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Research Article

In Vitro Seed Germination and Shoot Growth of *Nepenthes jamban* Chi. C. Lee, Hernawati & Akhriadi, A Unique Pitcher Plant from Indonesia

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ABSTRACT

The study to optimize *in vitro* propagation of the Indonesian native and critically endangered species, *Nepenthes jamban*, in order to support the *ex situ* conservation efforts has been done. Using Murashige and Skoog (MS) as a basal medium, disinfected seeds of *N. jamban* were germinated on five types of germination media, viz. ¼ MS, ½ MS, MS, ¼ MS+benzyl adenine (BA)+Biotin and MS+BA+Biotin. Afterwards, *in vitro* shoots with 6-7 leaves were inoculated on growing media, i.e., ¼ MS, ¼ MS 60 (3:1) (MS modification with a higher concentration of nitrogen), and ¼ MS+naphtalene acetic acid (NAA)+BA. The results showed that the germination of *N. jamban* seeds was slow, indicated by the percentage of germination being less than 20% after six months of being planted on germination media. The highest percentage of germination at the sixth month and the greatest pitcher development at the tenth month were obtained on ¼ MS medium. Furthermore, shoot growth and pitchers development consistently increased for twelve months in ¼ MS 60 (3:1) medium while other media resulted in a decrease in pitcher formation. It seemed that low concentrations of nutrients in the medium proved to be more effective to induce *in vitro* seed germination and enhance shoot growth which was also supported by a higher nitrogen (nitrate) concentration in the medium. This study provides information that supports *ex situ* conservation action of native and critically endangered *Nepenthes* species from Indonesia.

INTRODUCTION

Nepenthes spp. are locally known in Indonesia as "Kantong Semar" due to the modification of the leaf tips into a pitcher structure that resembles Semar's belly, a Javanese traditional puppet character (Dinarti et al. 2010). More than 180 species of *Nepenthes* are distributed from Madagascar to South China and New Caledonia, where Indonesia, Malaysia, and the Philippines are considered the centres of *Nepenthes* diversity (Cross et

al. 2020; Mansur et al. 2023). In Indonesia, Sumatra Island has the most *Nepenthes* species by 39 species and 34 among them are endemic (Hernawati et al. 2022; Mansur et al. 2023).

Although *Nepenthes* is a carnivorous plant, it can also be a prospective commercial ornamental plant because of the attractive variation in the shape, size, and colour of the pitcher (Handayani 2021). It also has ethnobotanical utilisation by some local people as a potential herb plant since it contains phytochemical and phytopharmacological activity from its extracts (Sanusi et al. 2017).

Nepenthes jamban is one of nine new species of *Nepenthes* spp. (Nepenthaceae) reported from Sumatra between 2002 to 2022 (Hernawati et al. 2022) and one of the new species found by Lee et al. (2006) in North Sumatra. Lee et al. (2006) reported that *N. jamban* was distributed around Bukit Barisan in North Sumatra with the habitat of mossy forest in the upper mountainous area and scrub vegetation on the top of the mountain. The word "jamban" refers to a toilet in Bahasa Indonesia due to the resemblance to the pitcher (Lee et al. 2006).

According to the International Union for Conservation of Nature (IUCN) report, *N. jamban* is not listed on the red list (IUCN 2023). However, a study on the conservation of carnivorous plants classified this species as critically endangered (Cross et al. 2020). Furthermore, the documentations about conservation attempts, plant propagation, or other potential conservation approaches have not been reported. On the other hand, the majority of natural habitats inhabited by carnivorous plants, such as *Nepenthes* species are confined to areas that have been significantly disturbed and degraded (Cross et al. 2020). Certain species have come perilously close to extinction as a result of the constant conflict between development and conservation, which includes industries and housing. Therefore, it is critical to establish a dependable tissue culture technique in order to prevent the extinction of these native and endemic species (Siti-Suhaila & Norwati 2021).

The propagation of *Nepenthes* spp. can be done using seed, stem cutting, stem grafting (air layering), and ground layering, however, stem cutting and grafting methods are time laborious with low yields and difficult to carry out (Sukamto et al. 2011; Meinaswati et al. 2022). Moreover, according to Dwiati et al. (2023), performing stem cutting without *in vitro* techniques resulted in just two shoots in the propagation of *N. gymnamphora* and *N. adrianii*. *In vitro* techniques have been used in various species of *Nepenthes* spp. by shoot tip culture (Sukamto et al. 2011), callus induction (Novitasari & Isnaini 2021), micro-cutting technique from the shoot (Yelli 2013; Budisantoso et al. 2018), and seed culture (Isnaini & Handidi 2007; Khuraijam & Roy 2015; Meinaswati et al. 2022; Joshi et al. 2022). *In vitro* seed culture is an appropriate biotechnological approach for the massive propagation and conservation of many scarce and endangered species. Moreover, this method can maintain the genetic diversity as the seed is heterozygous (Nongrum et al. 2009).

