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RESPONSE OF SEED GERMINATION AND GROWTH OF *Nepenthes gymnamphora* Nees TO MS MINERAL SALT, PEPTONE, AND THIDIAZURON

Respons Perkecambahan Biji dan Pertumbuhan Kecambah *Nepenthes gymnamphora* Nees terhadap Garam Mineral MS, Pepton, dan Thidiazuron

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ABSTRACT
Nepenthes gymnamphora *Nees is a Java's rare endemic species. Ex situ conservation of this endangered species can be done through in vitro culture technique. The aims of this study were to determine (1) the mineral salt concentration of MS basal media and addition of peptone (P) on* N. gymnamphora *seed germination and seedling emergence and (2) the effects of TDZ in ½MS medium on seedling growth. Seeds were surface sterilized and cultured on four media formulations (½MS, MS, ½MS+P, MS+P) for 8 weeks. In the second experiment, ten-week-old seedlings, 0.25 cm in length were cultured on ½MS supplemented with 0, 0.5, 1.0, or 1.5 mg L^{-1} TDZ. Seedling growth was recorded at 8 weeks of culture. Results of this experiment showed that ½MS was the best medium for* N. gymnamphora *seed germination as indicated by the highest percentage of germination, the tallest seedling, and the fastest seedling emergence. Moreover, the best growth of* N. gymnamphora *was found on ½MS without TDZ.*

Keywords: *Pitcher plant, MS concentration,* Nepenthes gymnamphora*, organic supplement, tissues culture*

ABSTRAK
Nepenthes gymnamphora Nees. merupakan spesies endemik Pulau Jawa yang tergolong langka, sehingga perlu upaya konservasi. Konservasi *ex situ* spesies ini dapat dilakukan dengan teknik kultur jaringan. Penelitian ini bertujuan untuk mengetahui konsentrasi garam mineral media MS dan pepton yang dapat mendukung perkecambahan biji dan menentukan konsentrasi TDZ untuk pertumbuhan kecambah *N. gymnamphora in vitro*. Pada percobaan I, biji *N. gymnamphora* disterilisasi dan ditabur di 4 kombinasi media, yaitu MS, ½MS, dengan dan tanpa penambahan 2 g L^{-1} pepton. Pada percobaan II, kecambah berukuran ± 0,25 cm dengan penambahan beberapa konsentrasi TDZ (0; 0,5; 1; 1,5 ppm) pada media ½MS. Hasil penelitian menunjukkan bahwa media ½MS menghasilkan persentase perkecambahan biji tertinggi (56%) dengan tinggi kecambah kecambah terbaik. Media ½MS tanpa TDZ menghasilkan pertumbuhan kecambah terbaik yang ditunjukkan oleh waktu tercepat munculnya daun, Media ½MS merupakan konsentrasi garam mineral terbaik untuk perkecambahan biji *N. gymnamphora*, tanpa TDZ.

Kata Kunci: Kantong semar, Konsentrasi MS, kultur jaringan, *Nepenthes gymnamphora*, suplemen organik

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INTRODUCTION

The tropical pitcher plant *(Nepenthes gymnamphora* Nees) is one of the typical Indonesian plants endemic to Java. This plant is generally used by the community for traditional medicine, daily needs such as food wrapping, rope binding, and as an ornamental plant (Dariana 2009) in Jeffri et al. (2017)). *Nepenthes*' high attractiveness is in the unique morphology of its pouch and its use in various aspects. This leads to an increase of hunting activities in the forest so that the tropical pitcher plant becomes rare (Rugayah et al. 2017, Kristianus et al. 2018). According to the Convention on International Trade of Endangered Species (CITES) report in 2020, *N. gymnamphora* is included in the Appendix II category which means threatened with extinction. This is in line with the regulation of the Minister of Environment and Forestry of the Republic of Indonesia No P.106/MENLHK/SETJEN/KUM.1/12/2018 which categorizes this species as a protected plant. Conservation efforts need to be carried out, but propagation using the stem cuttings method or conventional seed propagation takes a long time and a small number of tillers are produced (Devi et al. 2013). The tropical pitcher plants under normal conditions are able to produce a germination percentage of 17-83% in a long period (45-65 days) (Chanchula 2013). The germination rate of Nepenthes seeds is low because the food reserves contained in the seeds are very small (Dwiyani 2015).

