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Temporary Immersion Bioreactor System (SETIS™): Technology for Large Scale *In Vitro* Production of *Boesenbergia rotunda*

Nurul Hanis Yahaya[1, 2], Siti Suhaila A Rahman*[1], Mohd Hakiman Mansor[2] and Mohd Zaki Abdullah[1]

[1]Forest Research Institute Malaysia (FRIM), 52109, Kepong, Selangor, Malaysia
[2]Faculty of Crop Science, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia
**Email: sitisuhaila@frim.gov.my*

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ABSTRACT

Boesenbergia rotunda is a well-known Southeast Asian herbal that has been used as a condiment, traditional medicine, and more recently in certain modern pharmaceutical goods. At Forest Research Institute Malaysia (FRIM), mass production of *B. rotunda* was obtained using tissue culture technique known as temporary immersion bioreactor system (TIBs), SETIS™, an efficient plant production system. However, it was observed that the young leaves were unable to open or unfurl due to lack of space in the vessel. This created unhealthy plant development and caused high mortality during acclimatisation period, as the unfurled leaves might have insufficient cuticular layer on their surface. A technique to manipulate the plant height was needed to facilitate leaf development and enhance high survival rates. Therefore, this study aimed to investigate the effect of paclobutrazol (PBZ) in the manipulation of medium culture formulations towards *B. rotunda* plant height. Young shoots of *B. rotunda* were treated in MS medium supplemented with 2 mg/L BAP and different concentrations of PBZ using SETIS™. Results indicated a reduction in plant height, where samples treated with 0.1 mg/L and 1.0 mg/L gave a plant height of 16.93 ± 2.00 cm and 20.17 ± 3.20 cm, respectively, compared to 34.27 ± 2.73 cm in control. The photosynthetic rates showed an increment in samples treated with 0.1 mg/L and 1.0 mg/L PBZ (0.56 ± 0.09 and 0.56 ± 0.07 µmol/m^2/s), respectively, compared to the control (0.18 ± 0.04 µmol/m^2/s). The PBZ treatments successfully inhibited *B. rotunda* plant heights to facilitate the development *in vitro* and increase survival rates during acclimatisation process.

Keywords: Finger root, liquid medium, medicinal plant, paclobutrazol, photosynthetic rate.

INTRODUCTION

Boesenbergia rotunda is a medicinal and edible plant that belongs to the Zingiberaceae family. It is native to India and Southeast Asia. It is commonly known as finger root because of the form of the rhizome's roots, which resemble fingers sprouting from the center. *B. rotunda* rhizome contains enzymes with anti-inflammatory, antioxidant, antipyretic, and antimutagenic effects (Ren et al., 2017; Sureerat et al., 2023). In a recent investigation, *B. rotunda* extract and its bioactive constituent, panduratin A, were found to reduce the infection of SARS-CoV-2 and potentially became a novel treatment against COVID-19 (Kanjanasirirat et al., 2020). Conventional propagation of *B. rotunda* involves vegetative techniques utilising rhizome segments, which can be time-consuming for large-scale production. In addition, several Zingiberaceae plants are susceptible to various diseases, including rhizome soft rot diseases, and leaf spot diseases (Hui and Liu, 2023; Merga, 2021). A study by Merga (2021) reported that leaf spot diseases caused by *Phyllosticta zingiberi* can cost up to 66% yield losses. Furthemore, *B. rotunda* rhizome is perishable as it is prone to fungal attack and needs to be dry upon harvesting to retain its physical and medicinal properties (Fahrudin et al., 2020). Therefore, plant tissue culture technique provides an alternative way of ensuring adequate disease-free planting materials for large-scale cultivation. Other than the micropropagation method for plant regeneration, studies were also carried out to develop somatic embryogenesis and suspension cells of *B. rotunda* and extract its valuable phytochemical properties (Tan et al., 2005; Ng et al., 2016; Ghani et al., 2020). The dry and embryogenic callus was found to contain flavonoids and several other metabolites, which may facilitate as marker for embryogenic cells studies in *B. rotunda*.

