CONSERVATION OF *PARABOEA BAKERI* M. R. HEND. USING TISSUE CULTURE TECHNOLOGY

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ABSTRACT

Paraboea bakeri M. R. Hend. from Gesneriaceae family had been listed as critically endangered in the IUCN red list. Propagation through in vitro technique is required to ensure the survival and increase the population of this species. In this study, shoot multiplication, in vitro rooting and acclimatization experiment was done using different medium in order to select the best medium in propagating this species. For shoot multiplication experiment, it was observed that MS basal medium added with 0.5 mg/L BAP produced the highest mean shoots numbers and leaf length which are 7.79 shoots number and 0.42 cm leaf length respectively. As for in vitro rooting, ½ MS basal media added with 2.0 mg/L NAA produced the longest roots (0.285 cm) and the highest mean number of roots (10 roots). Transferring Paraboea bakeri from in vitro to ex vitro condition was a bit challenging because of its natural habitat is limestone area. Based on our observation, the best potting medium to acclimatize Paraboea bakeri was mixture of baked soil and peat moss with 86.7% survival rates. Observation showed that Paraboea bakeri growth improved when planted into high drainage and moist potting medium. In conclusion, propagation method using tissue culture technique had been successfully developed for Paraboea bakeri.

Key words: *Paraboea bakeri*, *in vitro* propagation.

INTRODUCTION

Paraboea is found in southern China, north-eastern India and the eastern Himalayas, Burma, Thailand, Cambodia, Laos, Vietnam, Malaysia, Philippines and Indonesia as far east as Sulawesi. The genus of *Paraboea* characterized by the abaxi-ally matted leaves with densely interwoven indumentum and flowers with flat-faced to shortly campanulate corolla and non-erect anthers (Middleton et al. 2010; Puglisi et al. 2016). In Malaysia, there are 36 species with majority grow on limestone area and only five of it grow on other rock types such as soils derived from quartz, sandstone, igneous rocks and granite. In this paper, we will just focus on one species of *Paraboea* which is *Paraboea bakeri*.

Kiew et. al. (2011) had stated in her paper that endemism is extremely high in *Paraboea sp*. *Paraboea bakeri* from Gesneriaceae family is endemic to Bukit Tenggek and Bukit Sagu located in Pahang region. Followed is the characteristic of *Paraboea bakeri*. This species is a perennial herb with its leaves congested in a rosette. The petioles can reach up to 5 cm long and are covered by brownish matted hairs. Its lamina is ovate with a rounded to acute apex and a serrate margin. The upper surface is covered in dense pubescent hairs while the lower surface is covered with brownish matted hairs (Mybis, 2020).

Limestone only cover 0.3% of Peninsular Malaysia and almost 14% of the seed plant flora grows on limestone including *Paraboea bakeri* (Chin, 1977). Limestone area are rich of minerals such as calcite and aragonite. The mineral from limestone area are used as building materials, road bases, and many more. Besides quarry activity, agricultural practices in the surrounding areas, tourist and recreational activities associated with caving or rock climbing and building temples in caves had contributed to endanger the flora. Kiew et al. (2017) had mentioned in its paper that there is an urgent need to develop a strategy to protect the maximum biodiversity of the sensitive limestone flora, particularly to protect exceptional rare limestone species that are facing extinction unless afforded legal protection.

Plant usually produced seed or rhizome to breed. For a plant that listed as critically endangered in the Red list, leaving them to self-propagate while its habitat was being disturbed is not an option. *Ex vitro* propagation is needed in order to prevent the species from extinction. Propagation using tissue culture technique is an option to solve this issue. There are several special advantages that's offered by plant tissue culture as it ensures a continuous supply of planting material in a short span of time and helps in multiplication, as well as, conserving wild germplasm (Mudoi et al., 2013).

