# *IN VITRO* AND *EX VITRO* PROPAGATION OF A WILD-EXTINCT FERN *Lygodium circinnatum* (BURN.F) SW. GROWN IN BALI

**Rindang Dwiyani***
Faculty of Agriculture, Udayana University, Jalan PB Sudirman, Denpasar, Bali, Indonesia, Post code 80232.
*Email : rindangdwiyani@yahoo.co.id

***Abstract***

*Research concerning of propagation of a wild-extinct fern Lygodium circinnatum had been done at Faculty of Agriculture, Udayana University, Denpasar Bali Indonesia. At some places in Indonesia, as well as Bali, this species is used as materials for making handicraft. In Bali, the species grows wildly in the forest and it is almost extinct due to over gathering. This study aimed to find out method for domestication of L. circinnatum, therefore this wild species can be cultivated, provided materials for making handicraft and might solve the problem of extinction. Various media for growing spores of L. circinnatum in vitro and ex vitro were trialed. In conclusion, full strength of MS media without sugar was the most appropriate media for growth and development of spores of L. circinnatum in vitro. While for ex vitro, the appropriate media were paddy silt-soil and decomposed leaf either with or without addition of foliar fertilizer. However, we suggested ex-vitro cultivation was more appropriate, the technique was much easier and the spores grew faster compared to those of in-vitro.*

*Keywords: propagation, ex vitro, in vitro, Lygodium circinnatum*

**Abstrak**

Penelitian mengenai perbanyakan spesies paku-pakuan langka *Lygodium circinnatum* telah dilakukan di Fakultas Pertanian Universitas Udayana, Denpasar, Bali, Indonesia. Di beberapa tempat di Indonesia, seperti halnya Bali, spesies ini digunakan sebagai bahan baku anyaman. Di Bali, spesies ini tumbuh liar di hutan dan hampir punah karena pencarian yang berlebihan. Penelitian ini bertujuan menemukan suatu metode perbanyakan untuk domestikasi *L. circinnatum* melalui perbanyakan secara *in vitro* dan *ex vitro*, sehingga diharapkan dapat mengatasi masalah kelangkaan bahan baku anyaman dari *L. circinnatum*. Berbagai jenis media untuk perbanyakan secara *in vitro* dan *ex vitro* dilakukan dalam penelitian ini. Hasilnya mendapatkan bahwa untuk perbanyakan secara *in vitro*, media MS (dengan konsentrasi penuh) tanpa gula adalah yang terbaik untuk pertumbuhan dan perkembangan spora secara *in vitro*. Sedangkan untuk perbanyakan di luar laboratorium (*ex vitro*), media yang baik untuk pertumbuhan dan perkembangan spora adalah lumpur sawah dan kompos dari daun-daunan, dengan ataupun tanpa penambahan pupuk daun.

*Kata kunci: perbanyakan, ex vitro, in vitro, Lygodium circinnatum*

## 1. Introduction

In the taxon of plants, a wild climbing fern *Lygodium circinnatum* is family of Lygodiaceae, class of Polypodiopsida and phylum of Pteridophyta (GBIF Backbone Taxonomy, 2015). The plnat produces spores for its reproduction. Research of genus Lygodium have been done in many aspects by many researchers. For example regarding of antioxidant properties of Lygodium (Jeetendra and Manish, 2011), anthiridiogens identification (Yamauchi *et al*. 1996), and also about biological control agents (Goolsby *et al.* 2013), however, there is only few research about the cultivation of Lygodium. In Bali (Indonesia), *L. circinnatum* grows in the forest, climbs big tree with their creeping shoots. People take the old creeping shoots for making handicraft by lifting the plants from the ground without any replace with new plant, therefore, this species becomes nearly extinct in the forest. This study aimed to find out method for domestication of *L. circinnatum*, therefore this wild species can be cultivated, provided materials for making handicraft in Bali and might solve the problem of extinction. Previous studies in Indonesia have been done by Raka, *et a*l.(1997) and Rahayu (2006). They cultivated *L. circinnatum* by splitting young plants from the forest, and then intensively cultivated them by adding fertilizer to stimulate their growth. Unfortunately, those methods of cultivation were failed. We believe this was in accordance with the opinion of Baligar and Duncan (1986), which stated that in the condition of a high amount of nutrient availability, wild plants show slower growth even these nutrients can be toxic to plants. Therefore, we suggested that the earliest life cycle of the plant was required to start cultivate the wild plant. In the recent study, we started to cultivate *L. circinnatum* from the earliest life-cycle i.e. spores, then we explored methods of cultivation of this species, *in-vitro* and *ex-vitro* to find out the appropriate method for cultivation.