The solution for effective germination of the tropical pitcher plants is through plant tissue culture with the addition of organic substances, one of which is peptone. Apriliyania and Wahidah (2021) stated that the factors that influence the success of tissue culture techniques are genetics, types of explants, media containing macronutrients, micronutrients, growth regulators, and the addition of organic substances such as peptones. According to Utami et al. (2017) peptone contains amino acids, proteins, vitamins, and nitrogen which are needed to construct cell structures and enzymes during the germination process. Hossain et al. (2010) reported that the germination of *Cymbidium giganteum* orchid seeds using a medium with the addition of 2 g L^{-1} peptone was able to increase a germination percentage up to 100%. The use of peptone 2 g L^{-1} in culture media can also increase root growth of orchid plants because the amino acid tryptophan contained in peptone may be used as a precursor to the hormone auxin (Krisdianto et al. 2020).

The use of peptone to support the germination of *Nepenthes* seeds *in vitro* has not been widely reported. The seeds of *Nepenthes* have very small endosperm, which resulted in a low percentage of germination (17-80%). The conventional germination of *Nepenthes* seeds takes about 2 months. This is one of the reasons why it is important to add organic supplements in tissue culture media to shorten seed germination period. Siriwardana et al. (2013) reported a study related to the *in vitro* germination of *N. mirabilis* seeds and was able to produce 41% sprouts at half the mineral salt concentration of Murashige and Skoog media (½MS).

Nepenthes embryo growth in *in vitro* culture medium requires growth hormones, one of which is cytokinins. The addition of exogenous cytokinins, including thidiazuron (TDZ) to sprouts can accelerate the growth of sprouts. According to Guo et al. (2011) TDZ is a plant growth regulator (PGR) that can induce rapid cell division and stimulate morphogenesis. The cytokinin activity of TDZ is stronger than that of 6-benzylaminopurine (BAP) (Bilal et al. 2011 in Restanto et al. (2018). The role of TDZ in supporting plant growth and development is shown in the emergence of shoots in shoot culture from alfalfa plants (Nurmaningrum et al. 2017).The addition of 0.5 ppm TDZ in MS medium was able to increase plant height, increase the number of shoots and produce the highest number of leaves on *Vanda douglas* orchid plants (Karyanti 2017, Loi et al. 2020).The use of 0.5 ppm TDZ on strawberry shoot culture also produced the highest number of leaves compared to the concentration of higher than 0.5 ppm (Raisya et al. 2020).This study aimed to examine the effect of the mineral concentration of MS media and peptone in stimulating seed germination and determine the concentration of TDZ which can stimulate sprout growth of *N. gymnamphora in vitro* culture, so that it can be used as a source of information and reference material in selecting the right media for the conservation efforts of the tropical pitcher plant *N. gymnamphora*.

MATERIALS AND METHODS

Location and time

The research was conducted from November 2020 to May 2021 at the Plant Tissue Culture Laboratory, Biology of Plant Structure and Function, Department of Biology, Faculty of Science and Mathematics, Diponegoro University. This study consisted of two sequential experiments, namely (1) *in vitro* germination of *N. gymnamphora* seeds in response to MS mineral salt concentration with or without peptone and (2) growth response of *N. gymnamphora* sprouts in ½MS medium with the addition of TDZ.

Seed germination

The research material in the first experiment was the seeds of *N. gymnamphora*. The seeds were sterilized by immersion in a liquid detergent solution for 3 minutes, then in a fungicide solution (10 g L^{-1}) for 15 minutes, then rinsed with sterile distilled water. Next, the seeds were sterilized in a laminar air flow cabinet (LAFC), by soaking in 70% alcohol for 3 minutes and then rinsing with sterile distilled water. The seeds were then soaked in sodium hypochlorite (NaOCl) for 3 minutes and rinsed with sterile distilled water. The seeds were planted in several treatment media: ½MS+0 g L^{-1} peptone, 1 MS+0 g L^{-1} peptone, ½MS+2 g L^{-1} peptone, and 1 MS+2 g L^{-1} peptone with the pH of each medium being 6, followed by sterilization using an autoclave for 15 minutes. The embryo culture media was then incubated at 600 lux for 8 weeks after planting (WAP) with 80% humidity and 18ºC temperature. Parameters observed including percentage of seed germination and germination height.

