# A preliminary study on *in vitro* seed germination and rooted callus formation of *Tetrastigma rafflesiae* (Vitaceae)

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ABSTRACT. *In vitro* seed germination and induction of rooted callus formation was investigated as a preliminary study on the propagation of *Tetrastigma rafflesiae* as potential host plant material towards a sustainable conservation effort for *Rafflesia*. Seed germination of *T. rafflesiae* is epigeal with seedling emergence ranging 30–60 days after planting (dap), regardless of light presence. After 60 days, 54–60% of seeds germinated in media treatments. Callus formation began 7 dap in MS medium + 2 mg/L NAA, and 21 dap in MS medium + 2 mg/L 2,4-D. Browning of this medium due to phenolic compounds resulted from cutting a part of the hypocotyl. Rooting of the callus was obtained after 21 days on MS medium + 2 mg/L NAA, but was not evident with addition of 2 mg/L 2,4-D.

***Keywords.*** *In vitro* seed germination, *Tetrastigma rafflesiae*, rooted callus

## Introduction

*Tetrastigma* (Miq.) Planch. (Vitaceae) includes c. 100 species throughout tropical and subtropical areas, in Asia and northern Australia. Some of those species are traditionally used for medication in Indonesia and Malaysia, especially as leaf poultice in treating fevers and headaches. In the Philippines, *Tetrastigma* is used to cure scabies. In Vietnam, the leaf extract is used either internally or externally to treat headaches and fevers. In addition, the fruits of some species can be eaten (Lemmens 2003).

*Tetrastigma* is also well known as the exclusive host of the parasitic *Rafflesia* R.Br. (Nais 2001). Most *Rafflesia* spp. in Sumatra subsist parasitically on *Tetrastigma rafflesiae* (Miq.) Planch., of which *T. leucostaphylum* (Dennst.) Alston ex Mabb. is a synonym; see Veldkamp (2008). A shortage of the host plant caused by, for example, habitat reduction, can potentially affect the natural establishment and survival of *Rafflesia* spp. (Attenborough 1995, Meijer 1997). The seriously damaged forest environment in Sumatra makes it likely that viable *Rafflesia* populations remain only in some protected areas (Zuhud et al. 1999, Sofiyanti et al. 2007). It may be possible to help preserve the existence of *Rafflesia* in Sumatra by carrying out *in vitro* propagation of the host plant, so that host-plant abundance and sites can be potentially increased.

As far as we know, there have been no reports about *in vitro* propagation of *Tetrastigma*. Tissue culture techniques for *Tetrastigma* can potentially be adapted from those used for the better investigated commercially important *Vitis* L. *In vitro* propagation of *Vitis*, particularly grape, has been commercially carried out since a long

time ago (Akbas et al. 2004, Salami et al. 2005, Alizadeh et al. 2010). Jaskani et al. (2008) added the auxin NAA (approximately 2 mg/L) to the Murashige-Skoog (MS) medium to induce rooted callus and embryo formation from grape leaf tissue.

This preliminary study aims to investigate the period of seed germination of *T. rafflesiae* on various media preparations, and the optimal conditions for *in vitro* seed germination. It also attempts to induce rooted callus from pieces of hypocotyls by using different types of auxin.

## Materials and methods

*Tetrastigma rafflesiae* fruits were collected from the Andalas Botanical Garden for use as an explants source.

The Murashige–Skoog (MS) basic medium was used, to which was added modified active carbon, kinetin, NAA (1-Naphthalene Acetic Acid), or 2,4-D (2,4-Dichlorophenoxyacetic acid), as required. Other substances added included 0.7% agar and 3% sucrose. The culture medium acidity was controlled at pH 5 ± 0.5. The culture medium was heated until it boiled before being poured into sterilised culture bottles. The bottles were then covered with aluminum foil and paper and secured with rubber bands. Culture bottles were autoclaved for 15 minutes at 121°C and 15 psi pressure.

Ripe fruits of T. *rafflesiae* were sterilised by soaking in 5% commercial detergent solution for 20 minutes and washing them in flowing water for 5 min. The fruits were then rinsed with 70% alcohol for 5 min, then commercial bleach (30%) with 2 drops of Tween 20 for 5 minutes, before being washed three times with sterile distilled water. Fruits were then peeled and seeds extracted and sterilised with 70% alcohol for 3 min, and with 10% hypochlorite mixed with 1 drop of Tween 20 for 3 min before finally being washed with sterile distilled water for 5 min. These sterilised seeds were then ready for implantation into the treatment media.

For *in vitro* seed germination experiments, *T. rafflesiae* seed was germinated on four media types: basic MS medium, MS + 1 g/L active carbon, MS + 0.5 mg/L kinetin and MS + 0.5 mg/L kinetin + 1 g/L active carbon. The seed-implanted culture media were then placed in two environments, a controlled incubation room (24°C ± 2°C) with alternate 12 hours lighting (1000–1500 Lux), and a dark room. Type of seed germination, period (range in days) of germination, and percentage germination were recorded.

