IN VITRO SHOOT PROLIFERATION OF *Begonia pavonina*: A COMPARISON OF SEMISOLID, LIQUID, AND TEMPORARY IMMERSION MEDIUM SYSTEM

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Abstract
The *in vitro* shoot proliferation of endemic *Begonia pavonina* in three culture conditions i.e semisolid medium (SM), liquid culture medium (LM) and in temporary immersion bioreactor system (RITA®) was analyzed in this study. To minimize contamination rates, seeds were surface sterilized and cultured on MS basal media. The clean raised shoots were then used as explants for inoculation onto the tested culture conditions. In this experiment, the explants were maintained in MS medium supplemented with $0.1 mgL^{-1}$ BAP for shoot multiplication. After 4 weeks of incubation, higher regeneration rates were observed in TIM as compared to other medium conditions. The maximum shoot number was obtained from TIM system with a mean of 5.30 shoots per explant, followed by LM (2.47 shoots per explant) and SM (1.2 shoots per explant). Shoot hyperhydration was also lowest in a TIM system. Overall, TIM was shown to produce higher shoot multiplications combined with healthy morphological characteristics of plantlets. Shoot cultures from the all cultures were successfully rooted *in vitro* and acclimatized well in the greenhouse.

Keywords: *Begonia pavonina*, *in vitro* multiplication, liquid medium, semisolid medium, temporary immersion system medium

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Introduction
Begonia L. (Begoniaceae) is one of the most widespread plant taxa and the fifth or sixth largest angiosperm genus, with over 1800 recognized species names (Phutthai & Hughes, 2016; Tian et al., 2017). Begonias are widely distributed throughout tropical and subtropical regions, from America to Asia. In Peninsular Malaysia, 57 percent of the 54 native Begonia taxa are threatened, with 24 taxa classified as Critically Endangered and 21 species only known from a single location (Chua et al., 2009). *Begonia pavonina* is endemic to Malaysia, and is only known to be found in the Cameron Highland, Malaysia. Thus, this endemic species is essential for setting conservation priorities.

Begonias are of great horticultural interest and mostly used as food, ornamental purpose and for medicine around the world. Some *begonia* leaves are used as a flavoring for mixtures of fish and meat (Burkill, 1935). Indigenous people in Malaysia use *B. pavonina* leaves for food. In Indonesia, *B. baliensis*, *B. lempuyangensis* and *B. multibracteata*, are eaten in salads or cooked with fish (Girmansyah, 2009). In

several countries *Begonia* species are used medicinally and to treat different ailments (Ramesh et al. 2002; Girmansyah, 2009; Rop et al., 2012; Thorat et al. 2018).

Micropropagation is an innovative method that serve to overcome barriers in multiplication of elite species (Sengar et al., 2010). This method has been widely used for conservation of endangered, rare and important plant species. The method produces larger number of disease free and true-to-type plant breeds and in a relatively shorter time. There are several advantages of micropropagation technique over conventional methods of plant propagation which are as follow:

- Only small pieces of plant tissues (explants) are used to produce a large number of identified plants within a small space
- A much smaller space is required to store large number of plantlets, less space and labor for maintaining these plants
- Plantlets produced are usually free of bacteria and fungi. In some cases, plantlets can also be free of known viruses
- Nutrient levels, light, temperature and other factors can be more readily controlled to accelerate vegetative multiplication and regeneration
- In most cases, micropropagation is independent of seasons
- *In vitro* plant requires minimal attention between subcultures

In vitro propagation of many *Begonia* species has been reported worldwide including *B. cheimantha* (Fonnesbech, 1974; Preil; 2003), *B. erythrophylla* (Romocea et al., 2010), *B. rex* (Arora et al., 1970), *B. franconis* (Berghoef & Bruinsma, 1980), *B. semperflorens* (Romocea, 2011). In addition, *in vitro* plant regeneration and somatic embryogenesis from leaf explants of *B. pavonina* has been established previously (Rosilah et al., 2014). Plant tissue culture involved various methods for plant regeneration including modification of media, various type of explants, culture conditions etc. In plant tissue culture, plantlets are multiply in various culture media conditions including bioreactor, agar medium (semisolid) and temporary immersion culture system.