## 2. Material and Methods

*Lygodium circinnatum* collected by Bali Botanical Garden "Eka Karya" was used as material in the current research. Both *In-vitro* and *ex-vitro* experiment were started with collecting spores which are in a structure, called sporangia produced by mature fertile leaves, underside of the leaves. Spore collection of *in-vitro* method begun with maintaining spores-producing plants. The plants, especially spore-producing leaves, every day for a week were sprayed with fungicide. The leaves then were picked and washed gently, not to damage the sporangium (spores-producing structures at the underside of leaves). Subsequently, the leaves were sprayed with alcohol 95% and put in the laminar. In the laminar, the leaves were air-dried and then put it in a sterile paper envelope and sealed. After that, the paper envelope was kept at room temperature outside laminar. About fourteen days, leaves dried. By lapping the paper envelope with hand, spores will be detached. Furthermore, the paper envelope (that was still closed) was sprayed with alcohol and put in the laminar. Spores then were collected in a sterile container and separated from the leaves. The spore preparation process was done to prevent contamination, because spores are very fine like flour and difficult to be surface-sterilized. Spore collection procedures of *ex-vitro* method was similar to those performed on *in-vitro*, just all the work was done outside of the laboratory and did not require prudence to maintain the sterile conditions of the spores. Spore-producing leaves were picked and put in paper envelopes. Furthermore, envelopes (with fertile mature leaves inside) were exposed to the sun, so that the leaves dried. If the leaves have dried, the envelopes were beat on the table or floor so the spores detached. Furthermore, leaves separated from the spores and discarded, while the spores were collected. Figure 1 shows the fertile leaf with sporangia and spores after collecting in the container.

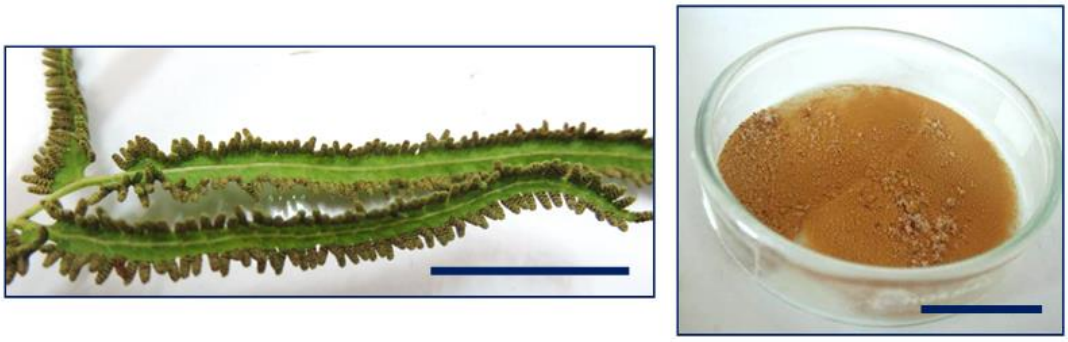

Figure 1. Fertile leaves with sporangia (left) and spores (right). Bar = 5 Cm

Both experiments, *in-vitro* and *ex-vitro* were conducted simultaneously during January to July 2014. The *in-vitro* experiment was done at The Laboratory of tissue culture, Faculty of Agriculture, Udayana University. The experiment was designed as Randomized Block Design. We used six (6) types of medium as treatments, i.e. M1 = Full strength of MS media without sugar; M2 = 1/2 (a half) strength of MS media without sugar; M3 = ¼ (fourth) strength of MS media without sugar; M4 = Full strength of MS media with 20 $gL^{-1}$ of sugar; M5 = 1/2 (a half) strength of MS media with 20 $gL^{-1}$ of sugar; M6 = ¼ (fourth) strength of MS media with 20 $gL^{-1}$ of sugar. Treatments were replicated 5 times. Volume of media was 30 cc per bottle. The media was sterilised with autoclave at 121$^{o}$C for 30 minutes. About 0.03 g of spores was sown in each treatment. Cultures were maintained in the culture room with temperature of 20$^{o}$C, and RH of 70%.

