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MICROPROPAGATION OF THREE ENDEMIC *BEGONIAS* USING VARIOUS HORMONES CONCENTRATION AND CULTURE MEDIA APPLICATION

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Abstract. *Three species of Begonias endemic to Java and Sumatra, namely Begonia leuserensis, Begonia atricha and Begonia scottii, were conserved in Cibodas Botanic Gardens as sources of germplasm for ornamental plant and/or medicines. However, the information on efficient hormones concentration and their culture media application through an in vitro propagation effort is still limited. Therefore, this study aimed to explain the growth response of three species of Begonias using various hormone concentrations and culture media through in vitro propagation. The culture media using Murashige & Skoog (MS) media that combined with 6-Benzyladenine (BA) dan Thidiazuron (TDZ) hormones in different concentrations i.e. 0.5 mg/L, 1 mg/L, 2 mg/L, and 3 mg/L. Observation parameters included shoot number, plantlets height, and leaves number. The data were analyzed using analysis of variance (ANOVA) with the F test at a 5% significance level. The results showed that three species of Begonias were observed to have different growth responses in the combination of MS+BA and MS+TDZ media. The combination of MS+TDZ media produces more shoots number, while the combination of MS+BA media influenced higher in leaves number. A concentration of 0.5 mg/L of hormone showed a good regeneration, therefore were recommended for in vitro propagation of Begonia species.*

Keywords*: Begonia atricha, Begonia leuserensis, endemic, micropropagation, plant conservation*

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INTRODUCTION

Begonia (Begoniaceae) is the fifth-largest flowering plant genus that comprises 2019 species (Hughes et al., 2021). In Indonesia, the number of *Begonia* species reaches 250 species with a high level of endemism (Ardi et al., 2014a; 2014b; Girmansyah & Susanti, 2015; Hughes et al., 2015; Undaharta & Ardi, 2016; Lin et al., 2017; Ardi & Hughes, 2018). Sumatra, for example, has an endemic level of up to 80% of the 65 species of Begonia that were recorded from this island (Hughes et al., 2015; Ardi & Hughes, 2018).

Three Indonesian endemic *Begonia*, namely *B. leuserensis*, *B. atricha* and *B. scottii*, are conserved in Cibodas Botanic Gardens (Figure 1). *B. leuserensis* and *B. scottii* are endemic species to Sumatra Islands (Hughes et al., 2015), while *B. atricha* has limited distribution in West Sumatra (Girmansyah, 2017) and Central Java Provinces (Efendi,

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2019). Both *Begonia* species have a unique leaves color pattern and flowers, therefore are used for ornamental plants (Hartutiningsih, 2017). Furthermore, *Begonia* is also widely used for medicinal plants due to their antibacterial compound (Hartutiningsih et al., 2018; Indrakumar et al., 2017).

Figure 1. A. *Begonia leuserensis*, B. *Begonia atricha*, C. *Begonia scotti*

Begonia can be propagated through tissue culture (Kaviani et al., 2015; Kumari et al., 2012), conventional technique by leaves cuttings (Ningsih & Wardisi, 2013), and by seeds (Efendi et al., 2019). Propagation technique through tissue culture in an ex-situ conservation is considered effective because fewer explants are needed than leaves cuttings. Tissue culture may be done at any time of year, without waiting for the fruiting season. Fruiting is usually a problem in ex-situ *Begonia* conservation (Efendi, 2018), particularly in species where male and female flowers do not develop at the same time.

Micropropagation techniques for several exotic *Begonias* species have been reported (Nada et al., 2011; Gergely & Cachiţă-Cosma 2011; Sara et al., 2012; Kumaria et al., 2012; Kaviani et al., 2015; Manuela & Carmen, 2013; Rosilah et al., 2014; Kumari et al., 2017; Warseno et al., 2018). Some of explants sources used during in vitro propagation of *Begonias*, e.g. apical shoot of tuberous *Begonia* (Mendi et al., 2009), somatic embryogenesis of *Begonia* x *hiemalis* (Awal et al., 2008), leaf explants of *B. homonyma* (Kumari et al., 2017; Hendriyani et al., 2020) and seed explants of *Begonia montaniformis* x *Begonia ningmingensis* var. *bella* (Lai et al., 2018).