In bioreactor, the propagation of plant tissues were done in which the cultures are completely immersed into the liquid medium. However, in several plant species the condition causes, physiological disorder and loss of material due to asphyxia and hyperhydricity. This is due to the low oxygen content and the high water retention capacity (Kevers et al., 2004). To overcome these difficulties and to improve efficiency, TIM method by flooding of plant tissue at intermittent and short-lasting time intervals in liquid culture medium may ensures a proper supply of nutrients and gas transfers, and decreases mechanical stress (Welander et al., 2014; Mosqueda-Frometa et al., 2014). Plant cultured in TIM also resulted in an increase of the total phenolic content (TPC) and in a lower value of the total flavonoid content (Ruta et al., 2020).

Nevertheless, to the best of our knowledge, there are no available studies concerning the application of liquid culture or TIM in *B. pavonina in vitro* propagation. In this experiment, BAP (6-Benzylamino purine) was chosen as plant growth regulator and was standardized in culture media for optimum result. BAP is a cytokinin that is widely used in plant tissue culture for shoot multiplication due to its stability compared to other cytokinins, less susceptible to light oxidation, less costly and easier to acquire (Bhosale et al, 2011; Teixeira da Silva, 2012).

Material and methods

Media preparation and shoot multiplication

Clean *in vitro* cultures were used as explants in this study. *B. pavonina* capsules containing seeds was firstly harvested from the stock plants maintained at Forest Research Institute Malaysia (FRIM) nursery, Kepong

Selangor. The capsules were cleaned with liquid detergent and kept under running tap water for 20 min. Once in the laminar air flow, the capsules were treated with 70% ethanol for 30 secs prior soaking in 20% commercial bleach, Clorox^R (7.4% sodium hypochlorite) for 10 min. The capsules were then rinsed several times with sterile distilled water and air dried for 10 min. Seed was removed from the capsules under aseptic conditions and placed on seed germination medium (MS basal media). All culture were grown under 16 hours light and 8 hours dark period in air-conditioned culture room, illuminated by 40W (watts) white fluorescent tubes. The intensity of light was regulated between 2500-3000 lux. The temperature of culture room was maintained at 25±2°C

Media preparation and shoot multiplication

B. pavonina shoots germinated from seeds after 4 weeks in culture. The generated shoots were excised and cultured into each of culture conditions for shoot multiplication. Shoot regeneration media used in this study was MS basal medium (Murashige & Skoog, 1962) supplemented with 3% sucrose and 0.1 mg/L BAP. pH of the media was adjusted between 5.7 to 5.8. Three different culture conditions were prepared for shoot regeneration of *B. pavonina* which were 1) semi solid media (SM) 2) liquid media (LM) and 3) temporary immersion system RITA System (TIM).

In the first culture condition (i.e SM), Difco-Bacto Agar (8g L^{-1}) was used to solidify the media and 30 ml of the media was poured into each jars. In second culture condition (i.e LM), the media was prepared without Bacto agar. LM was carried out in 250 ml Erlenmeyer flasks containing 50 ml of shoot multiplication media. Once the explants were cultured into the media, the bottleneck was wrapped with aluminium foil and kept on a gyratory shaker at 60 rpm. In the third culture condition (TIM), explants were cultivated in RITA apparatus (CIRAD Ltd., France) (Figure 1a) containing shoot multiplication media without Bacto agar. The cultures were submerged with 200 mL media with immersion frequency of 1 min flooding in every 30 min. All cultures were maintained at 25 ± 2 ^{0}C with illumination powered by cool-white florescent light (16-h photoperiod) and transferred onto fresh media every 4 weeks. The experiments were conducted in ten replicates of seven explants. The number of shoots and shoot length produced from each treatment were observed every week.

Rooting and acclimatization of plantlets

Microcuttings of plantlets sized around 3-4 cm long from all culture conditions were excised and individually cultured on MS basal medium supplemented with 1.0 mgL^{-1} NAA for *in vitro* rooting. For acclimatization, rooted plantlets sized more than 4.0 cm high were removed from culture medium and washed under tap water to remove excess agar. The plantlets were then transplanted on sand medium for acclimatized in a weaning chamber under greenhouse conditions.