*Ex-vitro* experiment was conducted at a shade house, located at Experimental Station of Faculty of Agriculture Udayana University. Eight (8) types of medium were used as treatments, i.e. T1 = fine grain soil (Indonesian = tanah halus), T2 = paddy-silt soil (Indonesian=lumpur sawah), T3 = decomposed leaves , T4 = moss ( made from root of *Asplenium nidus*), T5 = fine grain soil added with 1g $kg^{-1}$ of foliar fertilizer , T6 = paddy-silt soil added with 1g $kg^{-1}$ of foliar fertilizer, T7 = decomposed leaves added with 1g $kg^{-1}$ of foliar fertilizer , and T8 = moss added with 1g $kg^{-1}$ of foliar fertilizer. Foliar fertilizer used for T5, T6, T7 and T8 contains 20% of total nitrogen (N), 20% of available phosphoric acid (P2O5) and 20% of soluble potash (K2O). Treatments were arranged as Randomized Block Design, replicated 10 times. One and a half (1.5) kilos of each media was put in the plastic pot (top diameter was 15 cm). Spores of about 0.05 g were sown in each media, and then it sprayed with water and was cover with plastic sheet for maintaining high humidity. We also put a plastic tray with water in the bottom of the pot to maintain RH. At the shade house an average daily RH was 80% and temperature was 29.5$^{o}$C during a period time of experiment.

For both experiments, observation was done for variables of the time of spores germinated, the times of prothalia formation, the time of sporophyte formation, the percentage of prothalia formed, the percentage of sporophytes formed, and height of sporophyte. Prothalium is a heart-shaped structure (seen with microscope) that is formed after spores germinating (Figure 2) Sporophyte is a plant which formed from spore, subsequently after spore germination and prothalium formation.