Figure 1. *Nepenthes gymnamphora*

Sprout growth in vitro

A 10 weeks old *N. gymnamphora* that grew for ±2.5 mm in size (Figure 1) were used as explants and planted in treatment media, namely MS media containing various concentrations of TDZ (0, 0.5, 1, and 1.5 ppm). Sprout cultures were incubated for 8 weeks at 600 lux TL lamp, 80% humidity, and 18ºC temperature. Leaf emergence time and number of leaves were observed at 16 weeks of age in the treatment media.

Data analysis

This study consisted of two experiments using a completely randomized design (CRD). Experiment 1, seed germination of *N. gymnamphora* in response to the concentration of mineral salts in MS medium, without or with the addition of 2 g L^{-1} peptone (½ MS+0 g L^{-1} peptone; 1 MS+0 g L^{-1} peptone; ½MS+2 g L^{-1} peptone and 1 MS+2 g L^{-1} peptone). Experiment 2 was the growth of *N. gymnamphora* sprouts in response to MS medium with various concentrations (0, 0.5, 1.0 and 1.5 mg L^{-1}) of TDZ. Each treatment was repeated 3 times. Each experimental unit consisted of 3 culture bottles, each bottle containing 3 explants. Observations were made on cultures aged 10 to 16 weeks after planting in the treatment media, the observed data were analyzed using analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) using the SPSS 16 application.

RESULTS AND DISCUSSION

Seed germination

Based on the results of ANOVA, the treatment of media mineral salt concentrations (½ and 1 MS) with the addition of peptone (P) (0 and 2 g L^{-1}) was MS; 1 MS; MS+P; and 1 MS+P had a significant effect on the percentage of germinating seeds. Media with mineral concentration of MS without peptone (½MS) produced the highest percentage of

Table 1. The average time of emergence of *N. gymnamphora* sprouts in the treatment of mineral salt concentration of MS media and peptone, namely: ½MS, 1 MS, ½MS+P, and 1 MS+P

Treatment of mineral salt concentration in MS media (MS) and peptone (P)	Sprout's Emergence Time (WAP)			Average (WAP)
	Repeated for			
	1×	2×	3×	
½MS	3	4	3	3.33
1 MS	0	4	0	4
½MS+P	0	5	6	5.50
1 MS+P	5	6	0	5.50

Note: ½MS (½MS + 0 g L^{-1} peptone); 1 MS (1 MS + 0 g L^{-1} peptone); ½ MS+P(½MS + 2 g L^{-1} peptone), and 1 MS+P (1 MS + 2 g L^{-1} peptone)

Table 2. The average height of *N. gymnamphora* Sprouts in the treatment of mineral salt concentration in MS media and peptone, namely: ½MS, 1 MS; ½MS+P, and 1 MS+P at 16 WAP

Treatment of mineral salt concentration in MS media (MS) and peptone (P)	Height of sprout (mm)
½MS	2.67[a]
1 MS	2.47[a]
½MS+P	2.42[a]
1 MS+P	1.96[b]

Note: numbers accompanied by the same letter in the table column indicate that there is no significant difference based on the DMRT test at 95% confidence