As there is no previous report about *in vitro* propagation of *N. jamban*, a study to understand the effectiveness of various media to support the optimal germination and growth is required. This study is expected to add more knowledge about *in vitro* propagation of one native and critically endangered *Nepenthes* species from Sumatra, Indonesia.

MATERIALS AND METHODS

Seed Observation

The plant material used in this study was *Nepenthes jamban* seeds collected from Pasaman Regency (1200 masl), West Sumatra, Indonesia. Natu-

rally pollinated and mature seeds of *N. jamban* were collected from unopened seed pods in their natural habitat. The seed pods were stored in an envelope at room temperature until they naturally opened. These seeds were then utilised as the material for this study after being stored for one month.

Air-dried mature seeds were observed under a stereo microscope (Nikon SMZ 645) with 5× magnification. For further observation, observing the seeds under a scanning electron microscope (SEM) (JSM-IT200 InTouchScope™) proceeded after the middle cut of the seeds were placed on the stub and coated with gold using an EIKO IB-3 ion coater.

Seed Germination

Planting the seeds on solid MS basal media (Murashige & Skoog 1962) proceeded in a non-factorial completely randomised design with five replicates to observe their viability and growth. Containing MS macro-nutrient (NH_4NO_3, KNO_3, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4) and micro-nutrient ($MnSO_4.4H_2O$, $ZnSO_4.5H_2O$, $Na_2MoO_2.2H_2O$, $CoCl_2.6H_2O$, H_3BO_3), MS vitamins (nicotinic acid, thiamine HCl, pyridoxine HCl, glycine), iron source (NaEDTA, $FeSO_4.7H_2O$), 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, and 5 g L^{-1} agar as gelling agent, germination media consisted of five treatments as follows: i) quarter strength of MS macro-nutrient and full strength concentration of other components (¼ MS); ii) half strength of MS macro-nutrient and full strength concentration of other components (½ MS); iii) full strength of all MS components (MS); iv) ¼ MS supplemented with 0.5 mg L^{-1} benzyl adenine (BA) and 1 mg L^{-1} biotin (¼ MS+BA+Biotin); v) MS supplemented with 0.5 mg L^{-1} BA and 1 mg L^{-1} biotin (MS+BA+Biotin). The acidity was set at pH 5.7-5.8 by adding several drops of 1 N NaOH or HCl before adding the gelling agent. Sterilising the media proceeded at 121 °C for 20 min using an autoclave, then 25 mL sterile media was poured into petri dishes with a diameter of 10 cm. In this study, adding BA as a plant growth regulator and biotin, known as vitamin B7, in the germination media was expected to boost the germination rate of *N. jamban in vitro*.

The seeds were sterilised in laminar air flow cabinet by soaking in 0.25% NaClO (Bayclin®, active ingredient 5.25% NaClO) with a few drops of Tween 20 for 20 min, followed by rinsing with sterile distilled water three times. The planting of 20 sterile seeds on the germination media was repeated five times for each treatment so that the total number of seeds planted was 500. Seed cultures of *N. jamban* were then incubated in a culture room under dim conditions (without direct lighting from lamps) at a room temperature of 25±2 °C and 40% humidity. Observations of seed germination were carried out periodically until the embryos grew and formed leaves and pitchers. Non-germinated seeds and dead sprouts were observed as well. Transferring the explants to the same medium proceeded three times after the seeds germinated. The best medium was then used for shoot growing basal media.

Shoot Growth and Pitcher Formation

Designed using a non-factorial completely randomised design with ten replicates, the experiment used ¼ MS as basal media for growing media treatments including: i) ¼ MS; ii) ¼ MS 60 (3:1); iii) ¼ MS supplemented with 0.5 mg L^{-1} naphthalene acetic acid (NAA) and 0.5 mg L^{-1} BA (1/4 MS+NAA+BA). MS 60 (3:1) medium was a modification of MS medium using a total nitrogen concentration (N) of 60 mM with a nitrate and ammonium (NO_3^-:NH_4^+) ratio of 3:1, which was adjusted using the compounds KNO_3, NH_4NO_3 and $(NH_4)_2SO_4$ as sources of NO_3^- and NH_4^+ in

the medium (Handayani et al. 2021). The acidity level was set at pH 5.7-5.8 by adding several drops of 1 N NaOH or HCl before adding the gelling agent. As much as 25 mL sterile media was poured into a 300 mL jar prior to autoclaving at 121 °C for 20 min.