Statistical analysis

The experiment was conducted using 30 explants for every treatment. Data were subjected to ANOVA using the Least Significant Difference method at $P \leq 0.05$., and analyzed by Statistical Discovery Software (SAS). A Tukey's multiple comparison test was used to distinguish difference between treatments.

Result and discussion

Research and development of efficient protocols to improve large-scale micropropagation and reduced production costs through the use of innovative techniques are attractive alternative approach to traditional methods of plant production. Cultures are prone to developing glossy leaves and hyperhydricity when cultured in wrong medium. In addition, the occurrence of shoot tip necrosis on micropropagated shoots also limit micropropagation success. Therefore, mineral, hormonal composition of the medium and optimum culture condition must be carefully selected to prevent this disorder. Temporary immersion system has been used as an alternative to the conventional micropropagation process i.e semi-solid medium. The system ensures greater contact between explant and culture medium. It allows greater absorption of nutrients by

the explant and greater aeration in the *in vitro* environment allowing a larger number of plants to propagate in less time and space (Adelberg, 2006; Akin-Idowu et al., 2009). To date, there was no study reported on comparison of shoot proliferation of *B. pavonina* in SM, LM and in TIM. Thus, in the current study, we investigated the effect of semi-solid culture, liquid cultures and temporary immersion system on shoot multiplication of *B. pavonina* for development of high efficiency micropropagation protocols of the species.

To avoid contamination, clean cultures were first established by surface sterilized the seed upon cultured onto MS basal media. By using aseptic shoot cultures, contamination rate in shoot multiplication mediums was minimized. It was observed that direct transfer of surface sterilized seed onto mediums especially onto LM and TIM has produced 100% contamination after 4 weeks in culture (Table 1). Overall, by using aseptic seeds as an explant, contamination rates from Week 1 to Week 4 in SM, LM and TIM were 5% to 50%, 70% to 100% and 80% to 100% respectively. First sign of contamination appeared within one week of culture (Figure 1b) and the whole cultures was completely covered with contamination after 2 weeks in culture in LM and TIM. By using established aseptic shoot cultures as an explants, the percentage of contamination has drastically reduced to 10% in TIM, 20% in LM and 5% in SM (Table 2) (after 4 weeks of culture). Thus, it is important to established clean culture first before inoculation onto multiplication medium to avoid continuous contamination which will be time and effort wasted.