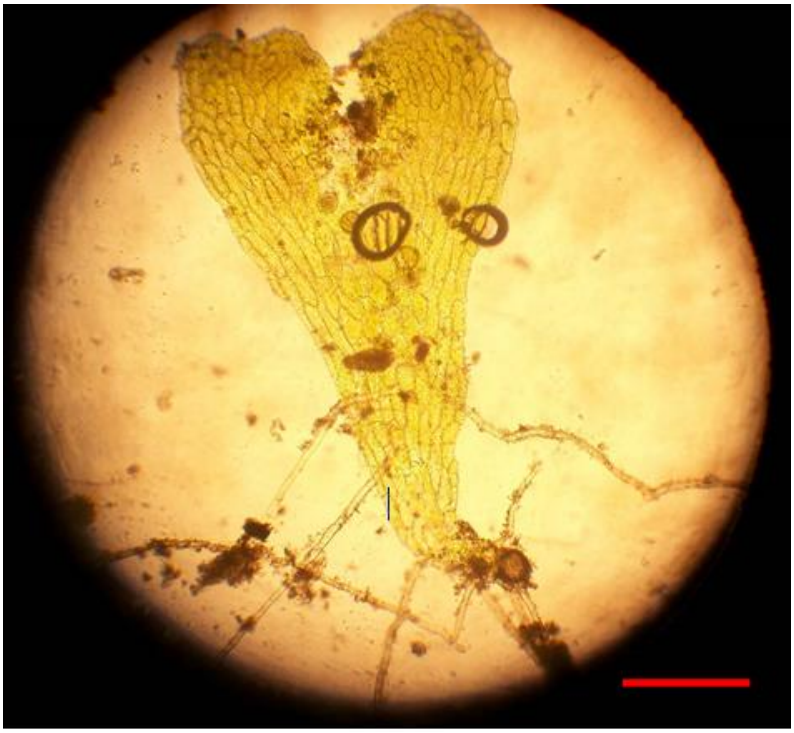

Figure 2. A prothalium (a germinated spore). Bar = 0.5mm

## 3. Results and Discussion

Up to 120 days after spores sowing *in-vitro*, cultures of M6 treatment were contaminated therefore only 5 treatments were left. The data was not statistically analysed because it was not complete. However, the data of *in-vitro* treatment was performed at Table 1. Prothalium and sporophyte were not formed on MS medium with sugar, indicating that the sugar inhibited the formation of prothalium, although spores can germinate. Sugar in plant *in-vitro* culture is a source of energy for the explants to do respiration, subsequently producing energy for the growth of the explant (Saad and Elshahed, 2011), however, it is also a source of energy for the growth of microorganisms as in the case of this study.

MS medium is the most frequently medium used in tissue culture (Saad and Elshahed, 2011), however, the amount of various ingredients in the medium vary for cultures of different species (Daud *et al*. 2011). This study found that for culturing spores of *L. circinnatum in-vitro*, full strength of MS medium resulted in the best growth (Table 1). The result was in line with Abou Dahab *et al*. (2005) who found that full strength of MS medium was better than ½ strength MS and ¼ strength MS for micropropagation *of Ruscus hypoglossum L.* Figure 3 shows growth of spores of *L. circinnatum* at full strength of MS medium without sugar. We suggested that for growing *L. circinnatum in-vitro*, medium of full strength MS without sugar was the most appropriate.

Table 1. Effects of media on spore germination, prothalus and sporophyte formation in *in-vitro* Experiment

| **Treatments** | **Time of spore germination (das)** | **Time of prothalia formation (das)** | **Time of Sporophyte formation (das** |
|---|---|---|---|
| M1 | 23.70 | 39.70 | 85.04 |
| M2 | 27.30 | 45.10 | 98.80 |
| M3 | 49.50 | 65.50 | 110.00 |
| M4 | 22.00 | Not yet formed | Not yet formed |
| M5 | 24.00 | Not yet formed | Not yet formed |
| M6 | contaminated | contaminated | contaminated |

M1 = Full strength of MS media without sugar; M2 = ½ (a half) strength of MS media without sugar; M3 = ¼ (fourth) strength of MS media without sugar; M4 = Full strength of MS media with 20 $gL^{-1}$ of sugar; M5 = ½ (a half) strength of MS media with 20 $gL^{-1}$ of sugar; M6 = ¼ (fourth) strength of MS media with 20 $gL^{-1}$ of sugar; das = days after spores sowing. Each value was averaged from 5 replications.