Plant tissue culture technique initially employed agar as a solidifying agent for growth medium. However, the non-nutritive nature of agar, coupled with its high cost, posed a challenge to cost-effective plant tissue culture. To reduce the expenses associated with agar usage, various gelling and non-gelling alternatives were evaluated as effective substitutes for agar commonly used in tissue culture media (Ullah et al., 2015). Consequently, using liquid medium is one of the alternatives to reduce production costs while improving propagation efficiency (Camargo et al., 2019), providing better growth for many species (Latawa et al., 2016) and also increasing nutrient diffusion rates (Prakash et al., 2002). Despite the benefits associated with liquid media for *in vitro* growth, technical issues that counter these advantages have been observed. One such issue is hyperhydricity, which arises due to the continuous immersion of plant tissues in liquid medium. A new commercial temporary immersion bioreactor system (TIBs), SETIS™ was used to overcome the obstacle. It is an advanced technique for micropropagation using liquid media in bioreactors. It is based on the periodic immersion of plant tissues into the liquid medium for a predetermined period of time, with alternating periods of aeration. This allows increased nutrient uptake, thus making TIBs an efficient system for large-scale micropropagation of plants (Georgiev et al., 2014; Ramírez-Mosqueda and Bello-Bello, 2021).

The SETIS™ bioreactor is based on the principle, using separated vessels for plant material and growth media, but with an optimised design and technology. The SETIS™ composed of seven components, such as culture and liquid medium containers, screw caps with silicone gaskets, filters for aeration and compressed air, and silicone tubes (Figure 1). However, the use of vessels in plant tissue culture limits the height of plant growth. Furthermore, plant growth regulators (PGRs) are used as retardants to strengthen and enhance plant quality and alleviate height limitations within the vessel.

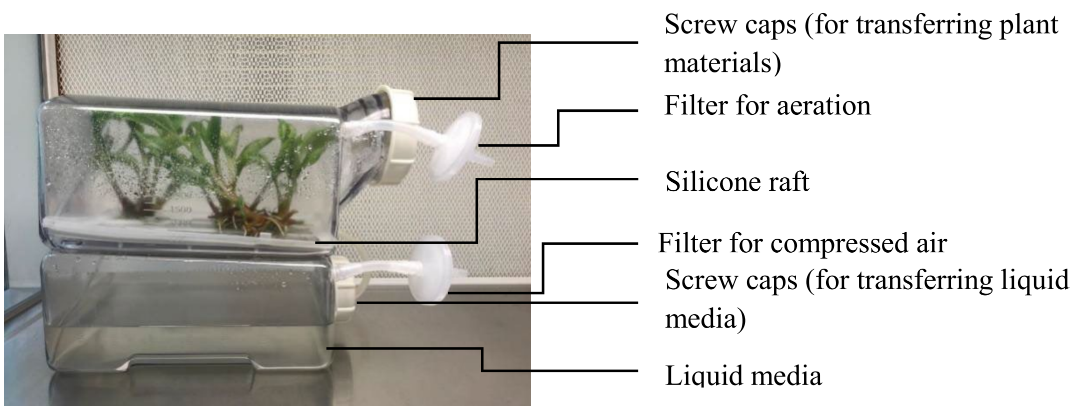


Figure 1. Structure of SETIS™ bioreactor system

Paclobutrazol (PBZ) has been shown to boost Zingiberaceae plant yield (Rusmin and Melati, 2018). PBZ when applied exogenously, showed a reduction of plant height in *Abies fraseri* (Crain and Cregg, 2017), increase the number of shoots in *Amethyst curcuma* (Jala and Bodhipadma, 2012), induce compact growth in *Malus domestica* (Kepenek and Karolu, 2011), and increase the photosynthetic rate in *Solanum tuberosum* (Tekalign and Hammes, 2005). However, the effect of PBZ on plants may vary depending on the species. Therefore, this study used a culture technique based on a temporary immersion bioreactor system (SETIS™) as an efficient system to determine the effects of different PBZ concentrations on the growth and quality of *B. rotunda*.

MATERIALS AND METHODS

Establishment of Aseptic Explants

The mature, healthy rhizomes of *B. rotunda* were purchased from a local herbal supplier in Selangor. The rhizomes were cleaned, rinsed with water to eliminate any residue, and air-dried before being placed in the dark to allow shoot sprouting. The sprouting shoot buds were collected and washed under running tap water for 5 min. They were then soaked in a 5% (v/v) Teepol (detergent solution) for 5 min, followed by 1% (w/v) Thiram (fungicide) for 5 min. Under aseptic conditions, the shoots were surface-sterilised with 50% ethanol for 2 min, followed by soaking in 50% (v/v) commercial sodium hypochlorite (Clorox®) solution for 5 min. After thoroughly rinsing with sterile distilled water, the shoot buds were surface-sterilised with 0.2% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) for 5 min and rinsed thrice with sterile distilled water. The sterilised buds were then placed vertically on a solid MS basal medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and 30% (w/v) Gelrite (Duchefa, Netherland) without hormone regulators. The explants were grown in a growth room under controlled conditions with 16/8 h (light/dark) regime under cool white fluorescent light at 22 ± 1 ºC, to obtain a clean culture with three shoots per explant.

