A preliminary study on *in vitro* seed germination and rooted callus formation of *Tetrastigma leucostaphylum* (Family : *Vitaceae*) a host plant for *Rafflesia*

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Abstract

Propagation *in vitro* of *Tetrastigma leucostaphylum* as a host plant for *Rafflesia* is one of the alternatives that must be explored in a sustainable conservation effort for *Rafflesia*. This research was conducted as a preliminary study on propagation of T. *Leucostaphylum*. Two step of research was *in vitro* seed germination and inducing rooted callus formation.

The result showed that T. *leucostaphylum* seed germination is epigeal with seedling emergence period ranging 30-60 days after planting (dap), regardless of presence or absence of light. Percentage of seed germinated after 60 days was 54-60% of the total seed that germinated in media treatments. Murashige-Skoog (MS) medium + 0,5 mg/L Kinetin was a better medium to promote seed germination. Callus formation began 7 dap on MS medium + 2 mg/L NAA. While, MS medium + 2 mg/L 2,4-D, callus formation began 21 dap. In this medium, a cutting part of hypocotyl resulted some phenolic that make the medium browning. Rooted callus formation was seen after 21 days on MS medium + 2 mg/L NAA and it was not evident with the addition of 2 mg/L 2,4-D. Callus producing roots is a better source for germination and infection of *Rafflesia* seed for the next research.

Key words : *in vitro* germination, *Tetrastigma leucostaphylum*, rooted callus

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I. INTRODUCTION

Tetrastigma (family: *Vitaceae)* covers 100 species and spreads throughout tropics and subtropical area in Asia and northern Australia respectively. Some of those species are traditionally used for medication in Indonesia and Malaysia especially the leaf poultice to treat fever and headache. In Philippine, *Tetrastigma* is used to cure scabies and diuretic but for external use only. In Vietnam, extract of the leaf is used either internally or externally to treat headache and fever. In addition, the fruit of some species of *Tetrastigma* can be used as foodstuffs (Lemmens, 2003).

Tetrastigma is well known as the exclucive host of the parasitic *Rafflesia* (Nais, 2001). Most of *Rafflessia* in Sumatra subsist parasitically on *Tetrastigma* type *Tetrastigma leucostaphylum*. Because of the shortage of the host plant, the natural propagation of *Rafflessia* becomes difficult which in turn lessen its existence in nature (Attenborough, 1995 and Meijer, 1997). The seriously damaged habitat of Sumatra is also attributable to this condition and makes *Rafflessia* to be only found in some protected areas (Zuhud, Hernidiah and Hikmat, 1997 and Sofyanti *et al*, 2007). *In vitro* conservation is so important that it enables us to preserve the existence of *Rafflessia* in Sumatra by carrying out *in vitro* propagation of the host plant.

As far as we know, there has been no or a few literature about *in vitro* propagation of *Tetrastigma* thus leaving difficulties in researching it. An alternative of propagation of tissue culture of *Tetrastigma* is an adaptation of technique employed in tissue culture of *Vitis* genus coming from the same family as *Tetrastigma* genus (family *Vitaceae*).

In vitro propagation of genus *Vitis*, particularly grape, has been commercially carried out since longtime ago as the needs of this fruit continue to grow (Akbas et al, 2004; Salami et al, 2005 and Alizadeh, Singh and Pantel, 2010). Jaskani *et al* added auxin, a growth regulator from NAA (*1-Naphtaleneacetic acid*) type to induce rooted callus formation and embryo of a cutting part of the grape leaf with NAA with concentration approximately 2 mg/L on Murashige-Skoog (MS) medium.

This preliminary study aimed at observing the period of seed germination of T. *leucostaphylum*, compatibility of the medium, and proper environmental condition for the seed germination. Induction of rooted callus formation later acts as the medium for infection sources of the *Rafflessia* seeds in its *in vitro* conservation.

II. MATERIAL AND METHOD

2.1. Plant Material

Plant materials of T. *leucostaphylum* were collected from The Andalas Botanical Garden area. The plant materials used as the explants source are those fruits with seeds.

