BIODIVERSITAS
Volume 21, Number 11, November 2020
Pages: 5373-5381

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d211143

In vitro spore germination and early gametophyte development of *Cibotium barometz* (L.) J. Sm. in different media

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Manuscript received: 2 October 2020. Revision accepted: 24 October 2020.

Abstract. *Isnaini Y, Praptosuwiryo TNg. 2020.* In vitro *spore germination and early gametophyte development of* Cibotium barometz *(L.) J. Sm. in different media. Biodiversitas 21: 5373-5381. Cibotium barometz* (L.) J. Sm. is known as the golden chicken fern and included in Appendix II of CITES. It is an important export commodity for traditional and modern medicine. Globally, populations of this species are under significant pressure due to overexploitation in the wild. In vitro culture is one of the technologies used for *ex-situ* propagation and conservation of rare and endangered ferns and lycophytes. This study's objectives were: (i) to observe in vitro spore germination and early gametophyte development of *C. barometz*, and (ii) to determine the best culture medium for rapid spore germination and early development of the gametophytes. The sterilized spores were sown in half-strength Murashige & Skoog (½MS) basal medium supplemented with combinations of 6-Benzylaminopurine (BAP) and α-Naphthalene acetic acid (NAA). A factorial combination of four BAP concentrations (0, 2, 4, and 6 mg L^{-1}) with four concentrations of NAA (0; 0.01; 0.03 and 0.05 mg L^{-1}) created 16 treatments replicated in a Completely Randomized Design. Spore germination of *C. barometz* was observed to be *Vittaria*-type, and its prothallial development was *Drynaria*-type. Spore germination started 7-14 days after sowing. Young heart-shape gametophytes consisting of 110-240 cells were formed in 45-61 days after sowing. The two best spore culture media for rapid spore germination and development of *C. barometz* gametophytes were ½ MS with or without 2 mg L^{-1} BAP.

Keywords: Ex-situ conservation, medicinal fern, propagation, spore culture

INTRODUCTION

Ferns and lycophytes are essential components of an ecosystem. They contribute 4% of the vascular plant diversity in the world (Mehltreter 2020). They are also vital components of the ecosystems of biological hot spots, exceptionally moist tropical forest and oceanic islands (Given 2002). These plant groups have received much attention as natural research material, as model organisms for studying plant evolution. The number of literature studies on ferns and lycophytes, especially in systematics and evolution, is relatively large (Ebihara and Nitta 2019), but ferns and lycophytes face an unprecedented threat due to human activities, such as fire and land-use change. By the year 2008, a global risk assessment using IUCN Red List had been completed for only 2% of the 11,000 species of ferns and lycophytes; 89% of these ferns were considered to be at risk (Mehltreter 2010). Rare and threatened ferns and lycophytes of Asia towards assessing globally threatened species have been published yet. Of the 886 taxa (835 species and 51 infraspecific taxa) enumerated, 577 occur in Southeast Asia, 215 in East Asia, and 101 in South Asia (Ebihara et al. 2012). We should well implement management strategies for ferns and lycophytes conservation in South Asia countries. *In-situ* protection of endangered fern species is recommended as a management strategy for fern conservation. However, *ex-situ* cultivation of endangered fern species may supplement *in-situ* protection efforts but should not replace them (Meheltreter 2020).

Cibotium barometz is a tree fern having economic value as an export commodity for both traditional and modern medicine (Praptosuwiryo 2003; Zhang et al. 2008; Praptosuwiryo et al. 2011a; Lim and Lim 2016). Before 1997, China was the first exporter and also the top user country of *C. barometz*. China exported *C. barometz*'s rhizomes to South Korea, the USA, and Canada with more than 500 tons in five years, over 1993-1997 (Zhang et al. 2008). The second exporter country of *C. barometz* was Vietnam, with its quota of 250,000 kg dry weight of sliced rhizome in 2009 and 2010 (UNEP-WCMC, 2010). Since 2006, under the regulation of CITES, Indonesia has been exported the dried hairs of *C. barometz* to French. The export quotas of *C. barometz* for Indonesia from the year 2006 to 2015 were 2500 kg (2006), 500 kg 2007), 450 (2008), 900 kg (2009 to 2016) (CITES Secretary 2006 - 2015).

