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Research Article

The Formula media *in vitro* Propagation and Conservation of *Ludwigia* sp.

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ABSTRACT
The aquatic plant "Red Malang" (*Ludwigia* sp.) has a fairly high economic value as an ornamental aquatic plant, so it has the potential to be developed. The growth of *in vitro* cultures in culture bottles is high-speed, so it is necessary to find a formula media to inhibit growth so that the frequency of subcultures is reduced. The current research aims to produce a formula media for shoot multiplication and *in vitro* culture conservation. The research was carried out at the ICABIOGRAD tissue culture laboratory from April 2020 to June 2021. Research activities included plant propagation, conservation, and regeneration after conservation. Plant material was using in the form of a culture collection in the ICABIOGRAD tissue culture laboratory, treatment media for propagation were BA (0; 0.1; 0.3; 0.5; 0.7 and 0.9 mg/L) + thidiazuron (TDZ) (0 and 0.1mg/L). For conservation were MS + BA medium (0 and 0.1 mg/L) + paclobutrazol (0; 0.1; 0.3; 0.5; 0.7 mg/L) and for shoot regeneration after conservation using MS medium without Plant Growth Regulator (PGR). Data analysis using the Anova SAS version 9.0 test program. Further test using DMRT test with alpha level 5%. There was no difference in the mean value between levels of TDZ treatment on the number of shoots and leaves. The difference in the mean value between levels of TDZ treatment was very significant on shoot height, the number of roots, and root length. BA treatment with a concentration of 0.7 mg/L is better because it gives higher results for each observation variable. For conservation, treatment with paclobutrazol 0.5 mg/L inhibited shoot and leaf count, and 0.3 mg/L inhibited shoot formation. Cultures stored for six months grew normally after being regenerated. The highest shoots and the highest number of leaves were obtained from the treatment of paclobutrazol without BA. This study indicated that the propagation media of aquatic plants *Ludwigia* sp. did not require high concentrations of BA. Cultures could be stored for over six months using paclobutrazol with 0.3-0.6 mg/L.

INTRODUCTION
Red Malang (*Ludwigia* sp.) is an aquatic water plant that has been widely developed, and its uses include beautifying aquariums. *Ludwigia* sp. aquatic plants are attractive for commercialization because they have economic value and are unique because of their bright red leaves. The benefits of aquatic ornamental plants are an addition to beautifying aquariums or fish ponds. It also protects fish from sunlight and improves water quality because they can produce oxygen and absorb toxins such as ammonia in the water and as a place for fish to lay eggs (Nugraha et al. 2018). Types of ornamental plants currently being developed include *Bacopa* genus, *B. australensis*, *B. caroliniana*, *B. ianigera*. *B. myriophylloides*. *B.*

monnieri, *B. rotundifolia*. *B. chamaedryoides* (Kunth) Wettst *(Syn. Herpestis chamaedryoides Kunth)*. *B. australis* (Nugraha et al. 2017).

Aquatic plants' micro-propagation is still conventional (Yunita et al. 2018). Constraints from conventional propagation for mass production of aquatic ornamental plants include non-uniform and non-sterile seeds produced. Developing tissue culture techniques to obtain significant and sterile seedlings is necessary to solve the problem. Tissue culture technology currently being applied has many benefits, including mass propagation of high-yield seedlings to assemble new varieties and preserve germplasm (Siddique et al. 2015). The formula media for the propagation of aquatic plants *B. australis* has been produced by Yunita et al. (2018), Nugraha et al. (2017), and Nugraha et al. (2018), using MS media + 0.5 mg/L Benzyl Adenine (BA)+ 0.5 mg/L kinetin and 0.5 mg/L BA + 0.1 mg/L TDZ.

BA, a growth regulator from the cytokinin group, has been widely used for shoot induction and propagation *in vitro* culture because BA has a vital activity for cell division and is more stable than kinetin and zeatin (Lestari 2011; M. et al. 2017). PGR, such as BA has a significant role in cell division during plant metabolism in shoot induction and multiplication (Ashraf et al. 2014). The exact concentration of BA for each plant is not the same depending on the type of plant, physiological conditions of the explant, type of primary media, environmental conditions of growth, and genetic factors (Lestari 2015). Propagation of ginger using ZPT 4.5 mg/L BA is the best concentration to stimulate shoot multiplication (Abbas et al. 2011). Besides PGR, minerals are essential elements in media culture (Kumar & Reddy 2011). TDZ is a compound belonging to diphenyl urea and has almost the same activity as cytokinins, often used to stimulate cell division in shoot proliferation, especially in woody plants (Lestari 2015). Using a combination of TDZ with BA has succeeded in increasing the ability of cell proliferation in various plants, for example, *Plumbago zeylanica* (Syahid & Kristina 2008; Lestari et al. 2013).