Treatment of PBZ in Liquid Medium

To prepare 3 L of liquid medium supplemented with different concentrations of PBZ, which were 0.1 or 1 mg/L, MS basal liquid medium compositions were measured and mixed in 1.5 L reverse osmosis (RO) water. The paclobutrazol (PBZ) stock was prepared by dissolving 100 mg of PBZ powder with 200 to 500 µL of denatured ethanol (96%) and adding double-distilled reverse osmosis (RO) water to make up 100 mL stock solution, while 6-benzyladenopurine (BAP) was prepared by dissolving 100 mg of BAP powder with 200 to 500 µL of NaOH (1 M). Both were kept in an ambient bottle at 4 °C. Then, 0.3 or 3 mL of PBZ stock solution (1:1) and 6 mL of BAP stock solution (1:1) were aliquoted into the mixture, followed by pH adjustment to 5.8. Once the pH was determined, RO water was added until the total volume reached 3 L. Approximately 1.5 L of medium was poured into individual temporary immersion bioreactor system (SETIS™). The medium was sterilised by autoclaving at 121 °C at 1 kPa for 1 h. The medium was kept in the media room at ambient conditions (18 °C) overnight before use.

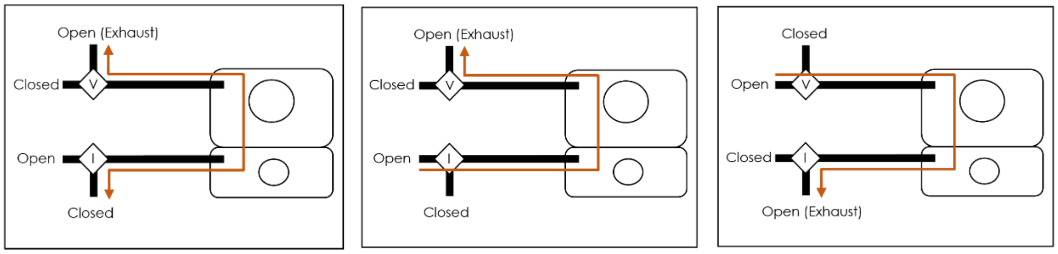
Seedlings Temporary Immersion Bioreactor System (SETIS™)

Working principles of SETIS™ bioreactor are based on three phases. The first phase is the stationary phase, where the liquid media stays in the media vessel and the plant material ventilates during this long phase. The second phase is the immersion phase, where the liquid media is transferred by air compression from the medium container to the growth container. During this phase, plant materials will periodically absorb nutrients and plant growth regulators from the liquid media for mass production. The third phase is the ventilation phase, where the liquid media returns to the medium container. By opening or closing the V (ventilation) and I (immersion) electrovalves, the immersion and ventilation phases are created (Figure 2).

Experimental Design and Treatment

A total of 9 shoots of *B. rotunda* were used for the treatments of PBZ on *B. rotunda* with three replications. Explants (previously cultured in MS basal media) were aseptically transferred into TIBs (SETIS™) with different concentrations of PBZ (0.1 and 1 mg/L) and the control (without PBZ); with immersion times of

10 min for every 4 h. The cultures were incubated at 22 ± 1 ºC with 16/8 h photoperiod (light/dark) at 2000 lux light intensity under 40% humidity room conditions for 3 months to reach maturity in culture.



a) Stationary phase b) Immersion phase c) Ventilation phase

Figure 2. SETIS™ Bioreactor working phase

Data Collection and Analysis

All plantlets that were 3 months old in TIBs (SETIS™) were observed for morphological changes through visual observation. Plant height, number of shoots, leaf length, and leaf width (fifth fully developed leaf from top) from each plantlet were measured using a meter ruler, and the number of shoots was counted manually. The photosynthetic rates of 3 months old cultures in TIBs (SETIS™) were determined using a portable photosynthesis system (GFS-3000; Walz, Germany, 2021). The portable GFS-3000 is a high-precision device for the assessment of plant photosynthesis (CO_2-absorption) or respiration (CO_2-release) and transpiration using leaf samples still attached to the plant. All data were analysed using variance ANOVA and Tukey's test (SPSS Ver. 20.1). Differences were considered statistically significant at a level of $P<0.05$.