The dry sliced rhizomes of *Cibotium* called "Gouji" are used for producing pills of "Zhuangyao Bushen Wan", a medicine which useful in maintaining the function of the kidneys (Zhang et al. 2008). Besides maintaining the function of the kidneys, *C. barometz* has been used to treat various illnesses. Polysaccharides of *C. barometz* is used for the treatment of osteoarthritis (Fu et al. 2017). An extract from the rhizome is also used as an anti-rheumatic, antioxidant, antimicrobial activities, antidermatophytic activity, and as a gastric ulcer (Praptosuwiryo 2003; Al-Wajeeh et al. 2017a,b; Wang et al. 2017a,b; Xie et al. 2017; Huang et al. 2018a,b; Sit et al. 2018; He et al. 2019; Heng et al. 2020; Musman et al. 2020).

In several countries, the *C. barometz* populations have decreased rapidly due to the overexploitation for trade for medicinal purposes (Zhang et al. 2008; Nguyen et al. 2009). Since 1976, this species was included in Appendix II of the Convention on International Trade in Endangered Species (CITES) for trade monitoring (Praptosuwiryo 2003; Zang et al. 2002; Praptosuwiryo et al. 2017; Liu et al. 2018; Schippmann 2018). It means that no export is allowed without a prior permit issued by the CITES committee under the regulation of each country's management authority. Therefore, various conservation efforts and strategies should be implemented to ensure that this species will sustain in nature.

Ex-situ conservation for rare and or exploited pteridophyte species can be supported by collecting live specimens from the wild, growing them in pots from spores, and propagating them from rhizomes (Mehltreter 2010). However, conventional cultivation of ferns from spores is generally more time-consuming. In vitro culture technology can be used as an alternative method for propagation and conservation of ferns and lycophytes (Makowski et al. 2016; Jang et al. 2019a). Many ferns and a few lycophytes have been grown in vitro (Moura et al. 2015; Luna et al. 2016, Jang et al. 2017; Kwon et al. 2017; Ballesteros and Pence 2018; Castilho et al. 2018; Johnson and Shibila 2018; Jang et al. 2019a,b; Romanenko et al. 2019; Jang et al. 2020; Park et al. 2020; Shelikhan 2020; Van Nguyen et al. 2020; Zhang et al. 2020). In vitro culture requires less space than whole plants' culture and can be kept free from disease. In vitro culture methods offer the chance to study the development stages of fern species from gametophyte to sporophyte (Simões-Costa et al. 2015; Ravi 2016; Cho et al. 2017; Park et al. 2019), and the opportunity to understand better their abiotic demands (Manonmani and Sara 2014). Therefore, in vitro culture can provide data to develop efficient propagation strategies for conserving ferns (Shelikhan 2020).

Studies dealing with the *Cibotium* propagation are only a few. Goller and Rybczyński (2007) studied in vitro *culture* of *C. glaucum* (Sm.) Hook. & Arn. and *C. schiedei* Schltdl. & Cham. using spores as explants. Chen et al. (2007) studied the gametophyte development and its diversity in *C. barometz* from China by culturing spores in Knop's solution and solid medium. Li et al. (2010) and Xu et al. (2010) reported their work on the in vitro culture and plant regeneration of *C. barometz* using Murashige & Skoog (MS) media. Yu et al. (2016a) investigated gametophyte development and apogamy of *C. barometz* by culturing sterile spores. Yu et al. (2016b) reported green globular bodies induction on half-strength MS media supplemented with Thidiazuron and α-Naphthaleneacetic acid (NAA). This paper reports our study on the in vitro propagation of *C. barometz* from Sumatra. The study aims were: (1) to observe spore germination and early gametophyte development of *C. barometz* grown in vitro, and (2) to determine the best spore culture media for rapid spore germination and early development of the gametophytes.

MATERIALS AND METHODS

Study taxon

Cibotium barometz (L.) J. Sm. is a tree fern belonging to the family Cibotiaceae (Smith et al. 2006). This species can be easily recognized by the existence of smooth, shiny, golden hairs covering its rhizome and basal stipes, with 2-10 or more pairs of cup-shaped sori on each pinnule-lobe (Holttum 1963; Rugayah et al. 2009; Praptosuwiryo et al. 2011; Praptosuwiryo et al. 2017). This species is distributed in the tropical and subtropical regions of Asia. It grows in warm and humid environments, in hilly or mountain forests, often in valleys, forest edges, and open places in forests at elevations ranging from 50 to 1600 m a.s.l. (Holttum 1963; Zhang et al. 2008; Rugayah et al. 2009; Praptosuwiryo et al. 2011; Praptosuwiryo et al. 2017). The national conservation status of *C. barometz* in Indonesia has been assessed using the 2012 IUCN Red List Categories and Criteria (version 3.1). This species is included in the vulnerable category (VU A4cd) (Praptosuwiryo and Rugayah 2017).