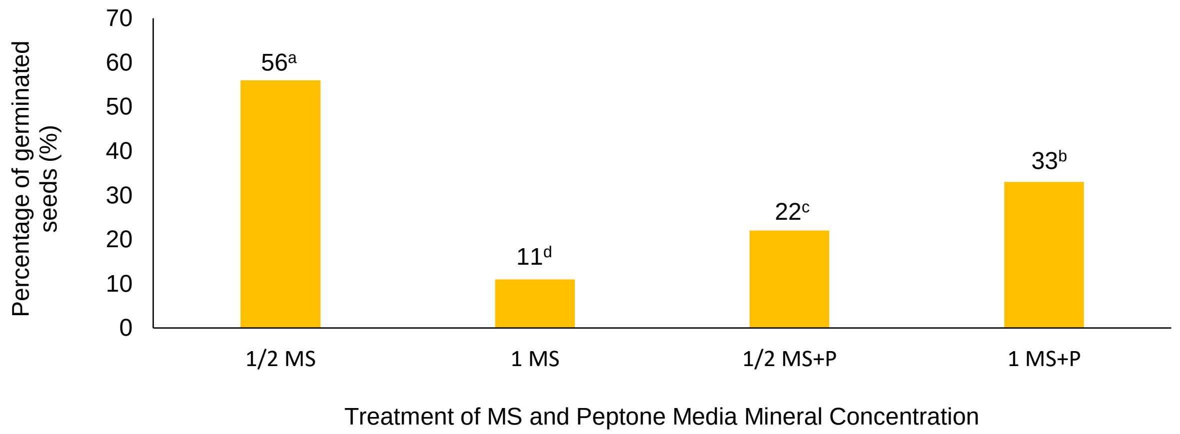

Figure 2. The percentage of *N. gymnamphora* germinated seeds at 8 WAP in the treatment of mineral concentrations of MS media and peptone, namely: ½MS, 1 MS, ½MS+P, and 1 MS+P (numbers accompanied by different letters on the histogram show a significant difference based on the DMRT test at 95% confidence)

germination, namely 56% (Figure 2) and produced germination in a faster time, namely 3.33 WAP (Table 1). This is possible because the media with low concentrations is in accordance with the original habitat of *Nepenthes* that lives in nutrient-poor conditions. Kunita et al. (2011) stated that optimal growth in *Nepenthes* occurred in media with ≤ ½MS concentration. Robinson et al. (2019) confirmed that the germination of *Nepenthes* in its natural habitat occurred around the mother plant with poor soil conditions and high humidity.

The increase in the percentage of germination and the speed of germinating seeds in the treatment of MS media and peptone concentrations of minerals was thought to be related to the ability of *Nepenthes* which was only able to absorb nutrients at low concentrations (Table 2). The ½MS media had a lower nutrient solution concentration, which was half of the MS mineral salt concentration, making it easier for seeds to absorb water compared to media with higher concentrations and the addition of peptone which tended to be more concentrated (1 MS, ≤ ½MS+P, and 1 MS+P). Optimal water absorption will help stop dormancy in seeds, and activate several hormones and enzymes needed during germination to stimulate the growth of plumules (prospective leaves) and radicles (potential roots). Parman (2015) stated that the absorption of water from the media with sufficient levels will stimulate the activation of gibberellins (GA_3) which is then accompanied by the activation of other hormones, such as auxins and cytokinins. Paramartha et al. (2012) confirmed that the presence of auxin will stimulate cell permeability to water to be high, so that the pressure on the cell wall will decrease and

