In vitro culture from internodes of Melastoma malabathricum L. on Murashige and Skoog (1962) modified medium with thidiazuron and 1-naphthaleneacetic acid

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Abstract. A study investigating explant response from internodes of Melastoma malabathricum L. cultured on Murashige and Skoog (MS) modified medium with a combination of thidiazuron (TDZ) (0, 0.1, 1 and 2 mg/L) and 1-naphthaleneacetic acid (NAA) (0, 0.1 and 1 mg/L) was conducted. The main objective of this study was to develop a protocol for M. malabathricum L. propagation using an in vitro technique. Results showed that explants could respond to all treatment media by forming calluses. Obtained calluses tended to be green in colour and to have a semi-compact texture. The optimal treatments for forming calluses were 0.1 mg/L TDZ, 1 mg/L TDZ, 0.1 mg/L NAA and 1 mg/L NAA and a combination of 0.1 mg/L TDZ and 0.1 mg/L NAA. The fast-growing callus was initiated upon treatments on MS without growth hormone (16.79 days after inoculation) and MS with 1 mg/L NAA (19.65 days after inoculation). Internode explants of M. malabathricum L. could also respond to the medium by forming calluses and roots on MS medium with 0.1 mg/L NAA and MS with 1 mg/L NAA. The optimal treatment for forming indirect roots was 0.1 mg/L NAA.

Keywords: Melastoma malabathricum L., TDZ, NAA, internode explant

1. Introduction
The Melastomataceae family comprises 4000–4500 species in 182 genera and is generally distributed in tropical as well as subtropical regions [1]. One of the Melastomataceae species on Java Island, Indonesia, is Melastoma malabathricum L. This species is a drought-tolerant plant that can be found on roadsides, riversides, secondary forests, grassland, fallow land [2] and wasteland [3].

M. malabathricum have several benefits, such as in traditional medicine [4], as a potential natural dye [5] and for phytoremediation [6, 7]. Therefore, there is a need for an in vitro propagation technique to develop and maximise its potential. However, to date, the source of explants of M. malabathricum L. has almost entirely been taken directly from its natural habitat. There are numerous risks associated with using explants taken directly from the species’ natural habitat, such as a high level of culture contamination. Therefore, there is a need for optimisation of medium for establishing an in vitro propagation protocol of M. malabathricum L.

The success rate of plant in vitro culture depends on several factors, such as the type of explant, medium components and the presence of plant growth regulator (PGR) in the culture medium. The types of explants that have been used for the in vitro culture of Melastomataceae include leaf [8,
9], node [10], internode [11] and axillary buds [12]. The medium that is often used for Melastomataceae culture is Murashige and Skoog (MS) (1962) [8, 9, 13]. Another plant-related factor for in vitro culture is the presence of PGR or hormone. Auxin and cytokinin are two groups of PGRs that are most commonly used in plant tissue culture [14]. Auxin–cytokinin addition is useful for regulating cell division, elongation and differentiation and organ formation [15].

Several studies have been performed for establishing the optimal concentration for propagating the Melastomataceae family [8, 9, 13]. Although efforts at optimising the medium for *M. malabathricum* have already been performed in many laboratories, it is unclear whether the same responses are achieved when this medium is used for internode explants. Therefore, the present study was conducted to find the best concentrations and combination of TDZ (0, 0.1, 1 and 2 mg/L) and 1-naphthaleneacetic acid (NAA) (0, 0.1 and 1 mg/L) on MS modified medium.

2. Materials and method

2.1. Plant material

The present study was conducted at the Laboratory of Physiology 2, Department of Biology, FMIPA UI, Depok, for 8 months (October 2016–May 2017). Seeds of *M. malabathricum* L. obtained from a parent plant near the greenhouse of FMIPA UI were used for initiating the study. Mature viable fruits were collected, scrubbed with detergent under tap water and then soaked in detergent solution for 5 min. The fruits were first surface-sterilised by immersion in 70 % alcohol for 1 min and then rinsed three times with sterile distilled water to remove all traces of alcohol. Subsequently, the fruits were soaked in Clorox solution (1.05 % NaOCl) (10 %) + three drops of Tween-20 (10 min), followed by fungicide suspension (0.3 %) + three drops of Tween-20 (10 min) and then antibiotic suspension (0.3 %) + three drops of Tween-20 (10 min). The process of immersion of the fruits was always followed by rinsing in sterile distilled water 3–5 times. The seeds were then dried using sterile Whatman filter papers and inoculated into MS basal medium with vitamins.

