on root formation at the cut site. Several studies have examined the use of auxins in cuttings for various *Nepenthes* species. Wahdani et al. (2022) reported that naphthalene acetic acid (NAA) could be used to induce rooting in *Nepenthes adrianii*. Additionally, Srilestari and Herastuti (2023) also reported that indole acetic acid (IAA) can be used to stimulate root formation in *Nepenthes* cuttings. Research on the effects of indole-3-butyric acid (IBA) on rooting success in other plants has also been reported. For example, the application of 500 mg L^{-1} IBA positively influenced rooting percentage, root number, and root length in blueberry (*Vaccinium* spp.) cuttings (Koyama et al., 2019). At the same concentration, IBA was more effective than IAA in *Morus alba* cuttings (Chen et al., 2023).

IBA is one type of auxin commonly used to induce rooting in various plants. IBA is more effective compared to other auxins because it is more stable, resistant to degradation by plant enzymes, and relatively unaffected by light and high temperatures (Nale et al., 2024). Information on the use of IBA in shoot cuttings of *N. reinwardtiana* is still limited, making research on IBA concentration important to support future conservation efforts. The objective of this study was to determine the best indole-3-butyric acid (IBA) concentration for inducing roots and shoots in *N. reinwardtiana*.

MATERIALS AND METHODS

Study area

This study was conducted from February to October 2023 at the greenhouse of the Faculty of Agriculture, Universitas Andalas, Padang, West Sumatra. The tools in this study included pots, hand sprayers, scalpels, measuring glasses, erlenmeyer flasks, micropipettes, plastic covers, labels, stationery, rulers, measuring tapes, strings, and cameras. The materials used *N. reinwardtiana* cuttings, IBA, distilled water, and sphagnum moss as the planting medium.

Experimental design and procedures

The experiment used a completely randomized design with IBA concentration treatments consisting of five levels: 0, 5, 10, 15, and 20 mg L^{-1}. Each treatment level was replicated three times, resulting in 15 experimental units, with 10 cuttings for each unit.

The cuttings, which were in healthy plants, were taken from Solok Regency. They were neither too old nor too young, with dark green-colored stems. Cuttings were made by slanting the top portion with three nodes. Each leaf on the cutting was trimmed to reduce transpiration.

The cuttings were soaked in IBA solution for 15 minutes to a depth of 3 cm, then planted in sphagnum moss, covered, and incubated for 20 weeks in the greenhouse. The moisture of the planting medium was maintained through regular watering.

Observed parameters included: 1) Shoot height increase measured from the top node to the growing point, 2) Leaf number increase calculated from the number of fully opened leaves minus the number of leaves at planting, 3) Leaf length and width measured from base to tip and at the midpoint of the leaf, 4) Number and length of pitcher, 5) Number and length of roots, and 6) Root spread measured the distance between the two longest roots growing from the main stem.

Data analysis

Data were analyzed statistically using analysis of variance (ANOVA) at a significance level of α = 5%. If the p-value was less than 0.05, Duncan's Multiple Range Test (DMRT) was performed. The data analysis used the Statistical Tool for Agricultural Research (STAR) software.

RESULTS AND DISCUSSION

Increase in shoot height, leaf number, and leaf length

Soaking *N. reinwardtiana* cuttings in IBA solution did not significantly affect shoot height, leaf number, and leaf length. The endogenous auxin hormone in the cutting tissue was likely sufficient to support vegetative growth. Shoot height ranged from 6.3 to 10.9 cm, leaf number ranged from 3.1 to 5.0 leaves, and leaf length ranged from 9.0 to 11.3 cm. However, the IBA concentration of 5 mg L^{-1} generally provided better responses than compared to other treatments (Table 1). This trend shows the vegetative growth response of *N. reinwardtiana* cuttings to IBA. At 5 mg L^{-1}, IBA enhanced shoot height, leaf number, and leaf length. However. At 10-15 mg L^{-1}, growth decreased, possibly due to hormonal imbalance. At 20 mg L^{-1}, growth improved again, likely due to an enhanced auxin signaling overcoming initial suppression. Generally, the dead cuttings showed signs of drying, softening, and fungal growth. Shoot initiates with the division and differentiation of meristematic cells, resulting in light green shoots (Shi & Vernoux, 2022).