RESULTS AND DISCUSSION

The effects of PBZ application at different concentrations (0.1 and 1.0 mg/L) on *B. rotunda* height were observed after 3 months of *in vitro* culture (Table 1). The overall result of the treated explant showed a rapid reduction in plant height compared to the control explant (Figure 3). In the third month, the control explant showed higher growth with a height of 34.27 ± 2.73 cm, almost double that of the explant treated with 1.0 mg/L PBZ. The plant height of the PBZ-treated explant at 0.1 mg/L was 20.17 ± 3.20 cm, showing a reduction of 51.8% in height compared to the control explant. In contrast, the explant treated with PBZ at 1.0 mg/L resulted in a height of 16.93 ± 2.00 cm, which exhibited a greater reduction of 71.8% compared to the control.

Table 1. Effect of different concentrations of paclobutrazol (PBZ) on plant height and the number of shoots of *B. rotunda* grown *in vitro*

Treatment	Number of sample	Parameters	
		Plant height (cm) (mean ± SD)	Number of shoots (mean ± SD)
Control	9	34.27 ± 2.73^{a}	10.67 ± 2.06^{a}
PBZ 0.1 mg/L	9	20.17 ± 3.20^{b}	12.67 ± 1.73^{b}
PBZ 1.0 mg/L	9	16.93 ± 2.00^{c}	4.33 ± 1.00^{c}

Values with different letters or an asterisk (*) is significantly different (one-way ANOVA, $P<0.05$).

In this study, the data showed that a higher concentration of PBZ resulted in a greater reduction in plant height. The reduction in plant height was probably due to the inhibition of cell elongation in the stem by PBZ. It has been proven that PBZ inhibits the production of gibberellic acid (GA), resulting in the depletion of these plant growth regulators within plant cells (Desta and Amare, 2021), which play important roles in regulating cell growth and elongation (He et al., 2020). PBZ functions by inhibiting the enzymes responsible for regulating the three steps of the terpenoid biosynthesis pathway associated with GA biosynthesis (Kumar et al., 2023). Previous studies also reported similar findings, where plant height reduced considerably with a high PBZ concentration (Kepenek and Karolu, 2011; Demir and Çelikel, 2019). Kumar et al. (2012) also reported that high PBZ concentration significantly decreased plant height (> 47.5%) of *Camelina sativa* when compared to the control. Additionally, the treated explants showed healthier and stronger characteristics than their control counterparts. The growth of control *in vitro* explants was rapid, and thereby affected their shoot tips due to the limited height of the container and needed to be sub-cultured to prevent shoot tip bending or damage (Figure 3(a)). Thus, these results suggested that PBZ successfully inhibited plant height and can be beneficial in alleviating these limitations within the container. At the same time, using PBZ reduced subculturing requirement of the species.

The number of shoots developing from explants treated with a low concentration of PBZ were observed to be relatively higher than those from high concentration PBZ-treated explants and control explants (Figure 4). This study showed that the highest shoot multiplication in *B. rotunda* was successfully achieved at low concentrations of PBZ (Table 1). The maximum shoot number per explant (12.67 ± 1.73) was obtained in MS medium supplemented with 0.1 mg/L PBZ, followed by the control with 10.67 ± 2.06 numbers of shoots. The lowest number of shoots was recorded at a high concentration of PBZ (1.0 mg/L), with 4.33 ± 1.00 numbers of shoots (Figure 4). A study by Indrayanti et al. (2018) partially agreed with this study, as they also observed a decrease in shoot number with an increase in PBZ concentration in the banana variant *cv.* Kepok grown *in vitro*.

A clear trend was observed in shoot numbers with increasing concentrations of PBZ, resulting in a decrease in the number of shoots per explant (but thicker, as shown in the picture). Hence, a low concentration of PBZ induced multiple shoots in *B. rotunda* grown *in vitro*. While a higher PBZ concentration may not be suitable for the explant. Therefore, strong correlations between PBZ concentrations and shoot multiplication in *B. rotunda* were identified. Different leaf sizes of *B. rotunda* became apparent 3 months after applying PBZ.