Spore collection and sterilization

Spores used as explants in this study were freshly collected from the plant growing in The Ecopark of Cibinong Sciences Center-LIPI (West Java). These plants were initially collected from the secondary forest at Soriak Hill, Lima Puluh Kota District, West Sumatra Province (Titien Ng Praptosuwiryo 3353). The spore collection procedure followed Praptosuwiryo et al. (2015): spore-bearing pinnae of mature sporophylls were cleaned in running water to avoid spore contamination from other species and airy drained in a clean newspaper envelope. The dried spore-bearing pinnulae were then placed in an envelope (22 × 11 cm^2) and kept at room temperature in a dry place. A few days later (7-10 days), most of the spores had released from sporangia and lying on the envelope. The spores were separated from the sporangia by tilting the envelope paper and removing them from the envelope to a piece of glassine weighing paper folded into a pocket. The spore collections were kept at room temperature until the sowing day (not more than two weeks).

In fern propagation by in vitro culture method, spore sterilization is required before the germination procedure. Spores sterilization procedures followed those described by Isnaini (2013). Spores were sterilized with a commercial Clorox at a concentration of 20%, 10%, and 5%, respectively, with 1-2 drops of tween-80 as a wetting agent in 20 ml of distilled water. The sterilized spores were rinsed three times in sterile distilled water for one minute each time to remove all traces of sterilant. All the sterilization works were carried out in sanitary conditions in a laminar flow cabinet.

Spore culture in vitro on a half-strength MS medium supplemented with BAP and NAA.

A completely randomized design with at least ten replications of a 4 × 4 factorial treatment arrangement was used in this experiment, which aimed to test the effectiveness of different media for spore germination and

early gametophyte development of *C. barometz*. The basal medium was a half-strength Murashige and Skoog (½MS) medium. This basal medium was supplemented with various combinations of 6-Benzylaminopurine (BAP) and (NAA). There were four levels of concentration of BAP (0, 2, 4, and 6 mg L^{-1}) and four levels of concentration of NAA (0; 0.01; 0.03 and 0.05 mg L^{-1}), combined in a factorial arrangement resulting in sixteen different spore culture media. Three percent of sugar was added to each medium as a carbon source. The pH of each medium was adjusted to 5.7 ± 0.01, then gel rite was used as a solidifying agent at a concentration of 0.2% weight/volume (w/v) before boiling them. The medium placed into the Erlenmeyer flask and sterilizing in an autoclave. The sterilized medium was poured into a Petri dish aseptically in a laminar airflow cabinet. The spores in sterile water were sown onto the media contained in 5 cm Petri dishes. Approximately 100-200 spores were sown for each replicate. The Petri dishes were sealed with strips of plastic wrap film to inhibit drying and contamination. For germination, spores were incubated in Petri dishes at 20-25^0 C at 2000 lux with a 16 h photoperiod.

Observation of spore germination and gametophyte development on ½ MS media without BAP and NAA

Spore germination was defined as the emergence of a rhizoid through the spore coat (Camloh 1993). Gametophytes were classified according to the development stages of fern gametophytes described by Rechenmacher et al. (2010), viz.: gametophyte with chlorocyte and rhizoid (chlorocyte and rhizoid stage), filamentous gametophyte (filamentous stage), and laminar gametophyte (laminar stage). In this study, the laminar stage was divided into two phases, the spatulate stage, and the early heart shape stage. Spore germination and prothallium development were observed from 10-13 to 55-61 days after spore sowing (das). The percentage of rhizoid, filamentous, spatulate, and young heart shape stages were determined on 13, 21, 37, and 61 das respectively.

Effect of BAP in combination with NAA on in-vitro germination and early gametophyte development of *Cibotium barometz*

Five replicate Petri dishes were observed for each of the sixteen media. A spore was considered germinated when the first emergent cell became visible (Chuter et al. 2008). The number of gametophytes with chlorocyte and rhizoid, filamentous gametophyte, spatulate gametophyte, and early heart gametophyte were counted under a binocular Nikon SM2-10A microscope (objective lens 4.9x). Five field-of-view for each Petri dish were observed under the microscope. An Olympus microscope U-TV0, 5XC-3 5H12344 (4× and 10×) connected to a computer was used to document spore germination and gametophyte development at every week of observation.