Although the tissue culture procedures and conditions for different *Begonias* are similar, the growth regulators required in the culture media varied. Several researches showed that different auxin (IBA, NAA) and cytokinin (BA, TDZ) concentrations and/or combinations are required in different species (Kumari et al., 2017, Kumaria et al., 2012, Espino et al 2004). Micropropagation study for native *Begonias* species in Indonesia is still limited. In practice, specific *Begonias* required different handling. Moreover, several problems in *Begonias micropropagation*, such as sterilization techniques (Kumari et al., 2017) and medium optimization (Kumari et al., 2017), as well as contaminants and browning of explants (Wati et al., 2020) are important to note. Therefore, this study aimed to explain the growth response of *B. leuserensis*, *B. atricha* and *B. scottii* using various hormones concentration and culture media through in vitro propagation. A simple and efficient protocol on micropropagation of *Begonia* using leaves explants are needed to facilities an ex-situ conservation, researches, and commercial planting for ornamental.

MATERIALS AND METHODS

Plant Materials

The study was conducted in Tissue Culture Laboratory, Cibodas Botanic Gardens, West Java. The explants of three *Begonia* species were collected from Cibodas Botanic Gardens in April 2017, namely *B. atricha* (No. collector ME489, originated from Mt. Slamet, Central Java), *Begonia scottii* (No. collector ME142, originated from Mt. Pesagi, Lampung), and *B. leuserensis* (No. collector ME458, originated from Mt. Ketambe, Mt. Leuser National Park, Aceh).

Media

The Murashige & Skoog (MS) medium was used as basic media (Murashige & Skoog 1962). It was added with 30 g/L of glucose. and 7 g/L of agar. Thus, hormones (BA and TDZ) at different concentration levels, i.e. 0.5 mg/L, 1 mg/L, 2 mg/L, and 3 mg/L were added to the medium for shoot induction. The pH of the medium was regulated with HCl or NaOH until it reaches 5.6 to 5.8.

Sterilization and Explant Initiation Stages

Sterilization of explants was conducted following sterilization methods by Ismaini et al. (2017). The fruit explants were cut transversely in a petri dish throughout the planting procedure, and the seeds were planted into each MS media without growth regulators. This initiation activity uses seeds as an alternative to obtain the contamination-free leaf explants. Next steps, fruit explants were washed using detergent, then soaked in a tween 20 solution for 15 minutes, immersed in a bactericide and fungicide solution for 30 minutes, immersed in a ppm solution for 30 minutes, immersed in a 20% bayclin solution for 15 minutes, and lastly immersed in 70% alcohol for 1-5 minutes. At each stage of sterilization, the explants were rinsed three times with sterile distilled water.

Shoot Propagation Stages

The *Begonia* explants, aged three months after culture, were used in the shoot propagation stage. Leaf explants of 0.5 cm were planted on MS+BA media and MS+TDZ, at concentrations of 0.5 mg/L, 1 mg/L, 2 mg/L, and 3 mg/L. Each treatment consisted of five replications. The parameters observed were shoot number, plantlets height and leaf number. The cultures were grown on a culture rack in an incubation room with 12 hours of continuous light exposure and a temperature of 20^{o}C.

Statistical Analysis

Growth data were analyzed using analysis of variance (ANOVA) with the F test at 5% significance level by MS. Excel and SPSS ver.16. If the F test has a significant effect, an intermediate test with the Duncan Multiple Range Test (DMRT) was performed at a 5% significance level.

RESULTS AND DISCUSSION

The Effectiveness of the Combination Hormone in Shoot Multiplication

The three species of *Begonia* produced shoot and different growth responses in the in vitro culture media. The combination of MS+TDZ media produced more shoots than MS+BA media on all of *Begonia*. The addition of BA and TDZ hormone increases cell division, stimulates shoot proliferation, and differentiates adventitious shoots (Arinaitwe et al., 2000). However, the best shoot multiplication rate was obtained with media containing combinations of MS+TDZ 0.5 mg/L in *B. atricha* species in this study (Table 1). This results in line with Kumari

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et al. (2017) and Kumaria et al. (2012) that additions TDZ effective to produce more in shoots multiplication *Begonia*.

Hormone concentration has an influence on shoot growth as well. *B. atricha* produced the most shoots with a medium combination of MS+TDZ 0.5 mg/L. Meanwhile, the higher the concentration of hormones tend to decrease in shoot growth (Table 1), excepts in *B. scottii*. The highest shoot number of *B. scottii* were produced in a combination MS+TDZ 3 mg/L (55.00 ± 2.88), however MS+TDZ at a level of 0.5 mg/L also produced a high shoot number (43.33 ± 3.79). George et al. (2008) suggested that the low concentrations of cytokinins can respond to the growth of axillary and adventitious shoots since the endogenous cytokinin concentration is sufficient, while applying high concentrations of cytokinins can decrease the number of shoots produced (Tiwari et al., 2000; Khawar et al., 2004; Kumaria et al., 2012).