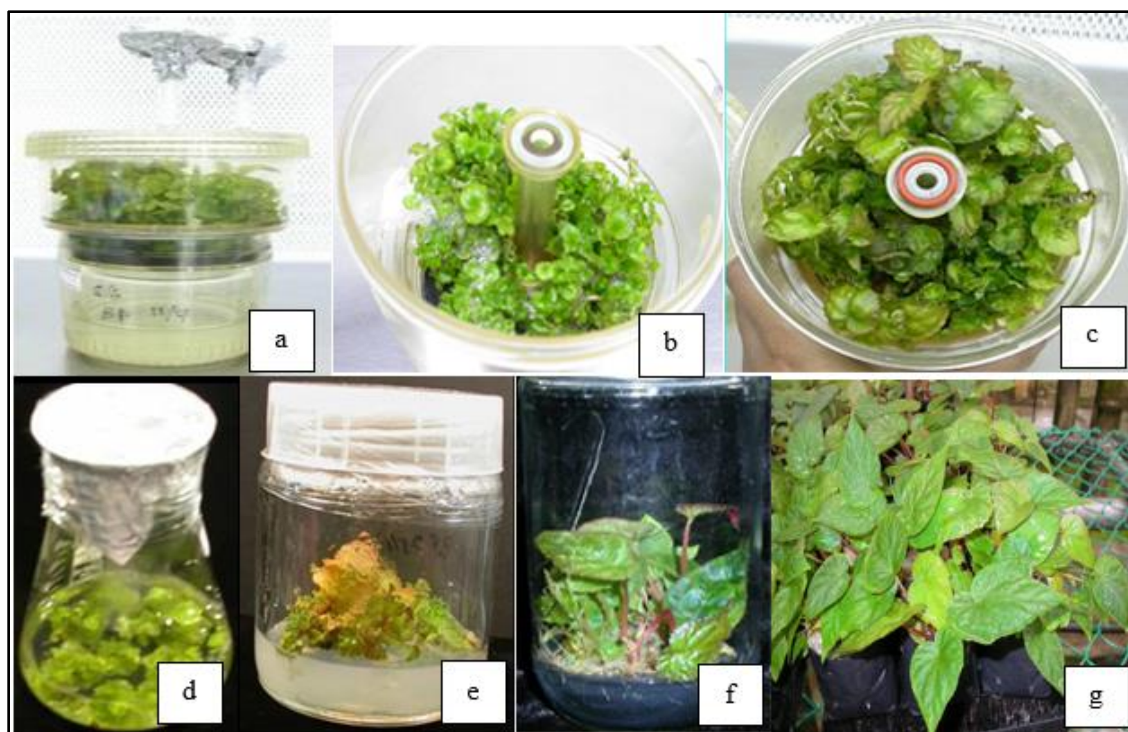

Figure 1. (a-g) *In vitro* culture of *B. pavonina*: (a) Shoot culture in TIM after 3 weeks in culture (b) Bubbles of contamination can be seen in TIM after one week of cultures (c) Proliferation of direct adventitious shoots grown on TIM supplemented with 0.1 mg/L BAP in TIM (RITA system) after 3 weeks in culture (d) Shoot culture in LM after 3 weeks in culture (e) Shoot culture in SM after 3rd sub cultured (f) Rooted plantlets cultured on MS supplemented with 1.0 mg/L NAA after 4 weeks in culture (g) Tissue-culture derived plant acclimatized in FRIM’s nursery (after 2 months).

Table 1. Contamination rates in different culture conditions after direct inoculation of surface sterilized seed explants onto multiplication medium

Culture condition	Number of explants	1st week (Mean %)	2nd week (Mean %)	3rd week (Mean %)	4th week (Mean %)
SM	30	5%	20%	30%	50%
LM	30	70%	100%	100%	100%
TIM	30	80%	100%	100%	100%

*SM = Semi solid medium
*LM = Liquid medium
*TIM = Temporary immersion medium (RITA SYSTEM)

Table 2. Contamination rates in different culture conditions in multiplication medium using clean shoot cultures firstly established from regeneration medium

Culture condition	Number of explants	Week 1 (Mean percentage)	Week 2 (Mean percentage)	Week 4 (Mean percentage)
SM	30	0%	5%	5%
LM	30	20%	20%	20%
TIM	30	10%	10%	10%

*SM = Semi solid medium
*LM = Liquid medium
*TIM = Temporary immersion medium (RITA SYSTEM)

Culture observation was made every day until the 4th weeks in culture. The results clearly indicated a significant increase in propagation rate in TIM as compared to LM and SM (Figure 2). The number of plantlets obtained in TIM was nearly doubled to LM and least plantlets were obtained in SM. This result indicates that liquid culture systems especially TIM improved the efficiency of *in vitro* propagation of *B. pavonine* as compared to LM and SM. In addition, no malformation of plantlets was obtained in TIM. Further development of the plantlets in TIM was also fast and without growth arrest, thus the use of TIM is recommended for this species. It is also noted that *B. pavonina* grew healthily with more stable morphological characteristics in TIM. No hyperhydric shoots was observed in the culture (Figure 1c).