In vitro culture conservation for active collection usually uses a formula media for propagation to only last for 2-3 months because the nutrients have run out (Dewi et al. 2016). For culture growth to be extended, it needs to be inhibited to prolong the time of sub-culture. Reducing the frequency of sub-cultures will lower maintenance costs and reduce the risk of contamination (Dewi et al. 2016; Mendes et al. 2021). Conservation of aquatic ornamental plants *B. australis* and *Alternatia reinecki* using MS media + 0.7 mg/L paclobutrazol showed inhibition until the 6th on shoot height, number of shoots, number of roots, root length, and number of leaves. In this conservation medium, the culture remains green and looks fresh (Lestari et al. 2021). In culture conservation, through *in vitro* culture, several techniques can be applied, including cryopreservation, simple conservation, and slow growth conservation (Dewi et al. 2016).

Conservation media techniques with slow growth techniques generally use chemical compounds to inhibit the growth of cultures, including paclobutrazol, cycocel (CCC), and osmotic compounds such as sorbitol and mannitol (Huang et al. 2014; Silva et al. 2019). Paclobutrazol is an active compound inhibiting the oxidation of kaurene to ent-kaurene as a precursor of the growth regulator of gibberellic acid in the apical meristem, causing inhibition of cell division at the growth point (Negi et al. 2017; Bisht et al. 2018). In slow-growth conservation, growth and cell division are conditioned to occur very slowly, or metabolism is stopped so that culture development stops and does not change the genetic nature of the plant (Lestari et al. 2021). The benefits of *in vitro* culture conservation include keeping germplasm accessions/collections from becoming extinct (Huang et al. 2014) by inhibiting cell growth and division from

becoming very slow (Indrayanti et al. 2018).

The advantages of *in vitro* culture for conservation include that it does not require a large/wide place/container, accessions, or germplasm stored in thousands and can be stored for more than five years (Dewi et al. 2016). To maintain accession, which is very valuable germplasm has a high economic value. In contrast, if it is stored conventionally, there is a risk of damage due to natural disasters or drought (Silva et al. 2019). The collection of germplasm accessions is also beneficial as genetic material in the assembly of new varieties (Arrigoni-Blank et al. 2014). Formulas media for the propagation of aquatic ornamental plants are still limited, namely *B. australis* (Nugraha et al. 2017; Yunita et al. 2018), as well as formula media for *in vitro* culture conservation.

Lestari et al. (2021) conducted a conservation study through *in vitro* culture of *B. australis* and *A. reinecki* using MS + paclobutrazol media. So many aquatic plants are currently being developed that research is needed to obtain formula media for *in vitro* propagation and conservation. The study aimed to produce the best formula media for the propagation and conservation of ornamental plant cultures.

MATERIALS AND METHODS

Materials

Plant material is a sterile culture of aquatic plants aged two months and collected of the Tissue Culture Laboratory.

Methods

The research was carried out at the tissue Culture Laboratory, the Indonesian Centre for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), at Cimanggu street No. 3 Bogor from April 2020 to July 2021.

The research consisted of 3 activities, namely (1) Testing the formula media for propagation, (2) Testing the formula media for conservation (3) Testing the regeneration ability of the culture after conservation. The media used were MS basic media plus macro salt, micro salt, vitamins from group B (thiamine, pyridoxine, nicotinamide acid), and myo inositol, two grams of agar gel were added as a compactor, and 30 g of sucrose as a carbon source.

The media's pH was 5.7 by adding 0.1 N HCl or NaOH solution. The media was sterilized using an autoclave with a pressure of 121 Psi for 15 minutes. Sterile culture bottles filled with 25 ml of treatment media

Test the media formula for propagation.