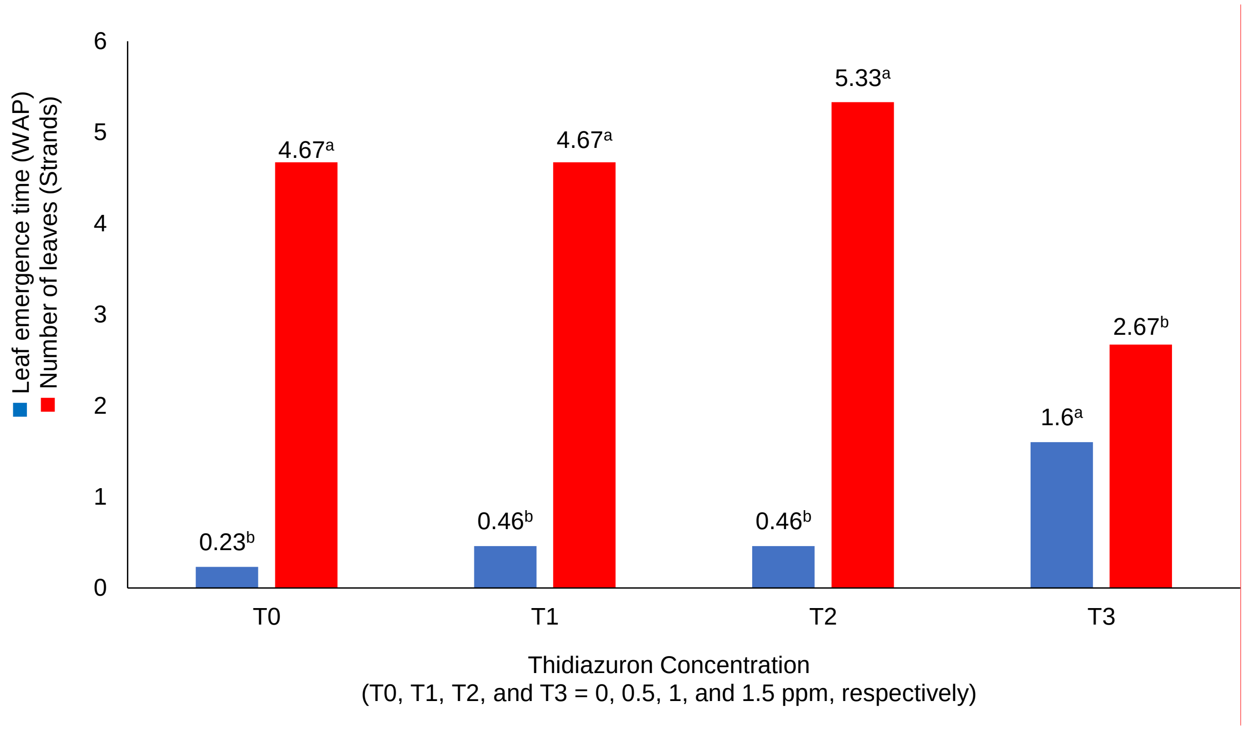

Figure 3. The average time of leaf emergence (WAP) and number of leaves (strands) of *N. gymnamphora* for 8 weeks at the thidiazuron concentration treatment, namely T0: 0 ppm, T1: 0.5 ppm, T2: 1 ppm; and T3: 1.5 ppm (a number accompanied by the same letter in each histogram shows a significant difference based on the DMRT test at 95% confidence)

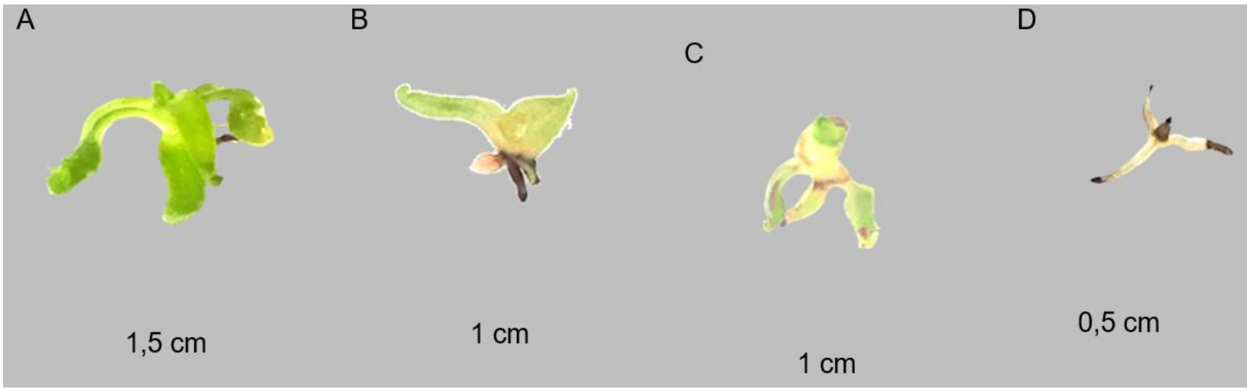

Figure 4. Growth of *N.gymnamphora* sprouts at several concentrations of Thidiazuron (A) 0 ppm (B) 0.5 ppm (C) 1 ppm (D) 1.5 ppm

will soften. This condition causes the seed coat to break, and water from it can enter the cell. The activity of auxin stimulates cytokinins to synthesize proteins to produce cells that will differentiate into new organs.