Sixteen-month-old in vitro grown *M. malabathricum* plantlets were used as a source of explants for this study. The second, third and fourth internode segments were cut into lengths of 0.5 cm and then used as explants.

2.2. Medium and environmental conditions

Seed explants were inoculated into MS basal medium with vitamins. The pH of the medium was adjusted to 5.6–5.8, and then the medium was autoclaved at 121 °C for 15 min. The cultures were incubated at 24 ± 2 °C and placed under continuous illumination for 24 h using a TL lamp at 20 W. The cultures were observed for 16 months after inoculation.

The internode explants were inoculated on MS modified medium with 30 g/L sugar; 8 g/L agar and various concentrations of PGR, TDZ and NAA. The pH of the medium was adjusted to 5.6–5.8, and then the medium was autoclaved at 121 °C for 15 min. The cultures were allowed to grow and were incubated at 24 ± 2 °C with a photoperiod of 16/8 h under white light (TL 20 W). The cultures were observed for 8 weeks after inoculation.

This study included 12 medium treatments with 20 explants subjected to each treatment. The measured variables were callus texture and colour, percentage of explants forming calluses, time required (days) for callus induction, callus formation score, percentage of explants forming indirect roots, average number of days to root induction and percentage of explants forming indirect shoots.
3. Results and discussion

3.1. Callus formation

The callus responses upon various treatments of *M. malabathricum* L. internode explants are presented in Table 1. Initial responses of callus induction observed at the 1st and 2nd weeks after inoculation are shown in figure 1. The responses were marked by elongation, followed by bending and then swelling of the explants. The side of the explants that had direct contact with the medium tended to elongate faster than the side that did not have direct contact with the medium.

The obtained findings showed that calluses formed on the wounded part of the explants, followed by the area around the wounded part. The presence of a wound on the explants is known to increase the absorption of nutrients and PGR within the culture medium [14]. The presence of PGR, particularly auxin, in the medium can increase cell division activity and induce callus formation [14, 16, 17].

Observation of calluses from the 4th and 8th weeks after inoculation showed variation in the colours of *M. malabathricum* calluses (figure 2). The colour of most calluses gradually changed along with the senescence of explants. The initial colour that appeared at the 1st and 2nd weeks after inoculation was white or light grey. The callus colour at the 3rd week after inoculation was greenish-white. The displayed colours then became more varied after the 3rd week of inoculation, including yellowish-green, green, light brown and brown.

The colour started to turn light brown or brown at the 6th week after inoculation. The results at the 8th week also showed that upon single NAA treatment, the callus tended to undergo more browning than upon single TDZ treatment. Calluses present upon single TDZ treatment tended to have a green colour up to the 8th week of inoculation. The results also showed uneven callus pigmentation on some explants.

Table 1. Effect of various treatments on internode explants that forms callus.

| Medium | Hormone | % Explants | Callus Texture | Callus Colour | Callus formation score | Average time required (days) for callus induction |
|--------|---------|------------|----------------|---------------|------------------------|-----------------------------------------------|
| M1     | -       | 65         | Semi-compact   | Green         | 4                      | 16.79                                         |
| M2     | 0.1     | 100        | Semi-compact   | Green         | 4                      | 20.90                                         |
| M3     | 1       | 100        | Semi-compact   | Green         | 4                      | 19.65                                         |
| M4     | 0.1     | 100        | Semi-compact   | Green         | 4                      | 22.25                                         |
| M5     | 0.1 0.1 | 95         | Semi-compact--compact | Green     | 4                      | 26.89                                         |
| M6     | 0.1 1   | 70         | Semi-compact--compact | Green     | 4                      | 31.50                                         |
| M7     | 1       | 100        | Semi-compact   | Green         | 4                      | 24.15                                         |
| M8     | 1 0.1   | 90         | Semi-compact--compact | Green     | 2                      | 33.44                                         |
| M9     | 1 1     | 35         | Semi-compact--compact | Green     | 3                      | 20.86                                         |
| M10    | 2       | 95         | Semi-compact   | Green         | 4                      | 26.47                                         |
| M11    | 2 0.1   | 90         | Semi-compact   | Brownish-green green | 4                      | 29.33                                         |
| M12    | 2 1     | 35         | Semi-compact   | Green         | 4                      | 32.14                                         |