To study the effect of two types of auxin on *in vitro* induction of rooted callus from excised hypocotyls of *T. rafflesiae*, the hypocotyls were cut and implanted on two types of media to induce rooted callus formation. Media employed were MS + 2 mg/L NAA + 0.5 mg/L kinetin, and MS +2 mg/L 2,4-D + 0.5 mg/L kinetin. The culture media with hypocotyls were placed in a room with 12 hours lighting and in a dark room, for 2 weeks, to induce callus formation. The time of callus formation, time of rooted callus formation, type and colour of callus, and percentage of callus and rooted callus formation, were recorded.

## Results and discussion

### *In vitro seed germination of Tetrastigma rafflesiae*

Absence of light during germination did not affect the period of germination. However, phenolic formation on the treatment media was higher in the presence of light (Fig. 1 A–B).

The type of seed germination is epigeal in which the hypocotyl is elongated and the cotyledons are raised above the growth substrate (Fig. 1). The plants with this type include cucumber, cotton, sesbania (Tischler et al. 2000), sunflower, pea and flax (Klicova et al. 2004).

Table 1 shows that the time taken to first germination is 30 days after being implanted on the medium for all treatments. The seed that germinated the highest was seen on the MS medium with 0.5 mg/L kinetin added. Thus kinetin is capable of promoting seed germination. Conversely, the lowest seed germination was seen on the MS medium without growth regulators added; statistical significance, however, was not tested in this preliminary trial.

The addition of active carbon does not affect the growth medium except that it can reduce phenolics production by the explants. However, the phenolic compounds produced do not appear to affect the *in vitro* seed germination of *T. rafflesiae*. Klicova et al. (2004) assert that sunflower seed germination requires cytokinin-type growth regulators to induce growth of the shoot with the use of 0.12% BA (6-benzyladenin).

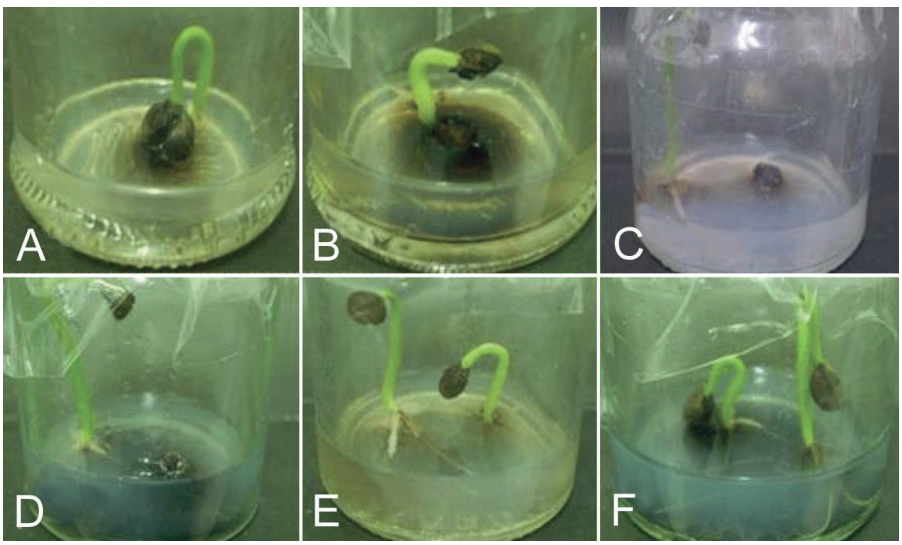


**Fig. 1.** *Tetrastigma leucostaphylum* seed germination in two environmental conditions in MS basic medium, after 8 weeks of treatment (A–B); and on four types of media with alternate 12-hours lighting, after 60 days (C–F). **A.** Complete darkness. **B.** Alternate 12-hours lighting. **C.** MS basic medium. **D.** MS + 1 g/L active carbon. **E.** MS + 0.5 mg/L kinetin. **F.** MS + 1 g/L active carbon + 0.5 mg/L kinetin. Photo by M. Idris.

**Table 1.** Time of seed germination (days after planting (dap) in the medium) and percentage germination after 60 days, in alternate 12 hours lighting.

| No | Treatment | Germination (dap) | % Germination | Tissue formation / medium |
|---|---|---|---|---|
| 1 | MS | 30–55 | 54 | roots formed, cotyledons raised, hypocotyls elongated / medium became brown |
| 2 | MS + 0.5 mg/L kinetin | 30–54 | 60 | roots formed, cotyledons raised, hypocotyls elongated / medium became brown |
| 3 | MS + 1 g/L active carbon | 30–52 | 56 | roots formed, cotyledons raised, hypocotyls elongated / medium normal |
| 4 | MS + 1 g/L active carbon + 0.5 mg/L kinetin | 30–52 | 56 | roots formed, cotyledons raised, hypocotyls elongated / medium normal |