Statistical analysis

Quantitative data of spore germination and gametophyte development were expressed as percentages and analyzed with SPSS 13.0. The data for spores germination and gametophyte development were tested by using the analysis of variance (ANOVA), and differences between means were tested by Duncan's test at 5% probability.

RESULTS AND DISCUSSION

Spore germination and early gametophyte development stages of *Cibotium barometz* on ½ MS media without BAP and NAA

Spore germination of Cibotium barometz

Germination of spore is defined as the set of mechanisms occurring in the dormant spore that culminates with the growth of cells to form a sporeling able to be established in the substrate (Gabriel y Galán et al. 2015; Steinbrecher and Leubner-Metzger 2017; Yan and Chen 2020). Spore germination can be easily detected by observing signs of the emergence of particular organs such as radicle, protonema, filament or rhizoid (Suo et al. 2015; Penfield 2017; Elgabra et al. 2019). Spores of *C. barometz* are trilete, tri-radially symmetrical, non-chlorophyllous, and golden-yellow with a perine. In our experiment, at 3-6 days after sowing, *C. barometz*'s spore became green in color (Figure 1.A.). The golden-yellow coloration of the spores changed to green, indicating that germination had started. It was followed by the breakage of spore walls to produce a prothallial filament, indicating the spores's successful germination. It is generally recognized that the criterion for spore germination is the emergence of the chlorocyte, or the rhizoid (Chuter et al. 2008; Marcon et al. 2015). Similar to Praptosuwiryo (2015) observations, the first cell produced from in vitro spore germination of *C. barometz* in this experiment was rhizoid. Therefore, it is called the rhizoid stage.

The second stage of gametophyte development was the chlorocyte-rhizoid or rhizoid-protocorm stage in which the spore bore both rhizoid and the first cell of a filament. The chlorocyte-rhizoid stage for *C. barometz* is illustrated in Figure 1.A. Spore germination started 7-14 days after sowing. It demonstrated that the period time for germination in vitro is not different from *C. barometz* spores grown conventionally in natural media. Praptosuwiryo et al. (2015) reported that *C. barometz'*s spores sown conventionally in natural media germinated 7 - 15 days after sowing; bearing gametophytes with chlorocyte and rhizoid and the early stage of filamentous gametophytes, the filament consists of 1 - 4 cells.

Referring to patterns of gametophyte development, Nayar and Kaur (1969) described seven different types of prothallial development in the homosporous ferns. In previous works of spore germination, Chen et al. (2017) reported that the germination of *C. barometz* from China belonged to the *Vittaria*-type and *Cyathea*-type. In our study, the spore germination of *C. barometz* from Sumatra also belonged to *Vittaria*-type. The first division giving rise to the rhizoid initial was perpendicular to the spore's polar axis, and the second division yielding the protonema initial was perpendicular to the first (Figure 1.B.). Deng et al. (2007) and Praptosuwiryo et al. (2015) also reported only

one type of spore germination of *C. barometz*, the *Vittaria*-type. A similar types of spore germination also reported in *Aglaomorpha cornucopia* (Huang et al. 2015), *Lepisorus nudus* (Singh et al. 2017), *Microgramma mortoniana* and *Pleopeltis macrocarpa* (Gorrer et al. 2018). Farfán-Santillán (2017) reported two types of germination of *Gleichenia* and *Cyathea*; and three types of prothallial development *Marattia*, *Osmunda* and *Drynaria*.

Figure 1. Spore germination and early development of *Cibotium barometz* on ½ MS media (NAA 0 $mgL^{-1}$and BAB 0 mg L^{-1}). A. Spore becoming green in color in the process of germination; rhizoid and chlorocyte (15 days after sowing). B. Early stage of filamentous prothallus composed of three cells, showing *Vittaria*-type of spore germination (16 days after sowing); C. Filamentous stage of prothallus composed of 11 cells and spatulate stage of prothallus composed of 29 cells (23 days after sowing); D. Early stage of young heart-shape gametophyte composed of 69 cells (43 days after sowing); E. Young heart-shape gametophyte composed of 115 cells (46 days after sowing); F. Young heart-shape gametophyte composed of 233 cells (56 days after sowing). Bar = 200 µm for all.