The successful formation of shoots in *N. reinwardtiana* cuttings is likely attributed to the presence of sufficient endogenous auxin hormones within the plant tissues. Endogenous auxins play a pivotal role in maintaining apical dominance by regulating the activity of the shoot meristem and suppressing lateral bud growth, thereby prioritizing upward growth. These hormones may also activate key physiological pathways required for root induction and shoot elongation, effectively supporting cutting establishment without the need for additional exogenous auxin application. The lack of significant differences between the control and various IBA treatments suggests that the endogenous auxin levels in the shoot were already adequate to stimulate these processes. This finding highlights the potential of *N. reinwardtiana* to rely on its intrinsic hormonal regulation for propagation (Koike et al., 2020).

Table 1. Increase in shoot height, number of leaves, and leaf length of *N. reinwardtiana* cuttings at 20 weeks after planting.

IBA concentration (mg L^{-1})	Increase in shoot height (cm)	Increase in number of leaves	Leaf length (cm)
0	9.9	4.6	11.2
5	10.9	5.0	11.3
10	7.8	4.2	10.0
15	6.3	3.1	9.0
20	10.6	4.6	10.3

Note: Data in the same column are not significantly different based on the 5% F test.

In general, IBA serves as a precursor or starting material for the synthesis of endogenous IAA, which is responsible for various morphogenetic processes, including cell elongation and the formation of adventitious roots (Nale et al., 2024). IBA is also known to enhance GA_3 synthesis, which regulates shoot length or stem elongation (El-Banna et al., 2023). Exogenous IBA application influences phytohormone activity in plants by augmenting or modulating auxin signals within cells (Chen et al., 2023). Its effects on morphogenesis depend on the relative balance with other phytohormones, particularly cytokinins and GA_3, which together regulate organ formation and plant growth patterns.

Cell elongation is influenced by auxins, which affect the synthesis of structural proteins, causing cell wall expansion and regulating cell elongation at the shoot tip (Du et al., 2020) Additionally, shoot induction is reported to be triggered by endogenous cytokinins synthesized in the roots. Endogenous cytokinins also play a crucial role in cell wall formation during shoot development. Several studies have reported that cytokinins act as transcription factors stimulating genes involved in meristem cell division and differentiation. For example in the molecular aspect, the *Wuschel* gene is expressed in the promeristem, promoting cell differentiation during shoot formation (Abubakar et al., 2023). García-Gómez et al. (2021) also reported that the *AHK4* and *CYCD genes* are highly expressed in meristematic tissues during cell division. Cytokinins can activate the expression of the *AHK* and *ARR* are genes that regulate shoot apical meristem development by promoting cell division (Terceros et al., 2020). Cytokinins are also known to play a role in cell proliferation, endoreplication, and mitosis by regulating the transition from GI (Gap1) to S (Synthesis) and G2 (Gap2) to M (Mitosis) phases involving Cyclin-dependent kinases (CDKs) and cyclins. Additionally, cytokinins stimulate shoot initiation by regulating proliferation in the Shoot Apical Meristem (SAM) (Wu et al., 2021)

Leaf width, number of pitchers, and length of pitcher

Soaking *N. reinwardtiana* cuttings in IBA solution did not significantly affect leaf width, number of pitchers, or pitcher length. The endogenous auxins in the cutting tissues were likely sufficient to support leaf and pitcher growth. The IBA might not affect leaf and pitcher because IBA primarily functions in stimulating root formation rather than promoting leaf organ growth. Leaf width ranged from 2.5 to 2.9 cm, the number of pitchers ranged from 1.4 to 2.0, and pitcher length ranged from 7.3 to 10.4 cm. However, the IBA concentration of 5 mg L^{-1} generally provided a better response as compared to other treatments (Table 2). This result is consistent with other research on *Nepenthes bicalcarata*, which also found that while IBA effectively enhances root development, its influence on shoot and picher growth may be limited (Ningsih et al., 2014)

Table 2. Leaf width, number of pitchers, and pitcher length of *N. reinwardtiana* cuttings at 20 weeks after planting.