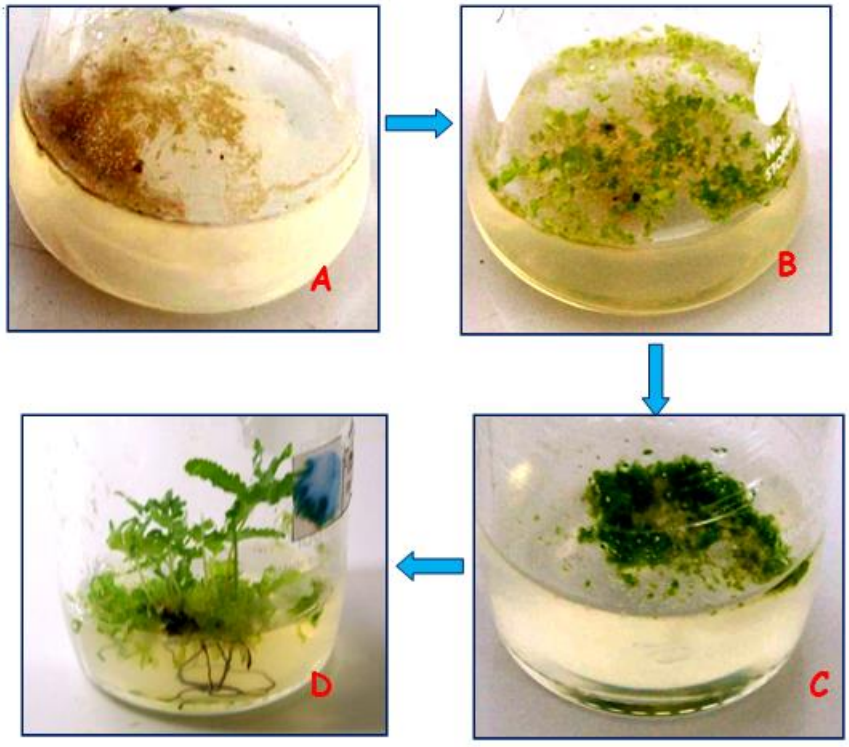


Figure 3. Growth of spores of *L. circinnatum in-vitro*
A= Spores after sowing; B= germinated spores; C=formation of prothalium; D=Sporophytes; Diameter of the bottom of the bottle = 4.5 cm

Table 2 shows results of the *ex-vitro* experiment. Up to 120 days after spores sowing (das), sporophytes were only formed on medium of paddy-silt soil and decomposed leaves either with or without addition of foliar fertilizer. We believed this is closely related to water content of the media. Analysis of water content was performed using method "water (%) by mass" (Icrisat, 2015) and can be seen in the Table 3. Paddy-silt soil had highest water content among types of medium used.

Lygodium is homosporous fern and has bisexual gametophyte. It has two free-living generation i.e. a haploid gametophyte and a diploid sporophyte (Lott *et al*. 2003). Sporophyte (young plant that formed from spores) of homosporous ferns can be produced through three types of mating, i.e. intragametophytic selfing (Soltis *et al*. 1988), intergametophytic crossing (Korpelainen and Kolkkala, 1996; Hooper and Hauffer, 1997) and mixing between both types (Soltis and Soltis, 1987). However, the mayority of them have intergametophytic crossing (Lott *et al*, 2003). In the current research we have no data about the type of mating of *L. circinnatum*, however, we believed that mating between an egg (usually in a structure of archegonium) and a sperm (in a structure named antheridium) in formation of sporophyte requires water, because sporophyte was not performed at the media of fine grain soil and moss, in which the soil water content was low. Although media of decomposed leaves had water content less than 100%, however, it might contain more nutrients, so that sporophyte can performed in this type of media. Besides that, the height of sporophyte increased faster in decomposed leaf media when fertilizer was incorporated (from 1.20 cm to 2.03 cm) compared to paddy-silt soil media (from 1.91 cm to 2.08 cm) (Table 2), it might be nutrient at media of decomposed leaf to be available after 120 days and stimulated the growth of plant.

Figure 4. shows growth of *L. circinnatum ex-vitro* 120 days after spores sowing.. There were no sporophytes formed at media of fine grain soil (T1, T5) and moss (T4, T8) either with or without addition of fertilizer, indicated that these types of medium were not appropriate for growing *L. circinnatum*. Although spores germinated (formation of green-globular structures) and produced prothalium (heart-shape structures under microscopes) at media of fine grain soil and moss, however, sporophytes were failed to be formed. We suggested that mating between eggs and sperms did not occur at these types of medium. If we compared between *in-vitro* and *ex-vitro* experiments, we suggested, *ex-vitro* propagation was more appropriate, the technique was much easier and the spores grew faster compared to those of *in-vitro*.

Table 2. The Effects of medium on the growth of spores of *L. circinnatum* growing ex-vitro

| Treatments | Time of spore germination (das) | Time of prothalia formation (das) | Time of Sporophyte formation (das) | Height of Sporophyte (cm) |
|---|---|---|---|---|
| T1 | 20.50 bc | 29.75 b | Not yet formed | Not yet formed |
| T2 | 19.75 bc | 28.13 b | 102.00 | 1.91 |
| T3 | 21.00 bc | 29.88 b | 108.58 | 1.20 |
| T4 | 26.00 a | 39.25 a | Not yet formed | Not yet formed |
| T5 | 21,25 b | 29.25 b | Not yet formed | Not yet formed |
| T6 | 19.00 c | 28.00 b | 111.75 | 2.08 |
| T7 | 21.00 bc | 29.50 b | 115.75 | 2.23 |
| T8 | 25.63 a | 36.13 a | Not yet formed | Not yet formed |