0 = no callus induction
1 = quantity of callus growth was very low
2 = quantity of callus growth was low
3 = quantity of callus growth was moderate
4 = quantity of callus growth was high
Browning on calluses may be caused by a decrease in nutrients within the medium or by degradation of chlorophyll, which indicates a senescence event in the explant [15-17]. Alternatively, browning can also be caused by the oxidation of phenolic compounds [18]. Callus browning upon NAA treatment was allegedly influenced by the effects of auxin and cytokinin on senescence. A certain concentration of auxin can increase ethylene production in plant cells or tissues. Ethylene is known to be a hormone that can trigger the senescence process [19]. In contrast, cytokinin is known to play a role in delaying the senescence process and stimulating chlorophyll production on calluses [16, 17].

The calluses upon treatment without PGR (M1), single TDZ (M4, M7 and M10) and single NAA (M2 and M3) tended to have a semi-compact texture with a watery surface. Meanwhile, the callus texture upon combination treatment (M5, M6, M8, M9, M11 and M12) tended to have a semi-compact texture with a dry surface. The increase in TDZ concentration upon treatment induced a semi-compact to friable callus texture, whereas that in NAA concentration induced a compact callus texture. The callus texture in combination treatment tended to be more compact when concentrations of TDZ and NAA were equal.

Callus formation was observed in all treatments of internode explants (table 1). The results showed that the application of single treatment with 0.1 mg/L NAA (M2), 1 mg/L NAA (M3), 0.1 mg/L TDZ (M4) or 1 mg/L TDZ (M7) was likely to generate explants, of which 100% form calluses. The results also showed that the addition of TDZ, in single or combined treatment, decreased the callus percentage. The concentrations of 0.1 mg/L TDZ and 0.1 mg/L NAA (M5) were the most effective for callus induction on combined medium. The addition of TDZ or NAA beyond the optimal concentration of M5 decreased callus induction. Based on the results, treatment without growth hormone (M1) can also result in callus formation, even though the percentage is lower than for other treatments. The finding of callus formation upon M1 treatment showed that the explants’ endogenous hormones are adequate for stimulating callus formation [20].

Variation in the results of the average time required for callus induction was identified (table 1). Internode explants generally started to grow on days 16–33 after callus inoculation. The fastest callus
Table 2. Effect of various treatments on internode explants that form indirect roots.

| Medium | Hormone | % Roots | Average time required (days) for callus induction |
|--------|---------|---------|--------------------------------------------------|
| M2     | -       | 0.1     | 28.05                                            |
| M3     | -       | 1       | 36                                               |

induction occurred on MS medium without additional growth hormone (M3) (16.79 days after inoculation). The second and third fastest callus inductions occurred on medium containing 1 mg/L NAA (M3) (19.65 days after inoculation) and on a medium containing a combination of 1 mg/L TDZ and 1 mg/L NAA (M9) (20.86 days after inoculation). The results showed that the induction of calluses tended to be slower upon combination treatment rather than upon single treatment.

The callus formation score showed that internode explants cultured with M8 treatment were likely to induce calluses with a score of 2, whereas those cultured with M9 treatment were likely to induce calluses with a score of 3 (table 1). Internode explants cultured with M1, M2, M3, M4, M5, M6, M7, M10, M11 and M12 treatments tended to induce calluses with a score of 4.

Based on the obtained results, numbers of calluses obtained and the differences in callus growth rate may have been caused by the explants’ physiological conditions, different types and concentrations of PGR used and interaction between endogenous and exogenous PGR in culture media [20]. All of these factors can be related to the speed and ability of cells to divide [17].

3.2. Indirect root formation
Indirect root formation was observed with two treatments of internode explants, namely, 0.1 mg/L NAA (M2) and 1 mg/L NAA (M3) (table 2). The results also showed that the roots formed with M2 treatment tended to elongate more than those formed with M3 treatment. M2 treatment was also associated with earlier indirect root induction (28 days after inoculation) than M3 treatment (36 days after inoculation). Therefore, in this study, M2 treatment proved to be the best for root induction from internode explants of *M. malabathricum*.