IBA concentration (mg L^{-1})	Leaf width (cm)	Number of pitchers	Pitcher length (cm)
0	2.7	1.4	9.6
5	2.9	2.0	10.4
10	2.8	1.4	8.6
15	2.5	1.5	7.3
20	2.7	1.6	8.4

Note: Data in the same column are not significantly different based on the 5% F test.

Increased leaf width and pitcher sizes are associated with increased root number and length, which optimize nutrient and water absorption. Several macronutrients, such as nitrogen, play a central role in plant metabolism as components of proteins, nucleic acids, chlorophyll, coenzymes, phytohormones, and secondary metabolites (Hawkesford et al., 2023). Sulfur is assimilated into amino acids like cysteine, which are used for synthesizing enzymes and coenzymes (Kopriva et al., 2019). Phosphorus is a structural element in nucleic acids and plays a crucial role in energy transfer as part of adenosine phosphate, and in carbohydrate transfer between organelles in leaf cells (Malhotra et al., 2019). Magnesium is necessary for photosynthesis, photoassimilate transport, and protein synthesis (Kwon et al., 2019). Calcium is important for cell wall stabilization and osmotic pressure regulation. Potassium regulates osmotic pressure, which is vital for cell expansion, stomatal movement, sucrose translocation, and water movement driven by mass flow in plants (Hawkesford et al., 2023). Furthermore, leaves are a source of endogenous auxins and carbohydrates, which provide the primary energy source during root formation. Auxins and carbohydrates are translocated from the leaves to the base of the cutting, where interactions between endogenous and exogenous auxins occur to initiate root primordia.

Root number, root length, and root spread

Soaking *N. reinwardtiana* cuttings in IBA solution did not significantly affect number of roots, root length, or root spread. Root number ranged from 4.8 to 8.7, root length from 4.8 to 6.5 cm, and root spread from 6.9 to 7.4 cm (Table 3). The effect of IBA may have been limited by sufficient endogenous auxin levels in the cutting tissues. Roots emerging from the cut surface were black and varied in number (Figure 1). Endogenous auxin is assumed to saturate plant tissue receptors, rendering IBA ineffective in further promoting root development. This saturation leads to a diminished response to external auxin application, as the plant's auxin receptors are already fully engaged (Frick & Strader, 2018). These results indicate that endogenous auxin content was sufficient to stimulate root growth. However, the synthesis of endogenous hormones and nutrient allocation in cuttings are also known to be influenced by the addition of exogenous auxin. This is evident from the increase in the number, length, and spread of roots when IBA was applied, compared to cuttings without IBA even though not significant.

Table 3. Number of roots, root length, and root spread of *N. reinwardtiana* cuttings at 20 weeks after planting.

IBA concentration (mg L^{-1})	Number of roots	Root length (cm)	Root spread (cm)
0	6.6	4.8	6.9
5	8.7	4.9	6.7
10	5.8	5.2	6.7
15	4.8	6.5	7.7
20	7.5	4.9	7.4

Note: Data in the same column are not significantly different based on the 5% F test.

Root induction consists of four stages: root primordial initiation, root pattern formation, root induction, and root emergence (Torres-Martínez et al., 2022). Root induction begins with swelling at the cutting site and from the node. The expression of endogenous hormones and nutrient allocation in the cuttings is influenced by the addition of exogenous auxins. For instance in the hormonal regulation aspect, Chen et al. (2023) found that the application of exogenous auxin (ABT-1) increased endogenous IAA levels and affected adventitious root formation. Besides cell division, cell elongation occurs when endogenous IBA levels increase (Shang et al., 2021). Additionally, auxins can affect endogenous ABA levels, a growth-inhibiting hormone. Auxin regulates ABA levels by modulating its biosynthesis, transport, and degradation, influencing plant growth and stress response (Emenecker & Strader, 2020). ABA levels rise during root primordia preparation and then decrease during root differentiation and formation (Liu et al., 2021).