Propagation using tissue culture method will involve surface sterilization, shoot multiplication, *in vitro* or *ex vitro* rooting and acclimatization in the nursery in order to produce tissue culture plantlet. It is not an easy job, as it is needed to be done in sterile condition and usage of special equipment such as laminar flow etc. and to develop tissue culture protocol for a certain plant sometimes need at least one year if we start from scratch. In this study, we will develop tissue culture protocol for *Paraboea bakeri* for conservation purposes.

MATERIAL AND METHODS

Plant material

In vitro Paraboea bakeri plants were produced and provided by FRIM Biodiversity Division. The plantlets were multiplied in MS basal media in order to get sufficient explants number to start the experiment.

Shoots Multiplication

Axillary bud sprouts from the explants were used as explants for shoot multiplication experiment. The explants were cultured into full strength MS basal medium supplemented with BAP of five different concentrations (0 mg/L, 0.1 mg/L, 0.25 mg/L, 0.5 mg/L, 1.0 mg/L and 2.0 mg/L). The medium pH was adjusted to 5.8 before autoclaving. Four bottles of medium were prepared for each treatment, and each bottle contained five shoots (±1.5 cm). The number of shoots induced per explants and shoots length was observed after 6 weeks of culture.

In vitro Rooting

Individual shoots from multiplication media were used for *in vitro* rooting experiment. Half strength Murashige and Skoog (MS) basal medium was used to induce roots *in vitro*. The MS basal medium was supplemented with eight different concentration of indole-3-butyric acid (IBA) and 1-Naphthaleneacetic acid (NAA) i.e., 1.0 mg/L, 2.0 mg/L, 3.0 mg/L and 4.0 mg/L. Ten replicates were prepared for each treatment. The number of roots and root length were recorded after four weeks in culture.

Acclimatization

In vitro rooted plantlets were used for acclimatization. Plantlets of about 3 cm in height were pulled out from the culture media and washed under flowing tap water to remove remaining agar medium. The plantlets were then dipped into Thiram (fungicide) for a few seconds to disinfect the plants. Then the plantlets were planted in different potting media (jiffy 7, sand and baked soil) with 15 replicates for each treatments. The plantlets were kept in a sealed glass chamber to maintain humidity. After one month, the seal was gradually released within several days to prevent heat shock. The plantlets were watered only when the sealed was opened. Plantlets that survived acclimatization process were transferred into poly bags. The survival rates of plantlets in the nursery were recorded.

Data Analysis

Data from the shoot multiplication and *in vitro* rooting experiment were analysed using SAS ver. 9.1 software. ANOVA was applied to compare values obtained for the different measured parameters ($P < 0.01$). Mean values were compared using Duncan's Multiple Range Test (DMRT).

RESULT AND DISCUSSION

Shoot multiplication experiment for *Paraboea bakeri* were observed after six week of culture. Observation showed that Murashige and Skoog (MS) basal added with 0.5 mg/L 6-Benzylaminopurine (BAP) produced the highest shoots numbers which is 7.79 ±0.36 followed by MS with 0.1 mg/L BAP (7.3 ±0.42) and MS with 2.0 mg/L BAP (7.08 ± 0.35). There are significance difference between MS basal medium with 0.5 mg/L BAP and without BAP.The results obtained clearly showed that BAP added medium produce better result. Another study done by Piriyavinit et al (2018) on *Paraboea doitungensis* supported our results as most mean shoot numbers can be produced by using MS basal with 0.5 mg/L BAP.

For the leaves size, there are no significance differences between all treatments. The plants cultured into MS basal with 0.5 mg/L BAP produced the largest leaf diameter followed by MS with 2.0 mg/L BAP and MS with 1.0 mg/L BAP. The leaves diameter ranges from 0.33 to 0.42 cm for all the treatments. According to Vinterhalter et al. (2001), sucrose also significantly increased leaf area and decreasing concentration of KNO3 and NH4NO3 caused a 3-fold decline of leaf area. From this statement, it is known that plant growth hormone (PGR) concentration do not affect the leaf size. Though, bigger leaf tends to give advantage to the plant during photosynthesis. In this study, BAP hormone were chosen as it is tested suitable to multiply rhizomous plant such as *Begonia sp.* And *Smilax myosotiflora*. (Kaviani et al 2015, Miswandi et al 2016)