**Table 2.** Period (days after planting, dap) and percentage of callus and rooted callus formation.

| Treatment | Period of callus formation (dap) | Period of rooted callus formation (dap) | % Callus formation | % Rooted callus formation |
|---|---|---|---|---|
| MS + 2 mg/L NAA + 0.5 mg/L kinetin | 7–10 | 21–30 | 100 | 80 |
| MS + 2 mg/L 2,4-D + 0.5 mg/L kinetin | 15–21 | (nil) | 100 | 0 |

***Auxins and rooted callus induction from excised T. rafflesiae hypocotyls***

MS medium augmented with 2 mg/L NAA was more effective at inducing rooted callus formation compared to the MS medium with 2 mg/L 2,4-D (Table 2). In the NAA-augmented medium, the elongation process and callus formation already began just 7 days after planting, and rooted callus formation began after 21 days after planting. Meanwhile, in the 2,4-D-augmented medium, there was browning and the tissue in fact shrank, and there was no sign of root formation (Fig. 2).

Jaskani et al. (2008) showed that addition of 2 mg/L NAA to the MS basic medium promoted callus formation and rooted callus formation from grape leaf tissue.

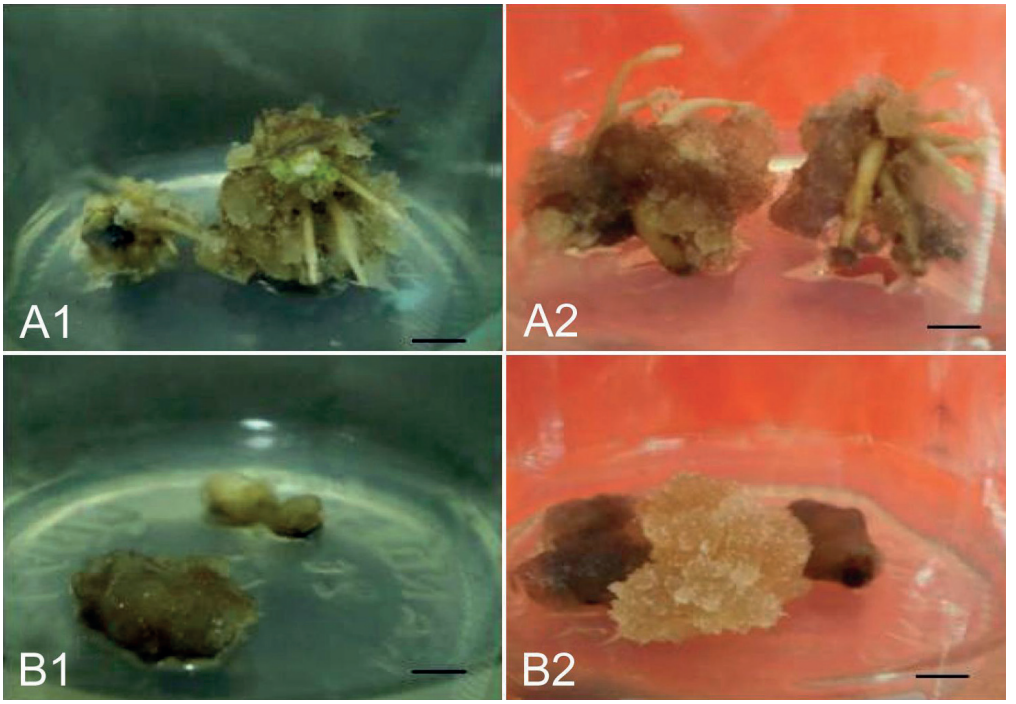


**Fig. 2.** Formation of rooted callus on excised *Tetrastigma leucostaphylum* hypocotyls in different media. MS + 2 mg/L NAA + 0.5 mg/L kinetin at 30 (**A1**) and 60 (**A2**) days after planting, dap; and MS + 2 mg/L 2,4-D + 0.5 mg/L kinetin at 30 (**B1**) and 60 (**B2**) dap. Scale bar = 0.5 cm. Photo by M. Idris.

Xu et al. (2005) added 1 mg/L 2,4-D to promote grape callus formation, but this did not initiate root formation. They also added 1 mg/L NAA and 0.25 mg/L BA which also formed callus without root formation. Akbas et al. (2004) added 1 mg/L NAA to the medium to induce shoot formation of grape.

Such rooted callus formation derived from *in vitro* manipulation can be a source of material to propagate the host plants of *Rafflesia*, and with which to investigate *in vitro* infection by *Rafflesia*. Zhou et al. (2004) used *Brassica napus* L. as an *in vitro* host material for infection by parasitic *Orobanche* L.. Kusumoto et al. (2007) used the root of *Trifolium pratense* L. for infection by *Orobanche minor* Sutton in their studies of the interaction between parasitic and host.

## Conclusions

The basic MS medium augmented with 0.5 mg/L kinetin was effective in promoting *in situ* seed germination of *Tetrastigma rafflesiae*. The formation of rooted callus can be produced on MS medium by adding 2 mg/L NAA + 0.5 mg/L kinetin.

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