Fourteen-month-old sterile shoots at the 6-7 leaf stage were planted in each medium treatment and incubated in a culture room for twelve months under a photoperiod condition of 16/8 h (light/dark) with a light intensity of 46 µmol m^{-2} s^{-1}, room temperature at 25±2 °C, and humidity of 40%. Transferring the shoots on the same media was carried out once every three months, as well as observing growth parameters including number of pitchers, number of leaves, and shoot height. The number of pitchers was counted on the leaves that formed pitchers as shown in Figure 2C. The height of the shoots was measured by taking out the shoots from the bottle and then measuring them on sterile millimetre block paper in the laminar air flow cabinet.

Data Analysis

Data processing and presentation were performed using Microsoft Excel and analysed using ANOVA and continued with the Least Significant Difference (LSD) test at $p \leq 0.05$ using R 4.1.0 software.

RESULTS AND DISCUSSION

Morphology of *Nepenthes jamban* seeds

Resembling orchid seeds (Orchidaceae), *Nepenthes* seeds have an embryo (dark brown) located at the centre and are coated with several layers of seed coat cells (testa) extending from either end of the embryo. *Nepenthes* seed is larger than that of dust-like orchid seed and 8-30 mm long, with the seed coat about six times longer than the embryo. With a distinct characteristic in the shape of a pitcher resembling a toilet (Figure 1A), *N. jamban* produces filiform and 14-20 mm long seeds that are transversely wrinkled at the centre (Figure 1B-E). The seed coat is light brown with a lighter structure than the embryo which is useful in the process of dispersal of seeds assisted by wind or water (Watson & Dallwitz 1992; Cheek & Jebb 2001; Barclay 2015). The seeds of *Nepenthes* from the humid tropics seem to be recalcitrant (Ellison & Adamec 2017). This kind of seed cannot tolerate dehydration during storage at the low water content or subzero temperatures. Therefore, the storage of recalcitrant seeds cannot use standard gene bank approaches (Pammenter & Berjak 2014).

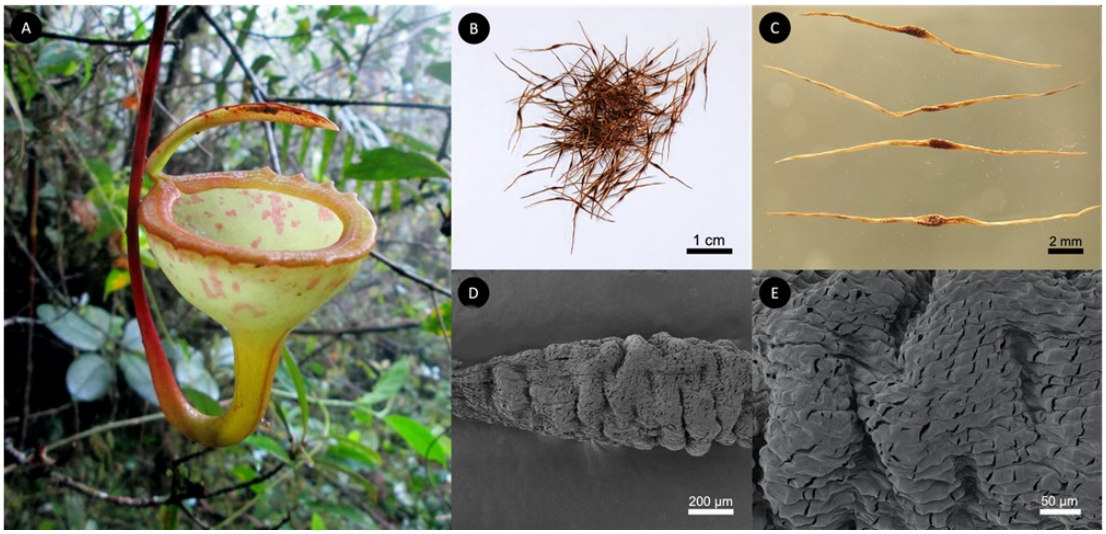

Figure 1. A) The pitcher of *N. jamban* in nature; B) Mature seeds; C) Seeds observed under a stereo microscope; D-E) The centre part of the seed observed under a scanning electron microscope.