Table 1: Effect of different BAP concentration on *P. bakeri* shoot multiplication and leaf diameter

Media	BAP conc. (mg/L)	Mean no. of shoots per explants	-Leaf diameter (cm)
MS	0.0	6.25 ± 0.33^{b}	0.33 ± 0.03^{a}
MS	0.1	7.30 ± 0.42^{ab}	0.35 ± 0.03^{a}
MS	0.5	7.79 ± 0.36^{a}	0.42 ± 0.04^{a}
MS	1.0	6.20 ± 0.52^{b}	0.38 ± 0.03^{a}
MS	2.0	7.08 ± 0.35^{ab}	0.40 ± 0.04^{a}

For *in vitro* rooting experiment, half strength MS basal medium were added with either NAA or IBA in order to promote root growth. The chosen of half MS as basal medium supported by Piriyavinit et al (2018) as his studies confirmed that half strength MS basal medium produced more number of roots compared to full strength of MS basal. Our observation showed that NAA produced better results of *in vitro* rooting compared to IBA. Half strength MS basal media with NAA can produce up to 10 ± 2.15 mean roots number (½ MS + 2.0 mg/L) while half MS with IBA only produced 6.05 ± 1.8 (½ MS + 4.0 mg/L) mean root numbers. In term of concentration, lower concentration of NAA can produced more roots compared to high concentration of NAA. On the other hand, higher concentration of IBA showed better result than lower concentration of IBA. There are significance differences for the number of roots produced by using NAA and IBA. For commercial production purposes, it is best to choose medium with lower growth hormone as it will reduce the production cost.

In term of roots length, the trend is similar to the root production. Half MS basal medium with 2.0 mg/L NAA still produced the longest root (0.29 ± 0.03 cm) compared to other treatments. Followed by half MS with 1.0 mg/L NAA (0.22 ± 0.03 cm), half MS with 3 mg/L (0.2 ± 0.02 cm) and half MS with 4.0 mg/L IBA (0.19 ± 0.05 cm). There are no significance difference between these four treatments.

Table 2: Effect of different IBA and NAA concentration on the mean number of roots and roots length

Medium	IBA conc. (mg/L)	NAA conc. (mg/L)	Mean no. of roots	Root mean length (cm)
½ MS	1.0	-	1.30 ± 0.72^{c}	$0.13\text{-} \pm 0.05^{ab}$
½ MS	2.0	-	0.13 ± 0.04^{a}	0.07 ± 0.02^{a}
½ MS	3.0	-	2.00 ± 0.98^{c}	0.07 ± 0.02^{b}
½ MS	4.0	-	6.05 ± 1.8^{abc}	0.19 ± 0.05^{ab}
½ MS	-	1.0	9.00 ± 2.09^{ab}	0.22 ± 0.03^{ab}
½ MS	-	2.0	10.0 ± 2.15^{a}	0.29 ± 0.03^{a}
½ MS	-	3.0	4.60 ± 0.9^{abc}	0.20 ± 0.02^{ab}
½ MS	-	4.0	3.55 ± 0.73^{bc}	0.14 ± 0.03^{ab}

For acclimatization experiment, using mixture of baked soil and peat moss as potting medium produced the highest survival rate (86.7%) followed by jiffy 7 (80%) and sand (70%). Kiew et al. (2011) had discovered that *Paraboea bakeri* grow on moss cushions in high shaded crevices in limestone hills where water seeps down. These conditions have the same criteria to the potting medium that we used during acclimatization process. From this observation, we can conclude that porous but high water retention potting medium is suitable for this species acclimatization process and growth. Peat moss is known to have this criteria and observation showed potting medium that contained peat moss produced higher survival rateOther than the acclimatization media criteria as mentioned above, calcium carbonate that is available at limestone area didn't affect the growth of this species. *Paraboea sp.* Such as *P. paniculata* was reported to have special secretion glands to discharge excess calcium carbonate for adaptation purposes (Soepadmo 1998).