Early gametophyte development stages of Cibotium barometz

Gametophyte development of *C. barometz* in vitro culture is of the *Drynaria*-type (Nayar and Khaur 1969). The same type of gametophyte development is reported in *Adiantum capillus-veneris* L. (Oloyede et al 2017), *Lepisorus nudus* (Singh et al. 2017), *Microgramma mortoniana*, and *Pleopeltis macrocarpa* (Gorrer et al. 2018). As also described by Praptosuwiryo et al. (2015), when at the fimentous stage (prothallus has 5-10 or more cells) one of the cells at the top margin of the prothallus (the anterior marginal cells) then divides obliquely across its width (Figure 1.C.). This results in obconical meristematic cells. Division by this type of cell is parallel to each other and perpendicular to the rest of the cells, forming rows. This results in the formation of a notch at the prothallus's anterior edge, giving it a roughly heart-shaped appearance. Young heart-shape gametophytes consisting of 110-240 cells were formed in 45-61 days after sowing (Figure 1.E-F).

Effect of BAP in combination with NAA on in-vitro germination and early gametophyte development of *Cibotium barometz*

A basal medium of ½ MS has often is used as a medium for in vitro spore culture in ferns such as *Polystichum craspedosorum* (Shelikhan 2020) and *C. barometz* (Li et al. 2010). The effect of BAP in combination with NAA on in vitro germination and early gametophyte development of *C. barometz* is shown in Table 1 and Figure 2. The highest spore germination, indicated by the percentage of the rhizoid stage (spores give rise rhizoids) and filamentous stage, was on ½ MS media with 2 mg L^{-1} BAP and 0 or 0.01 mg L^{-1} NAA. Visual observation at 13 days after sowing showed that most of the cultured spores grew well and looked green in color on ½ MS basal medium supplemented with 2 mg L^{-1} BAP or without BAP supplementation. Increasing with BAP to 4 and 6 mg L^{-1} did not result in better spore germination and early gametophyte development of *C. barometz.*

In a study of the fern *Asplenium nidus*, a basal medium of ½ MS supplemented with BAP and NAA growth regulators at concentrations of 2 mg L^{-1} and 0.5 mg L^{-1} respectively produced a significant increase in multiplication (Khan et al. 2008). Higuchi and Amaki (1989) also studied the effects of BAP and NAA concentration on the growth of in vitro plantlets of *Asplenium nidus*. The number of *A. nidus* plantlets were less at low concentrations of BAP and NAA. At higher concentrations, the explants started to show browning, which suggested the detrimental effect of higher doses. This reflects that plants need both auxins and cytokinins which should be supplied in the medium and the ratio between them seems to be very important for multiplication (Bertrand et al. 1999; Fernandez et al. 1999; Rocha et al 2015). Many reports in which the application of growth regulators either enhances or suppresses plant growth depending on the concentrations used and their relative proportions (Fernandez and Revilla 2003; Abbasi 2017; Smýkalová et al. 2019).

Table 1. The percentage (mean ± standard deviation) of the prothallus stage of *Cibotium barometz* observed along with the spore germination and developmental stages of gametophyte, between 10-61 days after sowing on media comprising half-strength Murashige & Skoog medium (½ MS) with 6-Benzylaminopurine (BAP 0, 2,4, and 6 mg L^{-1}) and 1-Naphthaleneacetic acid (NAA 0; 0.01; 0.03 and 0.05 mg L^{-1}).