The ex-plant was used as a single node with a size of ± 1 cm. The experimental design was factorial and completely randomized. consisting of two factors. the first factor was the concentration of BA (0; 0.1; 0.3; 0.5; 0.7 and 0.9 mg/L). Moreover, the second factor was the concentration of TDZ (0 and 0.1 mg/L). Each treatment was repeated in ten bottles. The observed variables were shoot height, number of shoots, number of leaves, number of roots, and root length. The basic medium commonly used for shoot induction and shoot multiplication is MS base media (Murashige & Skoog 1962).

Test the formula media for conservation.

Ex-plants are stem from sterile single-node culture ± 1 cm. The experimental design was factorial and completely randomized, consisting of two factors: two levels of BA concentration (0 and 0.1 mg/L), and the second factor was five levels of paclobutrazol concentration (0; 1; 0.3; 0.5;

0.7 and 0.9 mg/L). Bottles planted with ex-plants are placed on the culture rack under irradiation using a TL lamp. Illumination intensity 1500 lux for 16 hours in 1 day. The variables observed were shoot height, number of shoots, number of leaves, root length, and number of roots.

Culture regeneration after conservation.

Cultures stored for ± six months were transferred to MS 0 media (without PGR). Ex-plants in the form of stems of single nodes, one explant per bottle. The number of ex-plants from each conservation medium was planted in as many as ten bottles. The observed variables included shoot height, number of shoots, and number of leaves.

Data Analysis

The data were analysed using the Anova SAS version 9.0 test program, further testing using DMRT with an alpha level of 5%.

RESULTS AND DISCUSSION

Results

Induction of shoot multiplication

Analysis of variance on the variables of shoot height, number of shoots, number of leaves, and number of roots and interactions between TDZ and BA treatments are presented in Tables 1 and 2. Analysis of variance on all observed variables showed an interaction between TDZ treatment and BA.

Table 1. The variance of each observation variable on shoot proliferation.

Variable	F-Calculate treatment			CV (%)
	Thidiazuron (TDZ)	Benzil Adenin (BA)	TDZ*BA	
Shoot height	107.88**	8.30**	6.39**	27.90
Number of shoots	0.05ns	2.57*	3.51**	46.19
Number of leaves	3.55ns	1.59ns	1.81ns	36.82
Number of roots	175.06**	3.25**	17.89**	68.35
Root length	137.28**	5.16**	10.52**	79.07

Note: ns) not significantly different at = 5% based on F-test results. *) significantly different at = 5% based on F-test results. **) very significant difference at = 1% based on F-test results. tdz*BA Interaction between this treatment and BA. CV) Coefficient of Diversity.

Test formula media for conservation

Analysis of variance and Interaction between BA treatment and paclobutrazol on the variables of shoot height, number of leaves, number of roots, root length and number of shoots are presented in Tables 3 and 4.

Shoot regeneration after conservation

Analysis of the various effects of BA with paclobutrazol treatments on conservation media showed an interaction, and the results were significantly different for all observed variables (Table 5). The effect of interaction between BA and paclobutrazol during conservation on shoot regeneration ability is presented in Table 6.

The Interaction between TDZ and BA for shoot propagation showed that BA treatment of 0.7 mg/L without TDZ produced the highest shoots at 5.93 cm. However, for the number of shoots, the number of roots and root length were not significantly different (Table 2).

It is suspected that the *Ludwigia* sp. contains high levels of PGR, both cytokinins and auxins, so in the media, without BA and TDZ quite a lot of shoots were produced (Table 1). The results prove that the activity

Table 2. Interaction of Thidiazuron and Benzyl Adenine. on shoot height, number of shoots, number of roots, and root length five weeks after planting (MST).

Thidiazuron (mg/L)	Benzil Adenin (mg/L)						Mean
	0.0	0.1	0.3	0.5	0.7	0.9	
shoot height (cm)							
0.0	3.91^{b}	3.80^{b}	3.20^{bc}	3.10^{bc}	5.40^{a}	3.80^{b}	3.86
0.1	3.41^{b}	1.83^{d}	1.83^{d}	1.86^{d}	2.06^{d}	2.51^{cd}	2.25
Mean	3.66	2.81	2.51	2.48	3.73	3.15	
CV (%)	7.90						
Number of shoots							
0.0	4.60^{abc}	5.50^{ab}	5.80^{ab}	6.80^{a}	3.80^{bc}	5.40^{ab}	4.35
0.1	7.00^{a}	2.60^{c}	6.20^{ab}	5.70^{ab}	5.70^{ab}	4.10^{bc}	5.21
Mean	5.8	4.05	6	6.25	4.75	4.75	
CV (%)	46.19						
Number of roots							
0.0	7.50^{a}	0.00^{c}	8.02^{a}	7.00^{a}	0.00^{c}	7.90^{a}	5.1
0.1	3.20^{b}	0.30^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.58
Mean	5.35	0.15	4.01	3.5	0	3.95	
CV (%)	8.35						
Root length (cm)							
0.0	0.00^{c}	1.65^{a}	1.05^{b}	1.37^{ab}	1.43^{ab}	1.32^{ab}	0.94
0.1	0.37^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	0.37
Mean	0.18	0.85	0.5	0.68	0.7	0.66	
CV (%)	79.07						