A B C

Figure 3. The effect of different concentrations of PBZ in SETIS™ on heights of *B. rotunda* explants. A, Control, B, 0.1 mg/L PBZ, C, 1.0 mg/L.

A B C

Figure 4. The effect of different concentrations of paclobutrazol (PBZ) on the number of shoots of *B. rotunda* after 3 months – A, leaves from control culture (unfurl) leaf, B, 0.1 mg/L treatment, and C, 1.0 mg/L treatment.

The leaves of the PBZ-treated explants were relatively smaller than those of the control explants. This could be due to the inhibition of cell elongation in the leaf by PBZ. The inhibitory effect of PBZ was observed on the leaf expansion of *B. rotunda* (Figure 5). These characteristics (longer and broader leaves) in explants treated with PBZ were similar to those observed in *Petunia×hybrida* (petunia 'Dreams Midnight') and *Dianthus chinensis* (dianthus 'Super Parfait' Mix) that showed a significant difference in leaf size compared to control explants (Collado and Hernández, 2021). This study demonstrated that the longest leaf length (11.77 ± 0.32 cm) and broadest leaf width (4.34 ± 0.26 cm) were observed in the control. Meanwhile, the shortest leaf length (6.44 ± 0.18 cm) was obtained from the explant treated with 1.0 mg/L PBZ, while treatment with 0.1 mg/L PBZ produced a leaf length of 7.22 ± 0.91 cm. As for leaf width, the highest value of 4.34 ± 0.26 cm was observed in the control group, compared to treatment with 0.1 and 1.0 mg/L PBZ which resulted in 3.08 ± 0.48 cm and 2.96 ± 0.18 cm in width, respectively (Table 2). It was shown that 1.0 mg/L PBZ application produced the shortest and narrowest leaf. The results were consistent with previous reports on leaf size by Demir and Çelikel (2019), where the PBZ-treated samples in *Iris* x *hollandica* cv. Frans Hals and Blue Magic cultivars showed smaller leaves compared to their control counterparts. In this study, PBZ application successfully reduced leaf length by 47.9% to 58.8% and leaf width by 29% to 31.7% (Table 2).

Table 2. Effect of different concentrations of paclobutrazol (PBZ) on leaf length and width of *B. rotunda* grown *in vitro*

Treatment	Number of sample	Parameters	
		Leaf length (mean ± SD) (cm)	Leaf width (mean ± SD) (cm)
Control	9	11.77 ± 0.32^{a}	4.34 ± 0.26^{a}
PBZ 0.1 mg/L	9	7.22 ± 0.91^{b}	3.08 ± 0.48^{b}
PBZ 1.0 mg/L	9	6.44 ± 0.18^{c}	2.96 ± 0.18^{b}

Values with different letters or an asterisk (*) are significantly different (one-way ANOVA, P<0.05).

The longer and broader leaves of control samples in the SETIS ™ caused folded leaves due to limited and confined space within the container. Folded leaves can reduce the surface area of leaves, which can impact gas exchange, light interception, or other physiological processes that occur through the leaf surface. Fortunately, no abnormal leaves were produced following treatment with PBZ. It was previously reported that PBZ-treated *Syzygium myrtifolium* (Roxb.) Walp did not show any abnormal leaf formation 3 months after application (Ahmad Nazarudin et al., 2012).

Improvement of photosynthesis is widely recognised as the next significant breakthrough in agricultural crop yield. This study measured net photosynthesis based on the CO_2 assimilation of *B. rotunda* in PBZ-treated and control explants using a portable photosynthesis system (GFS-3000; Walz, Germany). The data showed that the photosynthetic rate increased by approximately 3-fold in the treated explant compared to the control (Table 3).

Table 3. Effect of different concentrations of paclobutrazol (PBZ) on photosynthetic rates of *B. rotunda* grown *in vitro*

Treatment	Number of sample	Assimilation (mean ± SD) (µmol/m²/s)
Control	9	0.18 ± 0.04^a
PBZ 0.1 mg/L	9	0.50 ± 0.09^b
PBZ 1.0 mg/L	9	0.56 ± 0.07^b

Values with different letters are significantly different (one-way ANOVA, P<0.05).