Fig 1 *P. bakeri* plantlets after 1 month of acclimatization

There are very few studies done on *Paraboea sp.* especially regarding its propagation methodology. Being able to produce *P. bakeri* plantlets through tissue culture will ensure the survival of the plants. The planting material produced can be out planted into its original habitat. Though, the survival rates of this plant outside nursery condition need to be tested and further study. Availability of *P. bakeri* plant can attract researcher to studies all the unknown about this plant.

CONCLUSION

Tissue culture protocol for *P. bakeri* were succesfully developed and can help in preventing this species from extinction. Compared to using seeds and rhizome of *Paraboea sp*., tissue culture protocol provide faster way to propagate this species. In spite of that, lack of reseach done on *Paraboea sp.* limit us in comparing and confirm our findings. But in order to successfully preventing this species from extinction, limestone quarrying need to be stopped. The authorities need to take action to prevent continuous destruction of the limestone hills.

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REFERENCES

Chin, S.C. (1977) The limestone flora of Malaya. 1. Gard. Bull. Singapore 30: 165–219.

Kaviani, B. Hashemabadi, D. Khodabakhshm, H. Onsinejad, R. Ansari, M.H. & Haghighat, N. (2015). Micropropagation of Begonia rex Putz. By 6-benzyladenine and α-naphthalene acetic acid.International Journal of Biosciences Vol. 6(8-15).

Kiew, R. Ummul-nazrah, A.R. & Chua, L.S.L. (2011). Conservation status of *Paraboea* species (Gesneriaceae) in Malaysia. Gardens' Bulletin Singapore 63(1 & 2): 433–450.

Kiew, R. Ummul-nazrah, A.R. Rafidah, A.R. and Ong, P.T. (2017). Limestone treasures: Rare plants in Peninsular Malaysia - What they are, where they grow and how to conserve them?. Retrieved from https://www.researchgate.net/publication/322569252.

Middleton D.J, Puglisi C, Triboun P and Möller M (2010). Proposal to conserve *Paraboea* against Phylloboea and Trisepalum (Gesneriaceae). Taxon 59:1603

Mudoi, K.D. Saikia, S.P. Goswami, A. Gogoi, A. Bora, D. and Borthakur, M. (2013). Micropropagation of important bamboos: A review. African Journal of Biotechnology Vol. 12(20), pp. 2770-2785.

Piriyavinit, P. Rukkid, P. and Kanjanawattanawong, P. (2018) Micropropagation technique of P*araboea doitungensis* for conservation. Thai Agricultural Research Journal.Vol. 36 (3).

Puglisi C, Yao TL, Milne R, Moller M, Middleton DJ (2016) Generic recircumscription in the Loxocarpinae (Gesneriaceae), as inferred by phylogenetic and morphological data. Taxon 65:277–292.

Rafidah, A.R. and Joanne, T.P.C. (2012) *Paraboea bakeri* M.R. Hend.. Malaysia Biodiversity Information System (MyBIS). Retrieved September 03, 2020, from https://www.mybis.gov.my/art/44.

Rasyidah, M. Aziah, M.Y. Nurhafizah, I. & Norakma, M.N.(2016) Direct shoot regeneration from Nodal segment of Smilax myosotiflora. UMTAS 2016-178/299

Soepadmo A. (1998) Limestone, quartzite and ultramatic vegetation. In The Encyclopedia of Malaysia Plants. Archipelago Press, Singapore, 26-27.

Vinterhalter, B. Vinterhalter, D. and Neskovic, M. (2001) Effect of Irradiance, Sugars and Nitrogen on Leaf Size of *In Vitro* Grown *Ceratonia Siliqua L*. Biologia Plantarum volume 44, pages185–188.