BAP (mg L^{-1})	NAA (mg L^{-1})	Percentage of prothallus stage along with the spore germination and developmental stages of gametophyte. 10-61 days after sowing (das)*			
		Rhizoid stage (10-13 das)	**Filamentous Stage (16-21 das)**	**Spatulate Stage (23-37das)**	**Heart Shape Stage (55-61 das)**
0	0	71.66 ± 7.84^{a}	16.44 ± 6.41^{b}	26.15 ± 8.85^{abc}	1.60 ± 0.07^{ab}
0	0.01	52.99 ± 8.37^{abc}	16.92 ± 9.41^{b}	27.07 ± 5.93^{ab}	1.72 ± 0.87^{ab}
0	0.03	32.99 ± 7.04^{cdefg}	21.88 ± 2.95^{b}	8.70 ± 5.79^{cd}	0.71 ± 0.51^{ab}
0	0.05	24.74 ± 5.65^{fg}	22.02 ± 6.99^{b}	2.73 ± 3.02^{d}	0.00 ± 0.00^{b}
2	0	71.63 ± 6.34^{a}	28.65 ± 2.86^{b}	35.37 ± 6.32^{a}	4.06 ± 0.11^{a}
2	0.01	58.20 ± 8.98^{ab}	55.88 ± 6.88^{a}	14.82 ± 6.90^{bcd}	3.03 ± 0.78^{ab}
2	0.03	50.86 ± 5.24^{abcd}	17.47 ± 4.07^{b}	28.53 ± 8.22^{ab}	1.92 ± 0.57^{ab}
2	0.05	45.32 ± 6.26^{bcdef}	17.20 ± 9.18^{b}	24.25 ± 3.46^{abc}	3.18 ± 0.65^{ab}
4	0	35.43 ± 7.99^{bcdefg}	16.15 ± 1.94^{b}	11.31 ± 2.63^{bcd}	4.33 ± 1.68^{a}
4	0.01	25.93 ± 4.02^{efg}	22.65 ± 7.30^{b}	5.47 ± 1.90^{d}	0.36 ± 0.12^{b}
4	0.03	28.46 ± 5.94^{defg}	9.78 ± 1.64^{b}	14.49 ± 6.77^{bcd}	4.04 ± 7.72^{a}
4	0.05	35.66 ± 2.27^{bcdefg}	10.52 ± 2.88^{b}	11.37 ± 7.87^{bcd}	1.21 ± 0.22^{77ab}
6	0	49.22 ± 4.73^{abcde}	20.25 ± 2.04^{b}	27.23 ± 7.80^{ab}	1.44 ± 0.73^{ab}
6	0.01	28.00 ± 1.14^{defg}	14.81 ± 9.84^{b}	11.33 ± 3.27^{bcd}	0.87 ±0.93^{ab}
6	0.03	24.07 ± 2.95^{fg}	18.52 ± 3.16^{b}	13.20 ± 2.23^{bcd}	1.42 ± 0.11^{ab}
6	0.05	40.97 ± 7.30^{g}	18.35 ± 7.71^{b}	1.93 ± 2.99^{d}	0.00 ± 0.00^{b}

Note: *Within a column, percentage values that are followed by the same letter are not statistically significant according to the Duncan test at a 5% level of significance.