In Vitro Seed Germination

Germination is a vital part of plant development. It is also a complicated physiological process that is driven by intrinsic factors (i.e., seed dormancy and food availability) and extrinsic factors (i.e., water, temperature, oxygen, and relative humidity) (Bhardwaj et al. 2014; Makena et al. 2018; Savaedi et al. 2019). From the research about seed germination in *Nepenthes*, we can assume that the characteristics and requirements of nutrient content to get the best germination rate are genetic-dependent. In this study, *N. jamban* seed coat became transparent after sowing on the germination medium so that the embryo in the middle part of the seed was clearly seen, however, there were also germinated seeds that failed to grow and eventually died (Figure 2A). Dead sprouts were indicated by the blackening of cotyledon leaves due to their inability to grow to the next stage of the growth. Viable seeds began to germinate indicated by a change in colour to green in the centre part of the seed, followed by the emergence of two cotyledon leaves. This stage is considered the sprouting seed stage, as shown in Figure 2B. The shoot grew and started to develop a pitcher on the fourth leaf (Figure 2B-C).

Nepenthes jamban seeds began to germinate six months after planting on *in vitro* media, except for MS and MS+BA+Biotin media (Figure 3). Figure 3A shows the seeds planted on ¼ MS and ¼ MS+BA+Biotin germinated with the same germination percentage, which was 15±4.4%. Seeds planted at ½ MS also germinated, but with a lower percentage, which was only 1±1%. Water is the main regulator of germination, as it is imbibed into the seed and initiates the germination process (Luna & Chamorro 2016). Moreover, the shortage of water availability will restrain seed germination. Since the lower concentration of MS medium indicates greater water availability in the medium, we suggest that this is why the germination rate on ¼ MS medium is higher than on ½ MS.

Due to the very small size of the endosperm in *Nepenthes* seeds, germination is extremely low. The conventional germination period for *Nepenthes* seeds is approximately two months (45-65 days), which is considered a long period of time (Meinaswati et al. 2022). Previous studies indicated there were variations in germination time on various *Nepenthes* species *in vitro*, i.e., 4 weeks after planting (WAP) for *N. gymnamphora* (Meinaswati et al. 2022), 5 WAP for *N. khasiana* and *N. mirabilis* (Nongrum et al. 2009; Dinarti et al. 2010), and 13 WAP for *N. distillatoria* (Siriwardana et al. 2013). These results were different from this study where *N. jamban* needed a longer time to germinate, around 6 months or

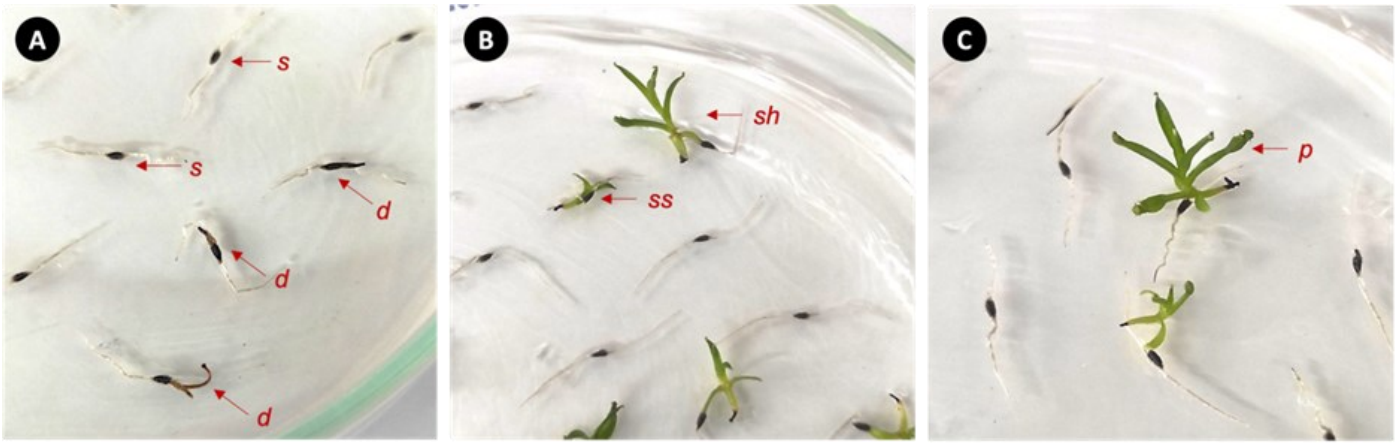

Figure 2. The growth and development of *N. jamban* from seeds. A) Non-germinated seed (*s*) and dead sprout (*d*); B) Sprouting seed (*ss*) and the shoot (*sh*) growing and starting to develop very young pitchers; C) The shoot with well-developed young pitchers (*p*).

