

IN VITRO CULTURE OF HOLOPARASITE *Rafflesia arnoldii* R. Brown

Kultur In vitro Holoparasit Rafflesia arnoldii R. Brown

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Abstract

Potongan kuncup bunga digunakan sebagai eksplan yang ditumbuhkan pada media dasar Murashige and Skoog (MS) dengan tambahan 0; 0.1; 0.5; 1 and 5 mg/l 2,4-D atau Picloram dan 2 g/l Phytigel. Eksplan tumbuh menjadi kalus pada media yang ditambahkan 0,1 dan 1 mg/l 2,4-D atau 0,5 dan 1 mg/l Picloram. Kultur kalus tersebut dipelihara pada media MS + 1 mg/l 2,4-D. Kemudian kalus ditumbuhkan pada medium dengan penambahan 1, 3, 5 dan 10 mg/l 2,4-D atau Picloram. Setelah dua bulan 66,67-100% kultur membentuk kalus. Semua kalus berstruktur kompak. Beberapa kalus yang diperlakukan dengan 5-10 mg/l 2,4-D menumbuhkan benang-benang putih pada permukaannya. Perlakuan Picloram menghasilkan kalus yang lebih banyak, tetapi 2,4-D menghasilkan kualitas kalus yang lebih baik. Kalus *R. arnoldii* tidak membentuk somatik embrio dengan penambahan 0,1 mg/l Zeatin dalam media kultur. Ini adalah laporan yang pertama kali tentang pembentukan kalus dari kultur *R. arnoldii* secara *in vitro*.

Kata kunci: *In vitro*, kultur, *Rafflesia arnoldii*, holoparasit, 2,4-D, Picloram, Zeatin

INTRODUCTION

Rafflesia arnoldii is a holoparasite in *Tetrastigma* spp. *Rafflesia* is achlorophyllous plant, the flower is the only part of the plant that can be seen. *Rafflesia arnoldii* is the largest single flower in the world with the diameter up to 150 cm.

Rafflesia arnoldii is a unique species among flowering plants, which has strategic values for both scientific and conservation. It was discovered by an Indonesian guide working for a physician Dr. Joseph Arnold who explored with Sir Stamford Raffles, the leader of the expedition in 1818 near Bengkulu city, Sumatra, Indonesia (Anonymous, 2009). *R. arnoldii* belongs to Rafflesiaceae family that derived from Euphorbiaceae. Most euphorbs produce minute

flowers but size evolution cause 73-fold increase in flower diameter (Davis *et al.*, 2006). Rafflesiaceae consists of 8 genera and about 55 species (Nais, 2001). Meijer (1997) and Nais (2001) reported that genus *Rafflesia* consists of 18 species, found in Southeast Asia, from Thailand, Malaysia, Indonesia, and The Philippines. *R. arnoldii* has a very narrow range of geographical distribution in Aceh, West Sumatra, Bengkulu, Lampung, West Kalimantan and West Sarawak (Meijer, 1997).

This holoparasite plant (obligate parasite) obtains water and nutrients from the host plant since the plant does not have any chlorophyll or capacity to assimilate carbon and inorganic nitrogen (Deeks *et al.*, 1999). The host plants of *R. arnoldii* are *Tetrastigma rafflesiae* (Veldkamp, 2007) and *T.*

curtisii (Nais, 2001). They are found in the primary rain forests on undisturbed lowland and lower montane forests, near rivers up to 1000 m asl. (Nais, 2001), especially 350–500 m in altitude (Zuhud *et al.*, 1998). *Rafflesia* is “similar” to fungi and it grows as thread-like strands embedded within host phloem tissue of *Tetrastigma* root or stem from which nutrients and water are obtained (Syahbuddin *et al.*, 1979). It eventually ruptures and forms buds with series of scales (bracts) at the base through the cortex of the host vine bark. The diameter of the flower could reach 43 cm. This process takes 498 days from 1.5 cm to 34 cm of bud diameter, the perigone lobes open one by one and takes 48 hours. From bud to anthesis it takes 18–21 months. The flower is uni or bisexual, perianth single (perigone), often tubular or saucer-shaped at base, consists of 5 corollas, 24–26 cm in length, bright red color. The flower is a unisexual, producing either male or female flower, which stretched up to 150 cm in diameter, 1.9 cm thick and 7 kg weight. Male flower contains 36–40 anthers. It is often called as corpse flower because of its smell. The blossom attracts carrion flies such as *Lucilia* spp. and *Sarcophaga* spp. which may bring pollen from male to female flowers (Syahbuddin *et al.*, 1979; Zuhud *et al.*, 1998; Nais, 2001; Nickrent, 2009). The fruit is large fleshy body with an internal labyrinth covered by tiny seeds (Heide-Jorgensen, 2008). The seed less than 1 mm long, covered by hard seed coat, often thickened or pitted and has undifferentiated few celled embryos. A single female flower may produce up to four millions seeds which dispersed by a variety of animals such as tree shrews, squirrels, wild pigs, and elephants (Wayne’s Word, 1997).