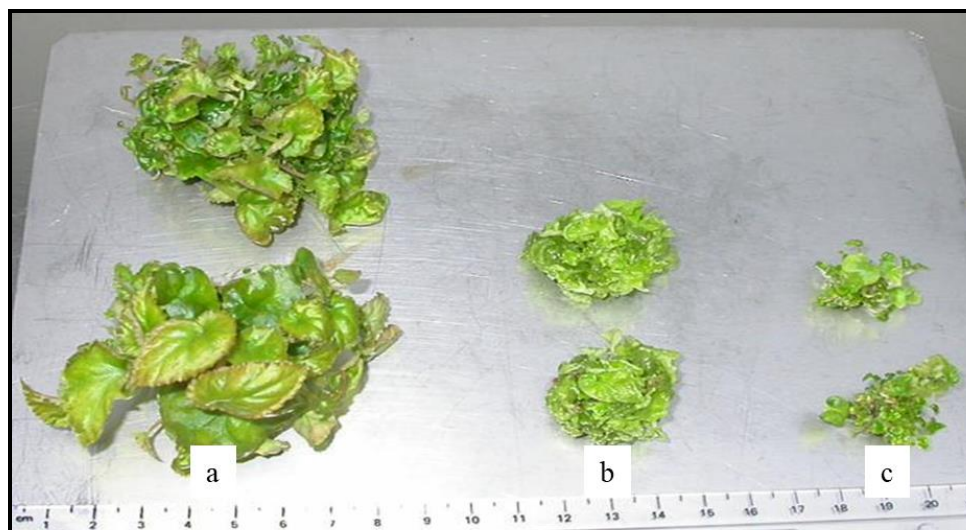

Figure 2. (a-c) Shoot clumps derived from different culture conditions after 4 weeks in cultures. All cultures were cultivated in MS supplemented with 0.1 mg/L BAP (a) Shoot culture in TIM. Each clumps sized around 8- 10 cm (b) Shoot culture in LM. Each clumps sized around 3-4 cm (c) Shoot culture in SM. Each clumps sized around 2-3 cm

On the other hands, shoots regenerated on SM were also typically of healthy appearance and did not display any symptoms of hyperhydration (Figure 1e). In contrast, the results obtained in LM were significantly different. The results showed that explant cultured on LM produced higher multiplication rate than in SM, but this was often accompanied by increased hyperhydricity (Figure 1d). The reason for the higher sensitivity of the cultures is possibly due to continuous access of the cultures to media in liquid media. In LM, greater surface area of the explant was in contact with the medium; whereas in SM, only the basal side of the explant was in contact with the medium and in TIM, the immersion of culture in the media is controlled. The experiment has also showed that stem segments showed more hyperhydricity than shoot tips in LM. Having said that, shoot multiplication rates in SM was lesser and smaller plants were produced in the same culture time as LM and TIM. The growth of plantlets was also slow in SM as compared to other medium.

The formation of vitrified shoots, which is one of the main drawbacks of a liquid medium, was also reduced using TIM system. Plantlets cultured on TIM showed continues favorable and positive effect during shoot growth every week. In the fourth week of culture, respond of plantlets in different media conditions were recorded. Plantlets cultivated in TIM gave higher shoot length, as well as, higher leaves diameter in comparison with plantlets cultured on LM and SM (Table 3). Leaves diameter in TIM ranged between 1.0 – 4.0 cm as compared to LM (0.5 – 2.0 cm) and SM (0.2 – 1.0 cm). Whereas the shoot length in TIM ranged between 2.5 – 9.0 cm as compared to LM (0.8 – 3.0 cm) and SM (0.1 – 2.0 cm). In addition, it is impossible to count the number of plantlets especially on LM and SM due to a numerous small and tiny shoots that were not suitable for further subculturing. However, after 3 – 4 times subculture onto the same media, cultures in LM and SM produced suitable plantlets size ready for the next stage (*in vitro* rooting). This result showed that cultures in TIM only need 3 - 4 weeks to produce suitable size of plantlets for rooting as compared to time needed by cultures in LM and SM (3-4 months). This result proved that culturing plants in TIM produced higher quality of plantlets in shorter time. Sizeable plantlets derived from different culture conditions were transferred onto MS semi solid supplemented with 1.0 mg/L NAA (Figure 1f) as previously described by Rosilah et al., (2014) for rooting. All plantlets rooted well and successfully acclimatized in greenhouse (Figure 1g).

Table 3. Comparison of *in vitro* shoot multiplication rate, leaves diameter and shoot length cultured on semi solid, liquid, and temporary immersion system medium after 4 weeks in culture.

Culture condition	Average shoot no.	Leaves diameter (cm)	Shoot length (cm)
SM	1.20 ± 0.08^{c}	$0.2 – 1.0^{c}$	$0.1 – 2.0^{c}$
LM	2.47 ± 0.05^{b}	$0.5 – 2.0^{b}$	$0.8 – 3.0^{b}$
TIM	5.30 ± 0.08^{a}	$1.0 – 4.0^{a}$	$2.5 – 9.0^{a}$

*SM = Semi solid medium
*LM = Liquid medium
*TIM = Temporary immersion medium (RITA SYSTEM)
*Different superscript letters in the same column indicate significant differences at $p \leq 0.05$.