From a molecular aspect, auxins are known to stimulate the expression of many genes involved in root induction For example, *Apetala2/Ethylene Response Factor* (*AP2/ERF*) transcription factors including *Abscisic Acid Repressor1* and *ERF109* is rapidly induced by wounding and serves as a proxy for wound signal to induce auxin biosynthesis (Ye, Shang, et al., 2020). *Auxin-resistant 1/like aux1* (*AUX/LAX*) influx carriers and *plant-specific PIN-FORMED* (*PIN*) efflux carriers can promote the local accumulation of auxin and induce the regeneration of adventitious roots (Hu et al., 2023), and the WOX5 gene, a regulator of stem cell niche and maintaining meristem activity during root development (Savina et al., 2020). Further, increased expression of *IAA-efflux* *genes* (*PIN1*), *IAA-influx genes* (*AUX1/LAX3*) (Yang et al., 2021), and *ASA* (*Anthranilate Synthase-Alpha*), *ASB* (*Anthranilate Synthase-Beta*) involved in IAA biosynthesis and adventitious root induction have been observed (Solanki & Shukla, 2023). As well as the *miR156 gene*, which also plays a role in adventitious root formation (Ye, Zhang, et al., 2020). Roots not only function in water and nutrient absorption but also support plant stability, store photosynthates, and synthesize cytokinin hormones that play a role in cell division and differentiation, vascular tissue development, and root morphogenesis (Papon & Caurdavault, 2022). Cytokinins stimulate *Cytokinin Response Regulator* (*RR*) genes, key transcription factors in root morphogenesis. More than 25 RR proteins are involved in this process (Zhang et al., 2022).

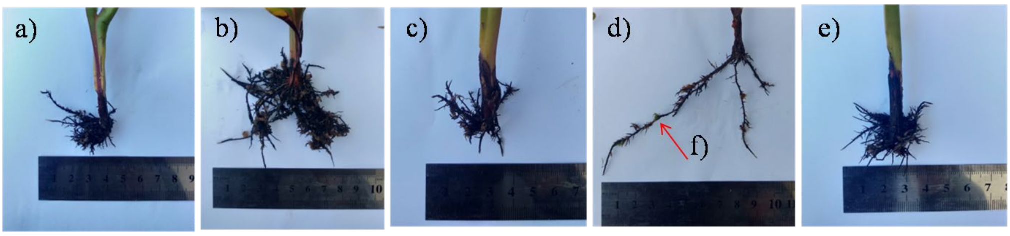

Figure 1. Root growth of *N. reinwardtiana* shoots cuttings from different IBA concentrations. a) 0 mg L^{-1}, b) 5 mg L^{-1}, c) 10 mg L^{-1}, d) 20 mg L^{-1}, e) 25 mg L^{-1}, f) root

CONCLUSIONS

This study provides information that the application of IBA did not have a significant effect on the growth of *N. reinwardtiana* shoot cuttings, indicating that endogenous auxin levels in the plant might already be sufficient to support rooting and shoot development. Nonetheless, the treatment with 5 mg L^{-1} IBA showed a notable trend toward better growth outcomes, producing the highest values for shoot height (10.9 cm), leaf number (5.0), and root number (8.7) compared to other treatments. These findings suggest that while *N. reinwardtiana* can grow effectively with minimal or no exogenous hormone application, specific concentrations of IBA may enhance certain growth parameters under controlled conditions. This highlights the need to investigate the physiological interactions between endogenous hormones and applied plant growth regulators. Future research should focus on understanding the underlying mechanisms and exploring the optimal hormonal balance for propagating *N. reinwardtiana* more efficiently, especially in large-scale conservation or cultivation efforts.

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