Based on the obtained results, the fact that indirect root formation was only achieved upon single NAA treatment may be related to the effect of cytokinins. Cytokinins are known to induce the formation of adventitious shoots while inhibiting the induction of adventitious roots [14, 19].

3.3. Indirect shoot formation
The explant that formed indirect shoots in this study was the fourth internode from the apical bud in MS medium without PGR. The percentage of explants that formed shoots with this treatment was 5 %. Shoot induction occurred on day 38 after inoculation. Shoot induction on medium without the hormone cytokinin may occur depends on explant position on the donor plant [14, 15]. The age of the explant is related to the organ’s position or order of growth in the plant donor. The explant farthest from the apical bud are older than the other parts. Cytokinins is naturally produced on roots. This cytokinin is then transported through the xylem to target cells. Explants with a position very close to the roots will have higher a cytokinin endogenous concentration than those positioned very close to the apical bud. The explants used in this work were the fourth internode from the apical bud. Therefore, the formation of indirect shoots may be caused by the presence of sufficient cytokinin within the explant [19].

4. Conclusion
The results obtained showed that explants could respond to all treatment media by forming calluses. Obtained calluses tended to be green in colour and to have a semi-compact texture. Optimal treatments for forming calluses were 0.1 mg/L TDZ, 1 mg/L TDZ, 0.1 mg/L NAA and 1 mg/L NAA and a combination of 0.1 mg/L TDZ and 0.1 mg/L NAA. The internode explants of *M. malabathricum* L.
could also respond to the medium by forming calluses and roots on MS medium with 0.1 mg/L NAA and MS with 1 mg/L NAA. The optimal treatment for forming calluses and roots is 0.1 mg/L NAA.

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References
[1] Rajendran M T 2010 *SEGi Review* 3 34-4
[2] Meyer K 2001 *Blumea* 46 351-98
[3] Plucknett D L and Stone B C 1961 *Pac. Sci.* 15 301-3
[4] Ong H C, Ruzalila B N and Milow P 2011 *Indian J. Tradit. Know.* 10 460-5
[5] Ahmad W Y W, Nor M A M, Saim N, Kadir M I A and Ahmad M R 2012 *Adv. Mater. Res.* 545 59-63
[6] Osaki M et al. 1998 *Plant Soil* 201 175-82
[7] Watanabe T, Osaki M, Yoshihara T and Tadano T 1998 *Plant Soil* 201 165-73
[8] Ghimire B K et al. 2016 *Plant Cell Tiss. Org. Cult.* 124 517-29
[9] Ma G, Li Y, Jiao G, Fu X and Lin Y 2007 *Floriculture Ornamental Biotechnol.* 1 27-9
[10] Adam N A M, Sidiq N J, Osman N I and Razak W R W A 2016 *Int. J. Pharm. Sci. Rev. Res.* 37 36-41
[11] Poosporagi R 2005 *Micropropagation and Effect of Growth Retardants on Selected Species of Melastomataceae* Ph.D Thesis (Selangor, Malaysia: Universiti Putra Malaysia)
[12] Ma G H, Li Y, Jiao G L, Fu X P and Lin Y R 2007 *J. Hortic. Sci. Biotechnol.* 82 428-32
[13] Zhang X, Dai S, Silva J A T d and Ma G 2015 *In Vitro Cell. Dev. Biol.* 51 482-7
[14] Pierik R L M 1997 *In Vitro Culture of Higher Plants* (Netherlands: Springer)
[15] Dodds J H and Roberts L W 1985 *Experiment in Plant Tissue Culture* (United States: International Potato Center)
[16] George E F and Sherrington P D 1984 *Plant propagation by tissue culture* *Handbook and Directory of Commercial Laboratories* (Basingstoke: Exegetics)
[17] Hopkins W G and Huner N P A 2008 *Introduction to Plant Physiology* 4th edition (London: John Wiley & Sons)
[18] Bidwell R G S and Bidwell R C S 1979 *Plant Physiology* 2nd edition (New York: MacMillan)
[19] Salisbury F B 1995 *Fisiologi Tumbuhan Jilid 3: Perkembangan Tumbuhan dan Fisiologi Lingkungan* (Bandung: Institut Teknologi Bandung)
[20] George E F, Hall M A and De Klerk G-J 2008 *Plant Propagation by Tissue Culture* 3rd edition, vol 1 (Netherlands: Springer)