Table 1. The shoot number during in vitro micropropagation of three species *Begonia* using different of media and hormone concentration combinatio

Medium	Concentration (mg/L)	Shoot Number (means)		
		B. scottii	***B. atricha***	***B. leuserensis***
MS+BA	0.5	10.00 ± 2.37	5.33 ± 5.35	2.33 ± 1.78
	1	32.67 ± 0.54	1.00 ± 1.00	6.33 ± 1.04
	2	5.67 ± 1.45	11.67 ± 2.48	4.67 ± 1.59
	3	9.67 ± 3.11	17.00 ± 2.56	4.00 ± 1.36
MS+TDZ	0.5	43.33 ± 3.79	65.67 ± 7.01	11.33 ± 1.02
	1	19.67 ± 3.63	43.67 ± 6.61	13.33 ± 3.59
	2	6.33 ± 1.84	34.00 ± 3.92	6.00 ± 1.73
	3	55.00 ± 2.88	14.33 ± 1.65	8.00 ± 0.71

Contamination: A Problem Still Unclear in This Study

Initial explants were used in this study are seeds, as an alternative to obtaining contamination-free leaf explants. Seed growth into good explants was shown in *B. leuserensis* grown on MS media without additional PGR (Figure 2). However, in this study, only *B. atricha* can develop well, so data on plantlet height and number of leaves only shown for *B. atricha*. In vitro cultures of *B. leuserensis* and *B. scottii* were discontinued because explants were contaminated by fungi. Contamination in both explants may be caused by endophytic contaminant (present within the explants), additionally, a lack of aseptic explant cutting fungal contamination also may occur from the air, or during culture. The richness and composition of fungal taxa varied according to the plant host. Correia et al. (2018) reported that fungi richness and diversity were greater in *Begonia fischeri* than in *Begonia olsoniae* and *Begonia venosa*. The discriminatory analysis demonstrated that fungal communities are structured according to hosts, implying that each plant species had its own endophytic communities, yet they were dominated by common fungal taxa. Ahmadi et al. (2012) also reported that contamination, especially biotic contamination, such as fungal and bacterial contamination, is the single most significant factor in plant in vitro production. Fungal contamination occurred in

all plantlets of *B. leuserensis* and *B. scottii*. It was indicated by the presence of white and green cotton or mycelium spores on the media and explants. Generally, contamination due to bacteria is indicated by the presence of yellow mucus on the explants or attached to the media in the form of wet lumps (Elfiani & Jakoni, 2015). In addition, the death of explants can occur due to injury when slicing plant tissue which causes cell damage to form a brownish color or browning. Browning can occur due to the presence of polyphenol oxidase enzymes which result in the oxidation of phenolic compounds to quinones which produce brown pigments when the tissue is injured (Queiroz et al., 2008).

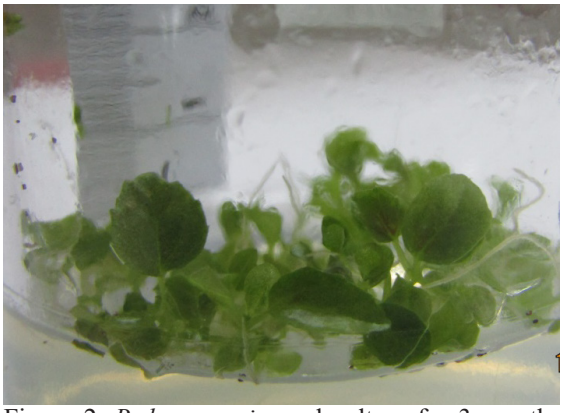

Figure 2. *B. leuserensis* seed culture for 3 months after planting

There are several factors that influence the tissue culture growth, including the type of culture media, freshness of explants, explant growing environment, and the frequency of subcultures (Hutami & Purmaningsih, 2003). The selection of explants is one of the important factors in determining the success of plant tissue culture multiplication. Explants with good physical condition, healthy and fresh will be able to survive on culture media with or without the addition of PGR (Sari et al., 2015). Wati et al. (2020) reported the propagation of *Begonia bimaensis* Undaharta & Ardaka using tissue culture was not able to grow properly due to fungal and bacterial contamination and browning occurred. The condition of tissue culture will be largely determined by the expertise of the implementer, working environment conditions, types of explants, sterilization methods, temperature, and climatic conditions at the time of culture.