Seeds that have germinated will go through growth and development. Based on the results of the DMRT test, ½MS, 1 MS, and ½MS+P media produced germination heights that were not different from each other, namely 2.42 mm – 2.67 mm, while 1 MS+P media produced the lowest germination height. The addition of peptone which contains several vitamins, such as thiamin and nicotinate should be able to increase respiration to produce the energy needed for metabolism. Thiamin is required for the oxidative decarboxylation process for the breakdown of pyruvic acid into Acetyl Co-A, and nicotinic acid is also used as a coenzyme for NAD+ and NADP+ which has a hydride ion (H) carrier role, but the result of the experiment show that *Nepenthes* sprouts had the lowest growth in full concentration MS medium with the addition of peptone. The addition of peptone to MS medium with full concentration can increase the concentration of solutes, so that the water potential of the media is lower. These conditions can make it difficult for *Nepenthes* sprouts to absorb nutrients from the media. In nature, *Nepenthes* generally lives in media with low nitrogen composition, so that at low media mineral salt concentrations (½MS) it is able to meet its nutritional needs. Syamswisna (2010) stated that a nitrogen concentration of 0.098% was sufficient to support the optimal growth of *Nepenthes*. Patti et al. (2013) also reported that the use of nitrogen concentration in ≤ ½MS media resulted in optimal growth compared to high nitrogen concentrations. According to Kunita et al. (2011), *Nepenthes* needs these conditions to form sacs at the tips of the leaves. Ubaidillah et al. (2020) added that the sacs produced in *Nepenthes* plants will absorb organic nitrogen from insects, while roots are used to absorb inorganic nitrogen in the form of NO_3^-. Some types of *Nepenthes* that live in nutrient-rich media cannot form sacs.

Growth of sprout

The ANOVA results showed that the ½MS medium that was added with TDZ was 0, 0.5, and 1 ppm resulted in leaf emergence time and the number of leaves, which did not differ from each other (i.e. time to the emergence of leaves 3 - 5 WAP and the number of leaves 2 - 5 strands). However, the addition of TDZ at a concentration of 1.5 ppm resulted in the longest leaf emergence, namely 1.6 WAP, and the least number of leaves, namely 2.67 leaves (Figure 3). Media without the addition of TDZ tended to cause faster leaf emergence time (0.23 WAP). This condition is possible because the endogenous cytokinin hormone in *Nepenthes* is able to suffice plants to support leaf formation in sprouts. The addition of TDZ actually inhibited growth due to too high cytokinin concentrations. This is supported by the statement of Rosmania and Aryani (2015) that root growth in *Nepenthes* takes a long time to grow due to several factors, one of which is endogenous hormone conditions in which auxin levels are small, and endogenous cytokinin concentrations are high. Sukamto et al. (2011) reported on *N. mirabilis* species containing high endogenous cytokinins at the tips of plant stems. This condition was characterized by explants planted on media with the addition of exogenous cytokinins, no shoot formation occurred. Yelli (2013) studied the growth of *N. ampullaria* and *N. mirabilis* in MS media with 0.5× mineral salt concentration produced the highest number of leaves compared to MS media with 0.25× and 0.625× mineral salt concentrations.

Sprouts grown on ½MS medium with 1.5 ppm TDZ produced the longest leaves, the least number of leaves, and the plants looked dry (Figure 4). The strong TDZ activity, at too high a concentration, allows stimulation of endogenous ethylene as an inhibitor of sprout growth. According to Restanto et al. (2018) the use of TDZ is only required at low concentrations in the range of 0.01-0.02 ppm. Various research results show that the activity of cytokinins on phenylurea-derived growth regulators such as TDZ is stronger than that of adenine-derived cytokinins, so the use of high concentrations of TDZ can actually inhibit plant growth. Sprout growth may be inhibited by means of TDZ stimulating endogenous ethylene production. Iqbal et al. (2017) stated that during leaf initiation the formation of S-adenosyl-L-Methionine occurs which is controlled by cytokinins for ethylene biosynthesis. The presence of ethylene will activate the Teosinte Branched 1/Cycloidea/PCF gene I. The next stage, PCF will bind to Retinoblastoma Related 1 (RBR1) to suppress the activity of the E2F promoter which is in charge of activating transcriptional genes during S phase division. This will inhibit cell division at the leaf formation stage. According to Arti and Manurung (2018), ethylene production in high concentrations causes leaf damage and chlorophyll degradation which is characterized by decreased growth and death in plants.

The use of ½MS media may be good to support the growth of *N. gymnamphora*. The ½MS media has the advantage of being able to produce seed germination and growth of *N. gymnamphora* with a faster time; and the lower concentration of media mineral salts allows for reduced handling costs, and efficient labor. Further research on *in vitro* culture of *N. gymnamphora* with modification of the concentration of mineral salts in basic media is needed to support the conservation of this ex-situ species.

CONCLUSION

The ½MS media without the addition of peptone resulted in the highest percentage of *N. gymnamphora* seed germination and higher sprouts. Sprout growth in ½MS media did not require the addition of TDZ.

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