There are many obstacles to propagate *R. arnoldii*. As a holoparasite, the species is grown entirely embedded within the body of the host and is only visible when it erupts into flower bud from the bark of the host. Flower bud mortality is 80–90%, male and female flowers is separated and very high in ratio, flower lifespan is 4–7 days (Nais, 2001), and even only 3–5 days (Syahbuddin *et al.*, 1979). This plant is rare. Its habitat mostly has been converted to farm land and timber concessions. The flower buds are often harvested and sold for medicinal purposes

(Barkman, 2003). The big pollinators become scare, its life cycle from seed until produced seed is predicted 4–5 years (Burkill, 1935; Syahbuddin *et al.*, 1979; Meijer, 1982; Dwiyoono *et al.*, 1992; Meijer, 1997; Zuhud, 1998; Nais, 2001). *In situ* conservation is difficult to maintain, whereas *ex situ* conservation has been collected at Bogor Botanical Garden in 1850–1929 (Meijer, 1997; Mogeia *et al.*, 2001).

Micropropagation of *R. arnoldii* is very important for both scientific and conservation purposes. There are nearly 40 species in 23 genera from 7 different families of parasitic flowering plants have been cultured *in vitro* using predominant seeds and embryos explants, but *R. arnoldii* is excluded (Deeks *et al.*, 1999).

Morphogenesis of holoparasite *Striga asiatica* culture is influenced by exogenous hormones such as 2,4-D, which could induce germinating seeds and forming calli of *S. asiatica in vitro* (Babiker *et al.*, 1994; Cai *et al.*, 1993). The response to hormone was dependent on an exogenous carbohydrate supply (Maheshwari *et al.*, 1980). Sugar is essential for the growth of dodder (*Cuscuta*) stem tip *in vitro* (Loo, 1946). Previously an attempt *in vitro* propagation of *R. arnoldii* flower bud for conservation from West Sumatra was unsuccessful because of improper handling and shipping (Sukanto, 2001).

Rafflesia arnoldii has been classified as a vulnerable plant. *In situ* conservation does not improved its population in natural habitat, therefore *ex situ* conservation through *in vitro* culture were carried out here.

MATERIALS AND METHODS

A bud of *R. arnoldii* about 2 cm in diameter was originally collected from Bengkulu, Sumatra. This bud was washed under running water, after the outer parts of the bud were removed it was then soaked in beaker glass containing 1% Teepol, shaken with magnetic stirrer for 10 minutes and washed with sterile water. The bud was disinfected in 10% Clorox inside laminar air flow cabinet for 10 minutes, repeated with 5% Clorox and then was rinsed three times in sterile water. The flower bud was cut horizontally and vertically about 0.5 cm x 1.5 mm as explants.

The explants were grown on MS media with 2,4-D or Picloram at the concentration of 0; 0.1; 0.5; 1.0; 5.0 mg/l and solidified with 2 g/l Phytigel. The pH of the media was adjusted to 5.7–5.8 and were sterilized at 121°C for 20 minutes. The cultures were incubated in the dark at 27°C. Callogenesis responses were recorded after one month in culture. Calli were maintained and subcultured every month on MS media with addition of 1 mg/l 2,4-D. Calli were treated with combination of 0.1 mg/l Zeatin and 2,4-D or Picloram (1; 3; 5 and 10 mg/l). The design applied was Completely Randomized Design (CRD). Every treatments consisted of five calli in petridish with three replications. Survival, structure, diameter, and color of the callus were recorded after one and two months. The calli survived then were subcultured on the same media after one month. Data were analysed with the Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT) of

Statistical Product and Service Solution (SPSS) 11.5 for Windows.

RESULTS AND DISCUSSION

Flower bud explants of *R. arnoldii* (Fig. 1a) produced a lot of tannin so they turned brown (Meijer, 1997). In order to alleviate the phenol, the cultures were incubated in the dark. The explants showed positive responses and produced whitish callus on 0.1 and 1.0 mg/l 2,4-D or 0.5 and 1.0 mg/l Picloram (Fig. 1b). Negative responses occurred on Control, 0.5 and 5.0 mg/l 2,4-D, and 0.1 and 5.0 mg/l Picloram treatments. The results indicated that morphogenesis of excised flower buds *R. arnoldii* resemble to *Striga asiatica* seeds, the response was associated with the exogenous and the endogenous phytohormones (Babiker *et al.*, 1994).