Responses of *Begonia atricha* in Various Media and Hormone Concentrations Combination: Leaves Number and Plantlets Height

Based on mean value, the combination of MS+TDZ produced higher plantlets height than a combination of MS+BA media, meanwhile the combination of MS+BA produces a higher in leaves number (Table 2). The hormone concentration of 0.5 mg/L was the best concentration for explants of *B. atricha*, for both hormones (Figure 3). PGRs such as cytokinins can regulate plant physiological processes even at low concentrations. Cytokinin activity is related to growth and development processes in the cell cycle, especially to carry out nucleic acid metabolism and protein synthesis (Reddy et al., 2014; Adds et al., 2004). This is also related to the process of leaf formation and also the elongation of cells on the stem or shoots. The use of PGR in tissue culture depends on the purpose or desired growth. BA (Benzyl Adenine) is a plant growth hormone that is commonly used to stimulate shoot propagation because it has a strong activity compared to kinetin (Zaer & Mapes, l982).

BA has the same basic structure as kinetin but is more effective for multiplication because BA has a benzyl group (George & Sherington, 1984). New leaves were more produced on media with the addition of BA than TDZ. Better explant growth can be seen from the number of leaves. In this study, direct

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organogenesis occurred, indicated by the emergence of shoots, stems, and leaves without going through callus formation. Meanwhile, Table 2 shows that the combination of MS+BA media produced more leaves than MS+TDZ media. The best concentration to produce leaves was 0.5 mg/L for both types of PGR, particularly on MS+BA 0.5 mg/L medium as presented in Figure 3.

Table 2. The plantlets height and leaves number of in vitro culture explants of *B. atricha* in differences of media and hormone concentrations combination

Media	Concentration (mg/L)	Plantlet Height (cm)	Means	Leaves Number	Means
MS+BA	0.5	1.70 ± 1.29	0.94	13.33 ± 9.35	6.92
	1	0.60 ± 0.69		7.33 ± 2.08	
	2	0.77 ± 0.61		3.33 ± 2.52	
	3	0.70 ± 0.37		3.67 ± 1.20	
MS+TDZ	0.5	1.33 ± 0.29	1.28	4.67 ± 2.19	3.67
	1	0.67 ± 0.82		2.00 ± 1.41	
	2	1.77 ± 0.08		3.67 ± 1.33	
	3	1.37 ± 0.34		4.33 ± 0.76	

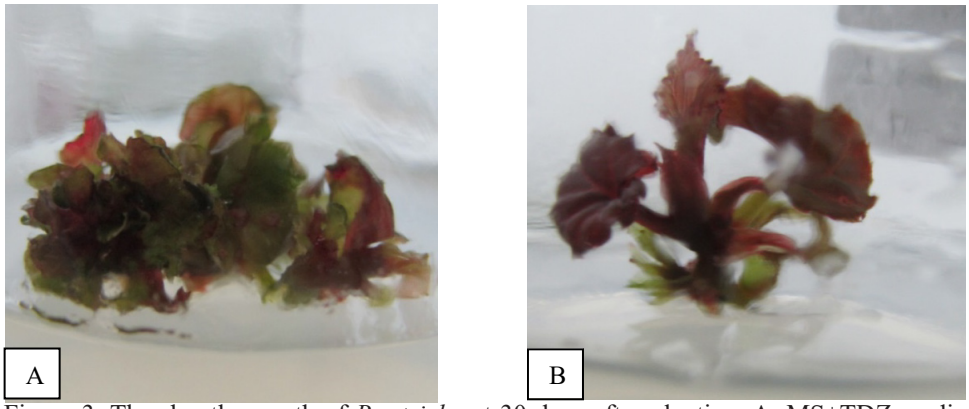

Figure 3. The shooth growth of *B. atricha* at 30 days after planting. A. MS+TDZ media with concentration of 0.5 mg/L; B. MS + BA media with concentration of 0.5 mg/L

*Begonia*s Growth in Various Hormone

The statistical analysis revealed that each species produced significant differences in shoot regeneration, plantlet height, and leaf number during in vitro culture (Table 3). The culture media of MS+TDZ and MS+BA produced significant differences in shoots number based on Duncan's analysis at 95%. Meanwhile, medium and hormone concentration did not produce a significant relationship on shoot growth, plant height, and the number of leaves.

The interaction and composition between hormones given in the medium and those produced by cells endogenously, as well as the different species and cultivar, determine the direction of the development of culture (Tores, 1989; Gunawan, 1992). This study used two synthetic cytokinins, both of which are predicted to stimulate shoot development in explants. Furthermore, the concentration of hormones also affected the growth phase of the explants. In the study of Kumari et al. (2017), *B. homonyma* shoots elongated when moved to media containing reduced concentrations of each hormone in the media.