26 WAP. The seeds used in this study were stored at room temperature after being harvested until they were used for *in vitro* germination experiments. The storage method was considered one of the factors causing the long germination time, as in the result from (Mao & Ranyaphi 2007) which stated that the seed of *N. khasiana* rapidly lost its viability after one year of storage. The germination time of *N. jamban in vitro* was longer than other *Nepenthes* species which germinated in *ex vitro* medium. For instance, the seed of *N. khasiana* planted in compost medium (Mao & Ranyaphi 2007) and coir media without fertiliser (Khuraijam & Roy 2015) started to germinate in 4 WAP. Additionally, the low germination rate of *N. jamban* seeds may be a contributing factor to its endangerment in natural habitat, a phenomenon observed commonly in rare and endemic species such as *Betula humilis* (Bona et al. 2022), *Manglietia crassipes* (Wang et al. 2021), and *Boswellia* spp. (Hamdiah et al. 2022).

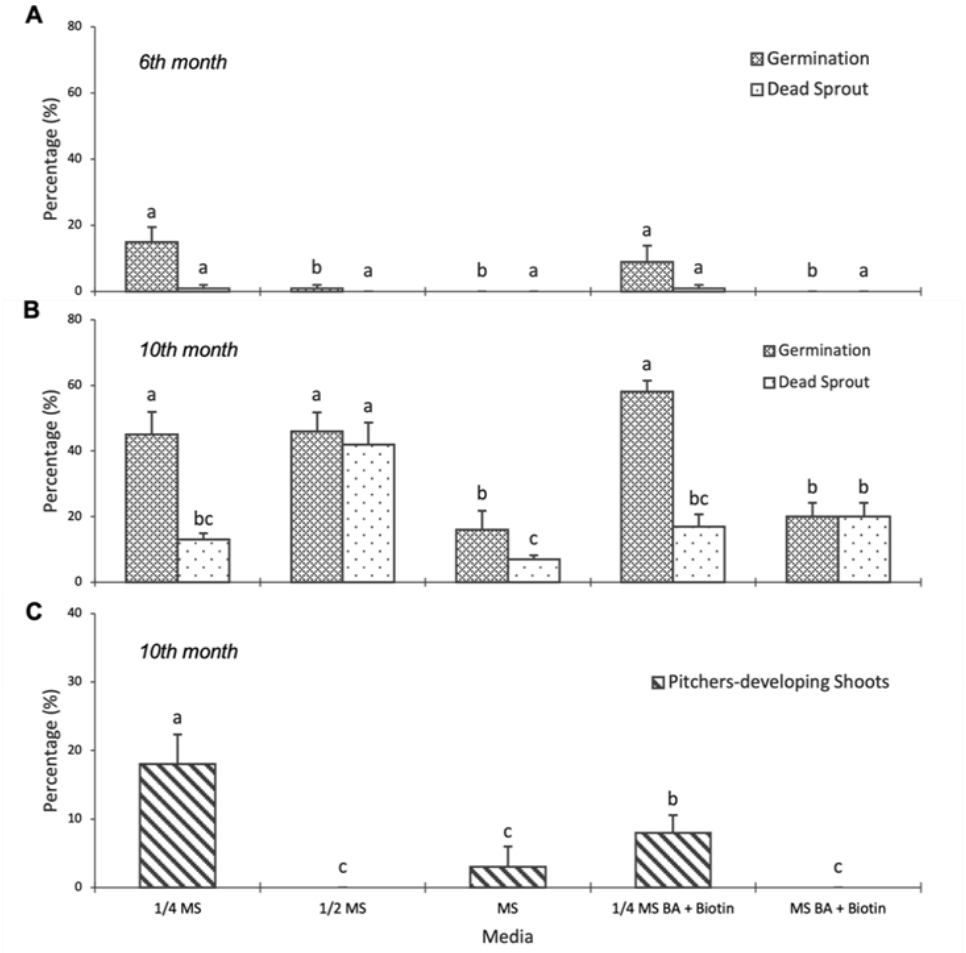

Figure 3. *In vitro N. jamban* seeds germination on five types of media: A-B) Germination percentage and death of sprouts at six and ten months after culture; C) The percentage of pitchers developing shoots at ten months after culture. The same letter on the bar for each parameter indicates no significant difference by LSD test at $p \leq 0.05$.