Fig 1a. Explant of *Rafflesia arnoldii* flower bud



Fig 1b. Explant started to grow callus



Fig 1c. Callus grew covering the explant

The explants produced calli on media with 0.5–1.0 mg/l Picloram but not for media with 0.0; 0.1; or 5.0 mg/l Picloram (Table 1). It showed that 0.5–1.0 mg/l of Picloram was better to initiation and production of callus. Explants showed negative responses at 0.5 mg/l of 2,4-D, but positive responses at 0.1 and 1 mg/l 2,4-D treatments, it might caused by heterogenous genotype of the explant tissues, even they originated from one bud material, as reported by Murashige (1974). Negative responses also occurred on explants treated with 5.0 mg/l of 2,4-D or Picloram. In this study, 2,4-D gave better result in inducing callus compared to Picloram. The callus grew covering the explant (Fig. 1c). The colour

of the callus was light yellow on 2,4-D treatment (Fig. 2a) and brownish on Picloram treatments (Fig. 2b). The best result was 1 mg/l 2,4-D treatment, which produced the most profuse and light yellow color calli. Some explants of root holoparasites, such as seeds of *Striga senegalensis*, *Alectra vogelii*, and *Aeginetia indica* requires host root exudates to germinate and growth, whereas *Cistanche tubulosa* and stem holoparasites of *Cuscuta trifolii* did not need of host shoot exudates (Okonkwo, 1966; Okonkwo, 1975; Deeks *et al.*, 1999; Bakos *et al.*, 1995). The explants of *R. arnoldii* could produce calli without addition of host exudates in the culture medium.

Table 1. The effect of 2,4-D and Picloram on callogenesis of *R. arnoldii* flower bud

| Doses of Hormone (mg/l) | Callogenesis | |
|-------------------------|--------------|----------|
| | 2,4-D | Picloram |
| 0.0 (Control) | - | - |
| 0.1 | + | - |
| 0.5 | - | ++ |
| 1.0 | +++ | + |
| 5.0 | - | - |

Note: - = no respon; + = little callus; ++ = bigger callus; +++ = biggest callus



Fig. 2a. Light yellow callus treated with 2,4-D



Fig.2b. Brownish callus treated with Picloram



Fig. 2c. Callus covered with white strands treated with 10 mg/l 2,4-D

Later the callus cultures were propagated and maintained on MS media with addition of 1.0 mg/l 2,4-D. The calli were treated with 0.1 mg/l Zeatin combined with 1.0; 3.0; 5.0 and 10.0 mg/l 2,4-D or Picloram for regeneration. The survival of the cultures were decreased to 93.33% and 66.67% respectively when the doses of 2,4-D treatments increased from 3.0 mg/l to 5 and 10 mg/l but not on Picloram treatments (Table 2). All calli produced are compact in structures. There was a different morphology of calli, which were covered with thread-like strands on the top (22.22%) occurred in the 2,4-D treatment the concentration of 10.0 mg/l (Fig. 2c). The cultures also producing calli on media with 5.0–10.0 mg/l 2,4-D, even though these calli have a poor growth, some of them covered by white strands on their tops, which also happened on cultures that had not been transferred for a long period. Probably the calli were under stress because of high concentration of 2,4-D treatment or the strands are the vegetative body of *Rafflesia*. This stress may induce a thread-like strands tissue of *R. arnoldii* that embedded naturally within host of *Tetrastigma* root or stem. The thread-like strands of calli may resemble a fungi to penetrate to host plant by using lytic enzymes (Mayer, 2006). These white strands

did not happen on calli induced by Picloram. This, it was showed that 2,4-D give more significant results compare to Picloram. As one of the strongest auxin hormones, 2,4-D was also used as a herbicide for killing wide leaf plants that belongs to *dicots*. The poor growth of *R. arnoldii* calli because of long period of subculture, may deprive nitrogen in medium culture. There were reports that nitrogen are important for seedling growth of root of parasites *Alectra orobanchoides*, *A. vogelii*, *Orobanche foetida* (Abbes *et al.*, 2009; Trautmann and Visser, 1987), and stem holoparasite of *Cuscuta reflexa* *in vitro* culture (Jeschke *et al.*, 1994; Srivastava and Dwivedi, 2003).

The growth of callus was represented by callus diameter. Calli diameter decreased as 2,4-D dose increased but it did not occur on Picloram treatments, at 10 mg/l Picloram produced the largest callus (Table 2). Callus color was influenced by the hormone. The colour of the callus was brownish on Picloram and light yellow on 2,4-D treatments. The callus treated with Picloram was more profused compared to those treated with 2,4-D but the quality of the callus was much better on medium added with 2,4-D compared to those of Picloram treatments.