Note: Data followed by the same letter in the same variable are not significantly different based on DMRT test level = 5%.

Table 3. Analysis of the variance of each variable in the treatment of BA and paclobutrazol Six months after planting.

Variable	F calculate			CV (%)
	Benzil Adenin (BA)	Paklobutrazol	BA*paclobutrazol	
Shoot height	17.9**	11.93**	29.8**	26.56
Number of leaves	8.99**	10.74**	6.55**	30.14
Number of roots	3.31^{ns}	3.50*	5.21**	35.23
Root length	22.62**	kon11.35**	0.52^{ns}	55.39
Number of shoots	7.12**	3.52*	11.48**	32.92

Note: ns) is not significantly different at = 5% based on the F-test results. *) is significantly different at = 5% based on the F-test results. **) is significantly different at = 1% based on the F-test results. BA*paclobutrazol) interaction between BA and paclobutrazol treatments. CV) Coefficient of diversity.

of growth regulators depends on the type of chemicals, chemical structure, and chemical concentration, plant genotype and physiological phase (Satyavathi et al. 2004).

TDZ can be given together with other growth regulators, such as cytokinins or auxins. At the initiation of mangosteen shoots, using BA plus TDZ is the best treatment for shoot formation (Lestari et al. 2015). TDZ stimulates shoot multiplication in several plants, including breadfruit (Supriati et al. 2005) and aromatic ginger, by adding 0.1 mg/L TDZ to MS + BA 5 mg/L (Lestari & Hutami 2005). However, not all plants responded to shoot multiplication in starfruit plants, TDZ only increased shoot height and number of leaves (Supriati et al. 2005).

Paclobutrazol is a growth inhibitory compound that has been applied to inhibit the growth of cultures in *in vitro* culture. and in various plants, including fruit trees (Hasan et al. 2013; Mendes et al. 2021). The

Table 4. Effect of Interaction between BA and paclobutrazol on the growth of shoot height number of leaves, number of roots, and number of shoots.

Benzil Adenin (mg/L)	Paclobutrazol(mg/L)				Mean
	0.1	0.3	0.5	0.7	
Shoot height (cm)					
0.0	2.50^{d}	4.67^{b}	2.95^{cd}	2.88^{cd}	3.25
0.1	6.80^{a}	3.30^{cd}	3.78^{c}	2.90^{cd}	4.19
Mean	4.65	3.98	3.36	2.89	
CV (%)	26.56				
Number of leaves					
0.0	29.30^{bc}	3.70^{b}	20.10^{d}	23.10^{cd}	26.3
0.1	47.20^{a}	25.90^{bcd}	25.67^{bcd}	30.30^{bc}	32.26
Mean	38.25	29.3	282.88	26.7	
CV (%)	30.14				
Number of roots					
0.0	18.30^{bc}	21.40^{bc}	15.00^{c}	21.50^{bc}	19.05
0.1	29.30^{a}	14.80^{c}	20.56^{bc}	23.40^{ab}	22.01
Mean	23.8	18.1	17.78	22.45	
CV (%)	35.23				
Number of shoots					
0.0	1.40^{b}	1.60^{b}	1.10^{b}	1.50^{b}	1.4
0.1	2.20^{a}	1.10^{b}	2.22^{a}	1.30^{b}	1.7
Mean	1.8	1.35	1.66	1.45	
CV (%)	32.92				

Note: Data followed by the same letter on the same variable are not significantly different based on Duncan's test with a level of = 5%.

Table 5. The variance of each variable of the effect of BA and paclobutrazol treatment media on shoot regeneration after conservation.