T1 = Fine grain soil, T2 = Paddy-silt soil, T3 = Decomposed leaves , T4 = Moss, T5 = Fine grain soil +1gkg$^{-1}$ of foliar fertilizer, T6 = Paddy-silt soil +1gkg$^{-1}$ of foliar fertilizer, T7 = Decomposed leaves +1gkg$^{-1}$ of foliar fertilizer, and T8 = Moss+1gkg$^{-1}$ of foliar fertilizer. The same letter behind values indicated not statistically different according to Duncan't Multiple Range Test (DMRT) at level of 5% and vice versa. Each value was averaged from 10 replications.

Table 3. Water Content (%) of Media at *Ex-Vitro* Experiment (Dry Weight-Base)

| Types of Media | Water content (%) *) |
|---|---|
| Fine grain soil | 59.85 |
| Paddy-silt soil | 210.81 |
| Decomposed leaves | 71.13 |
| Moss | 62.27 |

*) The value was averaged from 3 samples

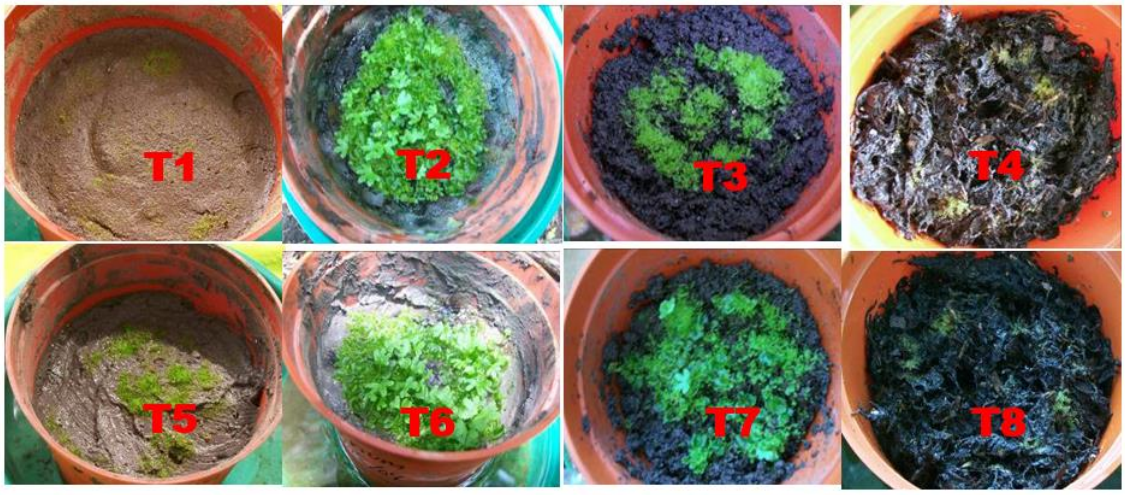


Figure 4. Growth of spores of *L. circinnatum* at several types of medium *ex-vitro*.

T1 = Fine grain soil, T2 = Paddy-silt soil, T3 = Decomposed leaves , T4 = Moss, T5 = Fine grain soil +1gkg$^{-1}$ of foliar fertilizer, T6 = Paddy-silt soil +1gkg$^{-1}$ of foliar fertilizer, T7 = Decomposed leaves +1gkg$^{-1}$ of foliar fertilizer, and T8 = Moss+1gkg$^{-1}$ of foliar fertilizer. Diameter of top of the pot = 15 cm.

## 4. Conclusion

Domestication of a wild fern of *L. circinnatum* was successfully done by using spores as planting materials. In conclusion, full strength of MS media without sugar was the most appropriate media for growth and development of spores of *L. circinnatum in vitro*. While for *ex vitro*, the appropriate media were paddy silt-soil and decomposed leaf either with or without addition of foliar fertilizer. However, we suggested *ex-vitro* propagation was more appropriate, the technique was much easier and the spores grew faster compared to those of *in-vitro*.

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