This study showed that higher concentrations of hormone for shoot propagation and lower concentrations for elongation were very important for in vitro regeneration of *B. homonyma* shoots.

B. atricha produced the highest average number of shoots, plant height, and a number of leaves compared to the other two *Begonia* based on a combination of hormone concentration and culture media. *B. leuserensis* produced the lowest number of shoots compared to the two species of *Begonia* (Table 4).

In this study, Murashige & Skoog (MS) basic media was used with good growth results in all three types of *Begonia* explants. MS media contains nitrate, ammonium, calcium, and other macro and microelements that can affect the growth of explants (Saad & Elshahed, 2012). MS media was combined with cytokinin group PGR, namely BA and TDZ. From table 5, the shoots number and height of plantlets in the combination of MS and TDZ media were higher than MS and BA. As explained by Schulze (2007), that the addition of TDZ on the media can increase the response of morphogenesis in explants in the form of frequency of shoot formation, the number of shoots and induction time which is faster than other types of cytokinins. However, according to Lu (1993) and Huetteman & Preece (1993), the application of TDZ in tissue culture also has a negative impact, for example causing hyperhydricity or vitrification of plants, namely physiological malformations, abnormal leaf morphology, short and compact shoots, and problems in elongation and rooting. Meanwhile, the effectiveness of BA in shoot propagation in vitro has been reported in the study of Mendi et al. (2009), where the combination of BA and NAA is needed for in vitro shoot propagation of *B. tuberous*. Higher concentrations of cytokinins combined with auxin were more effective in shoot regeneration than single cytokinins used.

Hormone concentration also affected the regeneration of explants during in vitro culture propagation. From the results of the study, it was known that the concentration of 0.5 mg/L of TDZ and BA were the best concentration to produce the number of shoots, the height of plantlets, and number of leaves on the three types of *Begonia* (Table 6). PGR from the cytokinin group plays a role in the process of cell division, organ formation, and the formation of plant buds (George et al., 2008). Determination of the right concentration of cytokinins is very important to produce maximum shoot multiplication. According to Pierik (1987), application of cytokinins 0.1-10 mg/L can induce shoot formation according to plant cultivar specifications. This is in accordance with the results of this study which used concentrations in the appropriate range so that growth could take place properly.

Table 3. Statistic data of *in vitro* regeneration of three *Begonia* species with combination of hormone concentrations and culture media

Parameter	Shoots Number	Plantlet Height	Leaves Number
Species (S)	*	*	*
Culture media (M)	*	ns	Ns
Concentration (C)	ns	ns	Ns
S x M	ns	ns	Ns
S x C	ns	ns	Ns
S x M x C	ns	ns	Ns

Notes: S x M: treatment combination of species and media, S x C: treatment combination of species and concentration, S x M x C: treatment combination of species, media dan concentration. *) significant difference, ns: not significant based on Duncan Multiple Range Test (DMRT) at 5% level of significance.

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Table 4. The average in vitro regeneration on three *Begonia* using combination hormone concentration and medium

Species	Shoots Number	Plantlets Height	Leaves Number
B. scottii	22.79b ± 4.59	0.00a ± 0.00	0.00a ± 0.00
B. atricha	24.08b ± 7.38	1.11b ± 0.15	5.29b ± 1.32
B. leuserensis	7.00a ± 1.04	0.00a ± 0.00	0.00a ± 0.00

Mean value followed by the same letters in the same column of each parameter indicate no significant differences based on the Duncan test at $P < 0.05$

Table 5. The influences on media combination and explants regeneration of *Begonia* species by in vitro propagation

Media	Shoots Number	Plantlets Height	Leaves Number
MS+BA	9.19a ± 1.82	0.32a ± 0.10	2.31a ± 0.97
MS+TDZ	26.72b ± 5.41	0.43a ± 0.12	1.22a ± 0.40

Mean value followed by the same letters in the same column of each parameter indicate no significant differences based on the Duncan test at $P < 0.05$

Table 6. The influence of hormone concentrations on explants regeneration of three *Begonia* species by in vitro propagation

Concentrations (mg/L)	Shoots Number	Plantlets Height	Leaves Number
0.5	23.00a ± 7.28	0.51b ± 0.20	3.00a ± 1.79
1	19.44a ± 7.41	0.21a ± 0.12	1.56a ± 0.84
2	11.39a ± 3.89	0.42ab ± 0.17	1.17a ± 0.53
3	18.00a ± 5.01	0.34ab ± 0.13	1.33a ± 0.51

Mean value followed by the same letters in the same column of each parameter indicate no significant differences based on the Duncan test at $P < 0.05$

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