As we assumed that the germination rate in *Nepenthes* is species-and genetic-dependent, this also applies to germination swiftness. A review by Carrera-Castaño et al. (2020) clearly explained that germination swiftness, one of the important traits of germination, is modulated by a continuous interaction between the plant genetic makeup and the environment from dormancy to germination stages. These can be achieved through regulation of metabolism and hormone signalling (Carrera-Castaño et al. 2020). Variations in germination time of *Nepenthes* seeds *in*

vitro was affected by the type of media used. In this study, the seed of *N. jamban* germinated faster in the non-full-strength media, i.e., ¼ MS, ¼ MS+BA+Biotin and ½ MS compared to other media. This result was in line with a previous study where the ¼ MS and ½ MS were the best media for *N. mirabilis* germination (Dinarti et al. 2010) and the ½ MS was best for *in vitro* germination of *N. gymnamphora* (Meinaswati et al. 2022), *N. khasiana* (Nongrum et al. 2009), and *N. ampullaria* (Sani et al. 2000) seeds. The addition of hormone (BA) and vitamin (biotin) apparently did not affect *N. jamban* germination in this study. Furthermore, the result indicated that *N. jamban* seeds tend to thrive in media containing low nutrients. This observation is consistent with the general habitat conditions of *Nepenthes*, which predominantly grow in areas that are poor in nutrients (Mansur et al. 2022).

The result in Figure 3A showed that not all the germinated seeds survived. Some of them failed to grow and then died. About 1±1% of dead sprouts were found in ¼ MS and ¼ MS+BA+Biotin media, while there were no dead sprouts in ½ MS, MS and MS+Biotin media at six months after planting.

Ten months after planting, seeds on MS and MS+BA+Biotin showed a low germination rate by 16±5.8% and 20±4.2%, respectively (Figure 3B). The same figure showed an increasing germination rate in ¼ MS and ¼ MS+BA+Biotin media by 30% and 49%, respectively, from the germination rate at six months after planting. The significantly increasing germination rate was also occurred in ½ MS medium by 45%, however the percentage of dead sprouts in this treatment also considerably elevated compared to other treatments, by around 40%. The sprout death in other media treatments was lower and significantly different from ½ MS medium treatment.

Nepenthes jamban pitchers started to develop on the fourth leaf, on average. Figure 3C indicated that even though all the seeds had already germinated in 10 months, the shoot in the ½ MS and MS+BA+Biotin had not developed the pitcher while the others did. The most pitchers developed in ¼ MS medium (18±4.4%) while on the ¼ MS+BA+Biotin and MS media were only 8±2.5% and 3±3%, respectively. In line with this result, *N. khasiana* seeds grew in MS and MS+BAP did not develop any pitchers in four months after planting (Nongrum et al. 2009). In general, *in vitro* shoot performance of *N. jamban* 10 months after planting was better in ¼ MS and ¼ MS+BA+Biotin media, which supported the shoot growth and the pitcher development (Figure 4A, D). Other media exhibited lower performance, with only a few sprouts were able to regenerate and more dead sprouts found (Figure 4B, C, E).

In Vitro Shoot Growth and Pitcher Formation

Germinated seeds on the germination media were transplanted to the growing media to observe further growth for twelve months. The results showed that ¼ MS 60 (3:1) medium produced the greatest number of pitchers whereas ¼ MS+NAA+BA yielded the least. *N. jamban* plantlets on ¼ MS 60 (3:1) kept forming pitchers for twelve months (12 pitchers) after transplanting, while those on ¼ MS only formed 8,6±0.7 and 10.8±0.5 pitchers for six and nine months, respectively, and those on ¼ MS+NAA+BA formed 9 pitchers followed by a decrease in number by the twelfth month due to senescence. Pitcher formation on *in vitro* media varied for different species. Previous studies reported that *N. ampullaria* and *N. mirabilis* on ¼ MS medium formed 4 pitchers by the twelfth month (Yelli 2013), while *N. khasiana* formed 3 pitchers on MS NAA medium and no pitchers on MS BAP medium in the third month after plant-