In this study, the explants exposed to PBZ treatments showed a significantly higher mean photosynthetic rate ranging from 0.50 to 0.56 µmol/m²/s compared to the control value of 0.18 µmol/m²/s. This was in line with previously published reports on *Paeonia lactiflora* (Xia et al., 2018), cucumber seedlings (Baninasab and Ghobadi, 2011), and *Stevia rebaudiana* (Hajihashemi and Ehsanpour, 2013) that reported PBZ application induced and increased the photosynthetic rate in these plants. In this study, the PBZ-treated leaves of *B. rotunda* gave higher values of photosynthetic rates compared to the control. However, no significant difference in the photosynthetic rate was detected among the plants treated with different concentrations of paclobutrazol in *B. rotunda*.

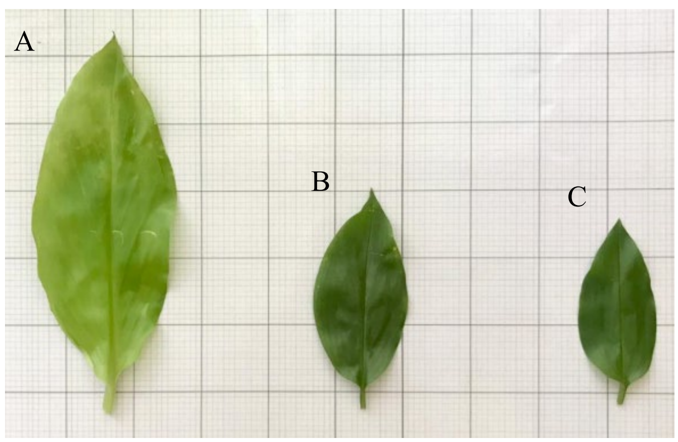


Figure 5. Morphological comparison of leaf size and a visual comparison of leaf colour in *B. rotunda* explants. Leaf for control explant (A), and explants treated with PBZ at 0.1 mg/L (B) and 1.0 mg/L (C).

In addition, visual observation of leaves from PBZ-treated and control explants showed a significant colour difference (Figure 5). Based on the observation, it was clear that paclobutrazol produced darker foliage in *B. rotunda* than that of control explants. This however, cannot be confirmed without proper assessment, such as using a colorimeter. In general, similar results were mentioned by Ahmad et al. (2015) and Mansuroglu et al. (2019). This finding was also supported by Xia et al. (2018), where an increase in PBZ concentration was accompanied by darker foliage in herbaceous peonies *(Paeonia lactiflora* Pall.). It was also reported that paclobutrazol intensified leaf colour in oil palm (*Elaeis guineensis* Jacq.) (Rahman et al., 2016) and in different cultivars of potato (Hamdani et al., 2018). Therefore, the direct cause of the leaf greenness of PBZ-treated *B. rotunda* cannot be concluded to be correlated with the increase of photosynthetic rates.

CONCLUSIONS

The *in vitro*-grown *B. rotunda* with desired traits has been successfully developed through PBZ treatments using temporary immersion bioreactor systems (SETIS™). This is an important finding, particularly for enhancing the *B. rotunda* micropropagation technique for mass production, as the plant offers many benefits and applications in pharmaceutical industries. In this study, the effect of PBZ on *B. rotunda* was observed in its morphological characteristics and photosynthetic rates. The results showed that the application of PBZ effectively inhibited the enzymes responsible for regulating the terpenoid biosynthesis pathway associated with GA biosynthesis, resulting in shorter plant height and leaf length, as well as narrow leaf width in *B. rotunda* compared to the control. The inhibition of plant height and leaf size can be beneficial for plants grown in small containers *in vitro*. In this study, a higher concentration of PBZ (1.0 mg/L and above) may not be suitable, as it significantly reduced the number of shoots. Therefore, the right combinations of PBZ application should be considered to enhance shoot regeneration and achieve preferable plant height. In addition, explants treated with PBZ showed an increase in photosynthetic rate and possessed darker green leaves compared to control explants. The presence of desired traits not detected in the control indicated that low concentration of PBZ could enhance *in vitro B. rotunda*. The repercussion of this study could be useful for larger scale *in vitro* cultivation with the supplementation of PBZ via bioreactor vessels to obtain continuous supplies of this valuable plant in the future.

ETHICAL APPROVAL

The *B. rotunda* is not native to Malaysia. Therefore, the sample was imported from Songkhla, Thailand and obtained at Pasar Borong Selayang, Selangor (Malaysia).

AUTHORS CONTRIBUTION

Conceived and designed the analysis: SSAR, MHM, MZA; collected the data: NHY, SSAR; data or analysis tools: NHY, MHM; performed the analysis: NHY; wrote the paper: NHY, SSAR.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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