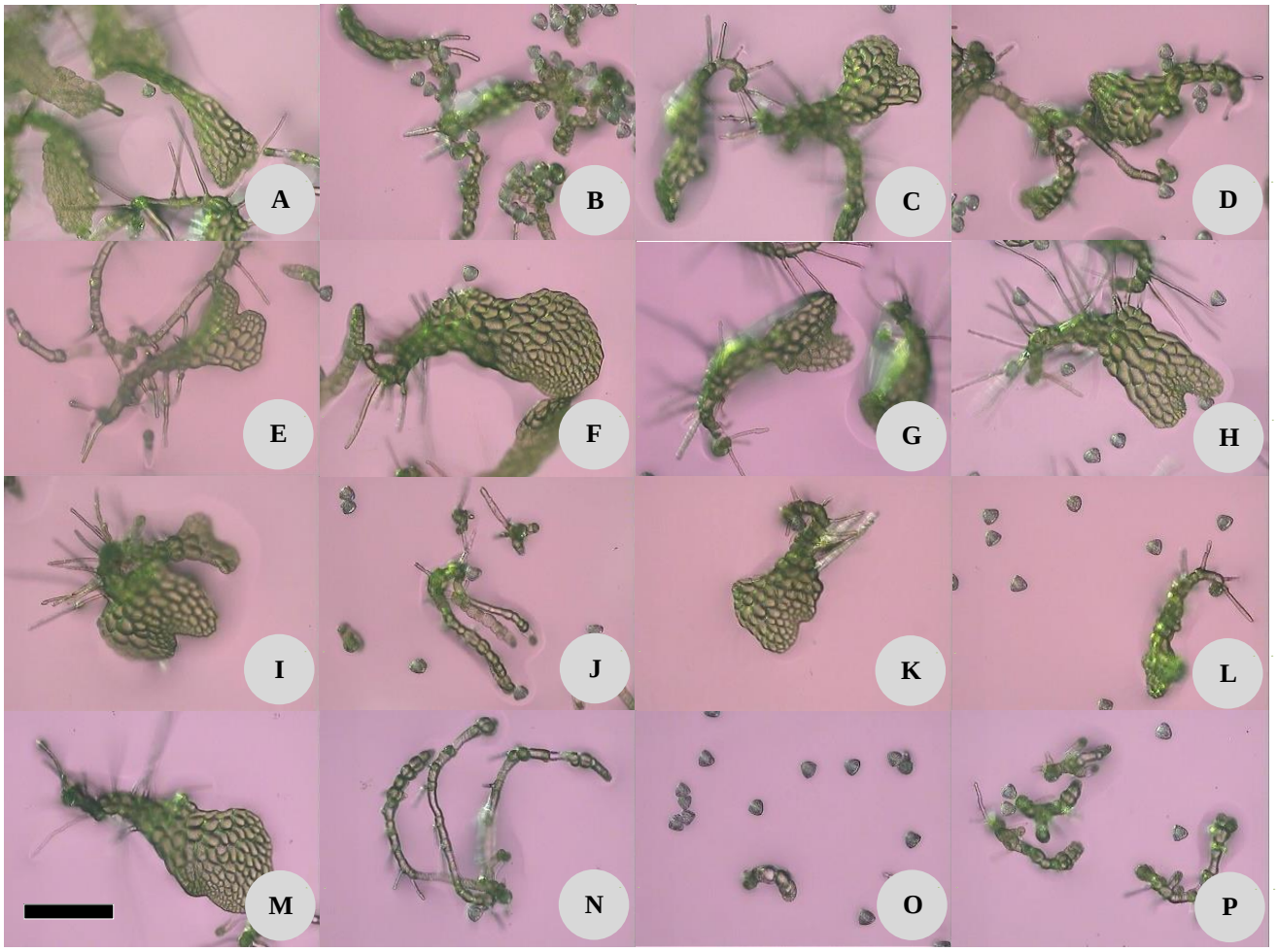

Figure 2. Microscopic appearence of the spores germination and early gametophytes development of *Cibotium barometz* (collection number: TNgP 3353) observed at 61 days after sowing on Petri Dishes containing MS media supplemented with BAP and NAA: A. 0 mgL^{-1} and 0 mg L^{-1}, B. 0 mg L^{-1} and 0,01 mg L^{-1}, C. 0 mg L^{-1} and 0,03 mg L^{-1}, D. 0 mg L^{-1} and 0,05 mg L^{-1}, E. 2 mg L^{-1} and 0 mg L^{-1}, F. 2 mg L^{-1} and 0,01 mg L^{-1}, G. 2 mg L^{-1} and 0,03 mg L^{-1}, H. 2 mg L^{-1} and 0,05 mg L^{-1}, I. 4 mg L^{-1} and 0 mg L^{-1}, J.4 mg L^{-1} and 0,01 mg L^{-1}, K. 4 mg L^{-1} and 0,03 mg L^{-1}, L. 4 mg L^{-1} and 0,05 mg L^{-1}, M. 6 mg L^{-1} and 0 mg L^{-1}, N.6 mg L^{-1} and 0,01 mg L^{-1}, O. 6 mg L^{-1} and 0,03 mg L^{-1}, P. 6 mg L^{-1} and 0,05 mg L^{-1}. Bar = 400 µm for all.

In conclusion, this is the first report that describes the in vitro germination and early gametophyte of *C. barometz*, one of the endangered species of tree ferns of Indonesia. In vitro spore germination of *C. barometz* from Sumatra is *Vittaria*-type, and the prothallial development is classified as *Drynaria*-type. Young heart-shape gametophytes consisting of 110-240 cells were formed in 45-61 days after sowing. The two best spore culture media for rapid spore germination and development of the gametophytes of *C. barometz* were ½ MS without supplementation, and ½ MS supplemented with 2 mg L^{-1} BAP. The basal medium of ½ MS, with or without BAP supplementation at 2 mg L^{-1} may be used for in vitro propagation of the closely related species to *C. barometz* and scaly tree fern species.

ACKNOWLEDGEMENTS

Two authors contributed equally to this work, discussed the results and commented on the manuscripts at all stages. This research was funded by a Grant-in-Aid for Scientific Research from Program Kompetitif LIPI TA 2013-2014 and *Program Unggulan* LIPI TA 2015. We are grateful to the staff of the Tissue Culture Laboratory of Bogor Botanic Gardens for their assistance. We would also like to thank Dr. Graham Eagleton (Australia) for reading and correcting the first manuscript.

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