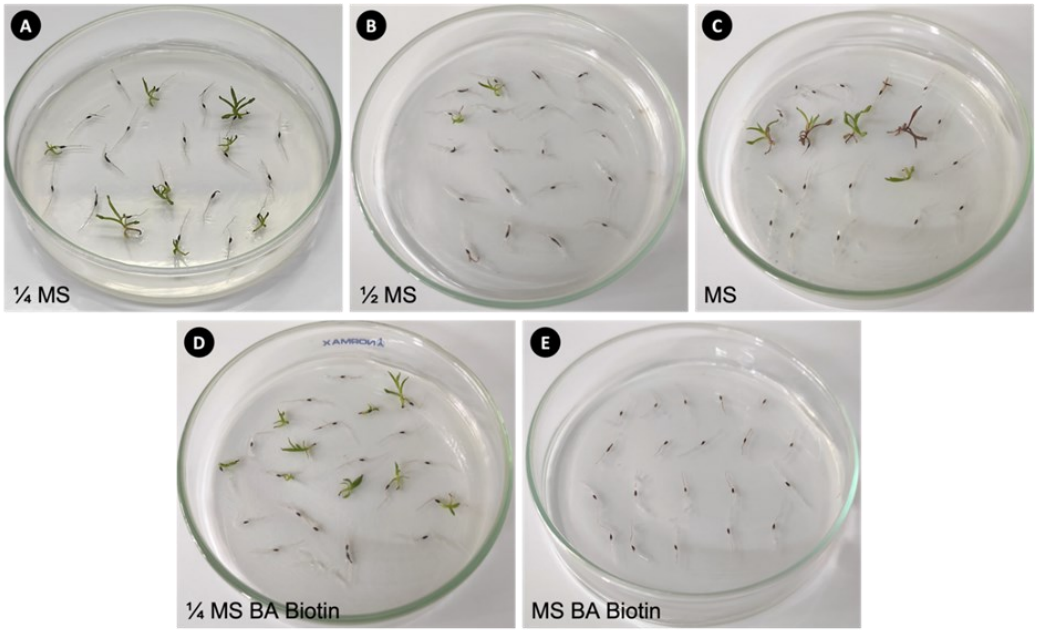

Figure 4. Shoot performance of *N. jamban* at ten months after sowing on germination media.

ing (Nongrum et al. 2009).

Number of leaves on the ¼ MS and ¼ MS 60 (3:1) increased for twelve months of culture (13.1±0.6 leaves), while that on ¼ MS+NAA+BA was increased only up to the ninth month (9.5±1.4 leaves), followed by a decrease in number (Figure 5B). A significant difference in number of leaves was only shown in the twelfth month after planting. Other *Nepenthes* species, *N. ampullaria* and *N. mirabilis*, grown in ¼ MS medium showed a different response compared to *N. jamban*, both of which developed 12 leaves by the twelfth month (Yelli 2013). The others were grown on ½ MS supplemented with cytokinin and auxin also showed different responses. An increase in the number of leaves in *N. ampullaria* occurred after twelve months of subculture (micro-cutting) in ½ MS supplemented with 0.5 mg L^{-1} BAP (Budisantoso et al. 2018). Another previous study also reported an increase in the number of leaves in *N. gymnamphora* (5 leaves) eight months after subculturing in ½ MS supplemented with 1 mg L^{-1} thidiazuron (Meinaswati et al. 2022).

The shoot height of *N. jamban* increased for twelve months in culture in all media treatments where the tallest shoots showed in ¼ MS medium with an average shoot height of about 2.57±0.1 cm (Figure 5C). However, the shoot height of another *Nepenthes* species, *N. khasiana*, grown on ¼ MS + 2.20-44.40 µM BAP medium reached 0.45-0.67 cm in the fourth month (Nongrum et al. 2009), 1.95 cm on ½ MS + 0.5 mg L^{-1} BAP medium in the twelfth month (Budisantoso et al. 2018), and 2.1 cm on ½ MS + 1 mg L^{-1} BAP medium in the eighth month of culture (Devi et al. 2013).

In general, the best growing medium for the three parameters observed in this study (number of pitchers, number of leaves, and plant height) was ¼ MS 60 (3:1) which was ¼ MS medium with a ratio of nitrogen sources of 3:1 (nitrate:ammonium). It showed that nitrogen sourced from nitrate (NO_3^-) gave a positive effect on the vegetative growth of *N. jamban in vitro*. Another previous study also reported that the combination of two nitrate sources (ammonium nitrate and calcium nitrate) as nitrogen sources was better for *N. khasiana*, *N. pervillei*, and *N. vieillardii* growth compared to adding only single source of nitrogen in the media (Mao et al. 2007). Schulze et al. (1999) also explained that there is a

transporter gene for ammonium localising ions in the *N. alata* pitcher glands associated with nitrogen uptake. Nitrogen is one of the most important minerals in plants as it is a part of amino acids and proteins. Moreover, it also regulates enzyme activities for energy metabolism (Hussain et al. 2016). In general, a high nutrient medium will significantly affect seed germination. However, excess levels of nitrogen and other nutrients will result in plant death as the plant experiences toxicity. On the other hand, improper composition and concentration of the basal medium can delay the germination rate (Jakovljević et al. 2017; Noorhosseini et al. 2018).