Table 2. The effect of 2,4-D and Picloram on *R. arnoldii* callus after one month

| Hormone concentration (mg/l) | Survival (%) | Callus (%) | Callus Covered with White Strands (%) | Diameter (cm) | Colour |
|------------------------------|--------------|------------|---------------------------------------|---------------|--------------|
| 2,4-D | | | | | |
| 1 | 100.00 a | 100.00 a | 0.00 a | 1.11 ab | Light Yellow |
| 3 | 100.00 a | 100.00 a | 0.00 a | 0.88 bc | Light Yellow |
| 5 | 93.33 a | 100.00 a | 0.00 a | 0.79 cd | Light Yellow |
| 10 | 66.67 b | 100.00 a | 22.22 a | 0.61 d | Light Yellow |
| Picloram | | | | | |
| 1 | 100.00 a | 100.00 a | 0.00 a | 1.05 ab | Brownish |
| 3 | 100.00 a | 100.00 a | 0.00 a | 1.16 a | Brownish |
| 5 | 100.00 a | 100.00 a | 0.00 a | 1.15 a | Brownish |
| 10 | 100.00 a | 100.00 a | 0.00 a | 1.26 a | Brownish |

Note: Means in the same group followed by the same letter in the columns are not significantly different at the 5% level.

Percentage of survival increased after they were subcultured to the same medium, especially when the media were added with 5.0 mg/l and 10.0 mg/l 2,4-D. After one month 93.33% and 66.67% of the culture were survived and after two months were increased into 100.00% and 80.00% (Table 2 and 3). After two months, percentage of calli covered by white strands decreased from 22.22% to 6.67% on

10.0 mg/l 2,4-D, even though there was 6.67% calli covered by white strands on medium with 5.0 mg/l 2,4-D (Table 3). The diameter of the callus were also increased after two months in culture. The colour of the callus did not change after two months, light yellow on 2,4-D and brownish on Picloram treatments.

Table 3. The effect of 2,4-D and Picloram on *R. arnoldii* callus after two months

| Hormone concentration (mg/l) | Survival (%) | Callus (%) | Callus Covered with White Strands (%) | Diameter (cm) | Colour |
|------------------------------|--------------|------------|---------------------------------------|---------------|--------------|
| 2,4-D | | | | | |
| 1 | 100.00 a | 100.00 a | 0.00 a | 1.33 a | Light Yellow |
| 3 | 100.00 a | 100.00 a | 0.00 a | 1.30 ab | Light Yellow |
| 5 | 100.00 a | 100.00 a | 6.67 a | 1.14 ab | Light Yellow |
| 10 | 80.00 a | 100.00 a | 6.67 a | 0.67 c | Light Yellow |
| Picloram | | | | | |
| 1 | 100.00 a | 100.00 a | 0.00 a | 1.10 b | Brownish |
| 3 | 100.00 a | 100.00 a | 0.00 a | 1.29 ab | Brownish |
| 5 | 100.00 a | 100.00 a | 0.00 a | 1.21 ab | Brownish |
| 10 | 100.00 a | 100.00 a | 0.00 a | 1.29 ab | Brownish |

Note: Means in the same group followed by the same letter in the columns are not significantly different at the 5% level.

Rafflesia arnoldii did not produce any somatic embryo with the addition of 0.1 mg/l Zeatin to the medium. The shape of the somatic embryo of *R. arnoldii* is still unknown, it is possible that the white strands grown in culture are similar to the

'haustorium' of *R. arnoldii* inside the host plant. *In vitro* culture is one of possible methods to conserve the species. In this study after three years, the callus is still survived in culture.

CONCLUSIONS

Young floral bud of *R. arnoldii* was grown successfully on MS media with addition of 0.1 and 1.0 mg/l of 2,4-D or 0.5–1.0 mg/l Picloram. This is the first report on callogenesis of holoparasite *R. arnoldii* in *in vitro* culture. The explant produced the most profuse and best quality callus on 1.0 mg/l 2,4-D treatment. When the concentration of 2,4-D were increased, the survival rate were decreased. Some callus cultures produced white strands, and inhibited callus growth, but the white strands was not found on Picloram treatments. Callus was more tolerant to high dose of 2,4-D after subcultured. The colour of the callus is influenced by kind of hormone added to the medium, brownish colour on Picloram and light yellow colour on 2,4-D treatments. *R. arnoldii* calli did not produce any somatic embryo with addition of 0.1 mg/l Zeatin to the medium.

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