According to Edward et al., (2017), liquid culture medium is more effective than solid culture medium for *in vitro* plant growth due to better accessibility of medium components for the plant tissue. Having said that, although liquid culture system in this study have shown to produce higher number of plant compared to semi solid media, the system also causes issues with plant hyperhydric and sometimes prohibits the growth (LM). This finding corroborated statement from Preil (2005) that plant grow faster in liquid culture systems but these systems are often of limited use due to shoots becoming hyperhydric with long-term continuous merged in liquid cultures.

This study showed that temporary immersion has improved plant material quality. It resulted in increased shoot vigour, high shoot length and increased leaves diameter. The morphology of *B. pavonina* cultured in

TIM were better as compared to LM and SM. It was shown that hyperhydricity, which seriously affects cultures in liquid medium, can be eliminated or controlled by using the immersion system. Temporary immersion system is one of few approaches available for rapid micropropagation of plant *in vitro* cultures. Many researches have highlighted the success of temporary immersion system for mass production of *in vitro* plants. Jime´nez et al., (1999) in their study on potato cultures reported that temporary immersion system produced three times higher stem length and internodes number compared to plant cultured in solid culture medium. *In vitro* production of hybrid hazelnut shoots also increased tremendously when cultured in temporary immersion system (Latawa et al., 2016). Temporary immersion system was reported to improved *in vitro* caper culture by promoting the proliferation, length, and vigor of the shoots (Gianguzzi et al., 2019). *Stevia rebaudiana* cultured on RITA bioreactor applications recorded healthiest plantlets and greatest number of shoots, nodes, leaves and root as compared to semi-solid medium (Bayraktar, 2019). Total biomass and the proliferation of shoots of *Lycium barbarum* is increased in TIM compared to the classical culture in semisolid medium (Ruta et al., 2020)

In addition, increased explant multiplication rate using temporary immersion system has been reported in other plant species including *Spathiphyllum* (Aka Kacar et al., 2020), *Vanilla planifolia* (Ramírez-Mosqueda and Iglesias-Andreu, 2016), *Quercus robur* (Gatti et al., 2017), *Gerbera jamesonii* (Mosqueda et al., 2017), *Dianthus caryophyllus* (Ahmadian et al., 2017) and *Anthurium andreanum* (Martínez-Estrada et al., 2019) *Musa* spp (Farahani & Majd 2012), *Stevia rebaudiana* (Vives et al. 2017) and *Chrysanthemum* (Hahn & Paek, 2005).

Temporary immersion systems have been said to give main advantage for reduction of the plantlets hyperhydricity (Pavlov & Bley, 2006). This technique provides short contact between the *in vitro* plant and the liquid medium rather than permanent contact, which based according to principle similar to mist bioreactors (Etienne & Berthouly 2002). Aguilar et al., 2019 also suggested that RITA system may reduce production costs in teak, since most of the multiplication would take place in the system. In addition, the submersion of plant in short duration in temporary immersion system will allow atmosphere renewal in the flask thus provides high aerobic system and photosynthetic activity needed for plant development (Akita & Takayama, 1994; Etienne & Berthouly, 2002; Ziv, 2005). This will lead to better culture condition for plant growth. However, the immersion times, i.e., duration or frequency, is the most decisive parameter for system efficiency (Alvard et al., 1993; Etienne & Berthouly, 2002). Thus it is recommended for further investigation for the most suitable time immersion to produce maximum growth of *in vitro B. pavonina.* Further improvements should be made by manipulating the immersion programs, culture conditions and various growth medium.

Conclusion

In this study, temporary immersion media (TIM), i.e. flooding of plant tissue at regular time intervals, have shown to combine high growth rates with optimal plant quality. This is the first demonstration of the production of *B. pavonina* plantlets from shoots cultured in liquid medium, and the protocol presented here shows good potential for application in large-scale propagation.This finding allows new opportunities for commercial laboratories dealing with the endemic *B. pavonina* production in future.

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