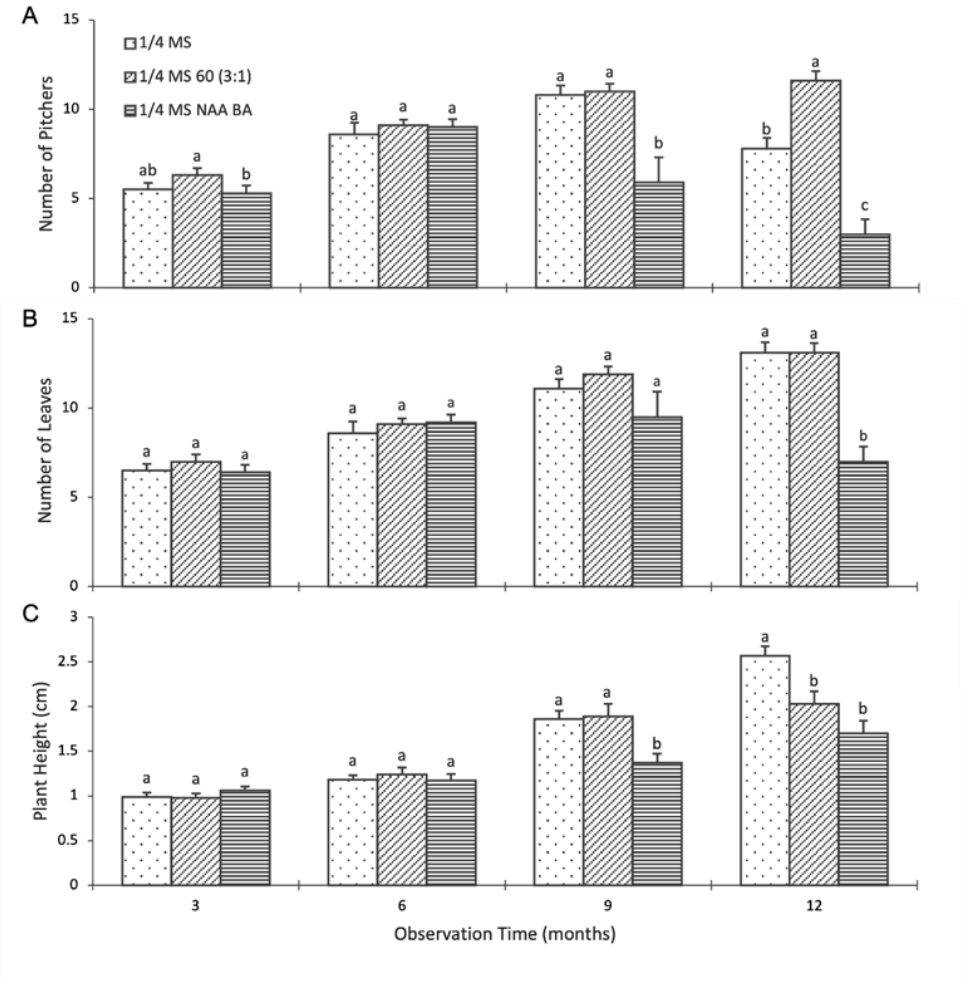

Figure 5. Shoot growth of *N. jamban* for twelve months observed on three types of growing media. The same letter on the bar in each time group showed no significant difference by LSD test at $p \leq 0.05$.

CONCLUSION

The concentration of nutrients in the medium proved to be more effective for inducing *in vitro* seed germination and enhancing shoot growth which is also supported by a higher nitrogen (nitrate) concentration in the medium. In this study, ¼ MS and ¼ MS 60 (3:1) media were the most suitable for *in vitro* seed germination and vegetative growth of *N. jamban*. This study provides information that supports the *ex situ* conservation action of native *Nepenthes* species from Indonesia. However, the germination started late at the sixth months after planting and attempts to make the germination faster should be done. The study about more effective and efficient culture media and its correlation to the seed preservation and dormancy might be advantageous for the conservation of *N. jamban*.

AUTHORS CONTRIBUTION

All authors have reviewed the final version of the manuscript and approved it for publication. ADP and RSR designed and performed the research and analyzed the data. All authors wrote and reviewed the manuscript.

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CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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