Variable	F-Calculate			
	Benzil Adenin (BA)	Paclobutrazol	BA*Paclobutrazol	CV (%)
Shoot height (cm)	6.59 *	1.74 ns	8.37 **	33.81
Number of shoots	3.32 ns	7.54 **	14.24 **	46.77
Number of leaves	9.97 **	4.76 **	8.62 **	38.40

Note: ns) is not significantly different at = 5% based on the results of the F-test. *) is significantly different at = 5% based on the results of the F-test. **) significantly different at = 1% based on the results of the F-test. BA*paclobutrazol interaction between the treatment of BA and paclobutrazol. CV=Coefficient of diversity.

effectiveness of paclobutrazol concentrate in inhibiting plant growth depends on the physiological conditions of each plant and environmental conditions (Mog et al. 2019). Its inhibitory effect is through the regulation of physiological processes such as inhibiting plant size from shortening, namely the presence of short internodes, and reduction of leaf size (Muengkaew & Chaiprasart 2016).

The research of Roostika et al. (2009) showed that *Pimpinella pruatjan* cultures conservation *in vitro* using paclobutrazol inhibitors also caused robust inhibition, so the conservation period could not be extended more than four months from the culture during recovery a rosette appears. In addition to using paclobutrazol inhibitors to inhibit culture, it can be done by diluting the primary medium to 50 and 75% in combination with the mannitol osmoregulator 20 g/L, as was done for conservation on *Carica papaya* Dieng. The culture can be stored in that media for 16 weeks (Rahayu et al. 2015).

Table 6. Effect of Interaction of Benzyl Adenine and Paclobutrazol on Culture Growth after.

Benzil Adenin (mg/L)	Paclobutrazol (mg/L)			
	0.1	0.3	0.5	0.7
Shoot height (cm)				
0.0	3.41^{e}	4.97^{abc}	5.23^{ab}	5.93^{a}
0.1	4.73^{bcd}	4.47^{bcde}	3.85^{cde}	3.78^{de}
Mean	4.07	4.72	4.54	4.85
CV (%)	33.81			
Number of shoots				
0.0	1.72^{c}	1.80^{c}	1.73^{c}	3.80^{a}
0.1	1.93^{c}	2.67^{b}	1.60^{c}	1.63^{c}
Mean	1.82	2.2	1.66	2.71
CV (%)	46.77			
Number of leaves				
0.0	21.95^{cd}	22.20^{cd}	25.93^{bc}	35.73^{a}
0.1	19.23^{cd}	29.20^{b}	17.20^{d}	20.13^{cd}
Mean	20.59	25.7	21.56	27.93
CV (%)	38.40			

Note: Data followed by the same letter on the same variable are not significantly different based on Duncan's test with a level of = 5%.

Cultures grown on media with paclobutrazol without BA showed lower yields on all observed variables other than the number of roots. Inhibition of shoot height growth and the number of leaves obtained from treatment with paclobutrazol of 0.5 mg/L and paclobutrazol of 0.3 mg/L. It is inhibited the formation of the number of roots, root length, and the number of shoots (Table 4).

The results showed that the paclobutrazol concentration up to 0.7 mg/L did not inhibit the culture when regenerated after conservation. The average shoot height was 4.85 cm, the number of shoots was 2.71, and the number of leaves was 27.9, but this concentration influenced growth inhibition during Conservation (Table 4). Shoots regenerated from the treatment of BA 0 + paclobutrazol 0.7 mg/L produced higher shoots than those from treatment paclobutrazol 0.1-0.5 mg/L, as well as for variables number of shoots and number of leaves except in the treatment paclobutrazol of 0.5 mg/L (Table 6). Shoots from paclobutrazol 0.7 mg/L + BA 0.1 mg/L treatment shorter shoots were obtained and fewer leaves. The study results by Mendes et al. (2021) showed that the citrus cultures stored for 12 months using a paclobutrazol growth inhibitor did not undergo genetic changes based on analysis using SSR markers.

CONCLUSIONS

Based on the results of this research, it can be concluded that the best media for the propagation of *Ludwigia* sp. is MS primary media without PGR. The best medium for culture conservation was MS + 0.5-0.7 mg/L paclobutrazol. Cultures stored in 0.7 mg/L paclobutrazol for six months produced the best response to growth after conservation.

AUTHOR CONTRIBUTION

All authors in this article have the same contribution as the main contributors both in research and in the preparation of the paper

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CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

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