2.2. Tissue Culture Media

Murashige–Skoog (MS) was used as the basic medium, added with modified active carbon, kinetin, NAA (*1-Naphtaleneacetic Acid*), and 2,4-D (2,4-*Dichlorophenoxyacetic acid*), and used properly according to the phase of the research. 0,7 % of agar and 3 % of sucrose were also added to the medium. Acidity of the culture medium was controlled until it reached pH 5, 5 ±0, 5. The culture medium was heated until it boiled before they were poured into sterilized-culture bottles. The culture medium was then covered with aluminum foil and paper and finally tied with rubber bands. The culture-bottles were sterilized by using autoclave for 15 minutes with 121 °C and on 15 lbs pressure.

2.3 Sterilization of Explants

The ripe fruits of T. *leucopstaphylum* collected from the field were physiologically sterilized. The sterilization includes soaking the ripe fruits in 5% of commercial detergent solution for 20 minutes and washing them on stream (flowing water) for 5 minutes. The fruits were then rinsed with alcohol 70% for 5 minutes, hcommercial

bleach 30% + 2 drops of Tween 20 for 5 minutes before washed with sterile distilled water for three times. The fruits were then peeled and the seeds were taken and sterilized with alcohol 70% for 3 minutes and with hypochlorite 10 % + 1 drop of Tween 20 for 3 minutes before finally washed with sterile distilled water for 5 minutes. The sterilized seeds were ready to be implanted on the treatment media.

2.4. Method of the Research

This research was conducted by using experimental method where the data were analyzed descriptively. The research comprises two stages: the *in vitro* seed germination of the *T. leucostaphylum* and the induction of two types of auxin on the cutting part of hypocotyl of *T. leucostaphylum* by *in vitro*. The phases of the research are explained as follows:

a. *In vitro* seed germination of *T. leucostaphylum*

   The seed was germinated on four media types: basic medium MS, 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-contained culture media were placed on two treatments, i.e. in a room with 12 hours light photoperiodism and in a dark room until the seed germinated. The parameters of observation included the types of germination, the period (range days) of seed germination, and the percentage of seeds that germinated.

b. The effect of the two types of auxin by *in vitro* in inducing the rooted callus on a cutting part of hypocotyl of *T. leucostaphylum*.

   The hypocotyl of shoot was cut and used as an explants source in this phase. The hypocotyl was then implanted on two types of media to induce rooted callus formation. Media employed in this stage 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 hypocotyl-contained media culture was placed on two treatment media, i.e. in a room

with 12 hours light photoperiodism and in a dark room fortnightly respectively to induce the callus formation. The parameters of observation included the time of callus formation, time of rooted callus formation, types and color of callus, and percentage of callus and rooted callus formation.

On every stage of the research (with an exception to the dark room), the explants were kept on incubation room with a controlled lighting and temperature. The temperature of incubation room was arranged ranging from 24°C to ± 2°C with photoperiodism 12L /12D (12 hours light and 12 hours dark) and the light intensity ranging from 1000 to 1500 Lux.

III. RESULT AND DISCUSSION

3.1. *In vitro* seeds germination of T. *leucostaphylum*

Having tested the *in vitro* seed germination of *T. leucostaphylum* on two environmental conditions, it was uncovered that the absence of light during germination did not affect the period of germination. Based on the result of this research, it can be concluded that the absence of light did not affect germination effectiveness. It means that germination of *T. leucostaphylum* seeds is not influenced by lighting exposure. However, phenolic formation on treatment media with the presence of light was higher compared to the media treatment with the absence of light. The former showed more phenolic than the later did (Figure 1 A-B).

The type of seed germination is epigeal in which the hypocotyl elongated and cotyledons raised from the growing media (figure 1). The plants with this type include cucumber, cotton, sesbania (Tschler, 2000), sunflower, pea and flax (Klicova, Sebanek and Vlassic, 2004).

The table 1 show that the range days needed by the seed to germinate are 30 days after being implanted on the media for all treatments. The seed that germinated the highest was seen on the MS treatment by adding 0,5 mg/L kinetin. This proves

that kinetin serves as growth regulator substance, which is capable of promoting the seed germination. Conversely, the seed, which germinated the lowest, was seen on the MS treatment without the growth regulator substance.

The addition of active carbon does not affect the growing media except that it can reduce the phenolic effect produced by the explants on the media. Generally, phenolic produced do not affect the *in vitro* seed germination of *T. leucostaphylum*. Klicova, Sebanek, and Vlassic (2004) assert the seed germination of sunflower considerably requires the growth regulator substance of cytokinin types to induce the growth of the sunflower shoot with the use of 0,12% BA (*6-Benzyladenin*).

3.2. The influence of the two types of auxin in inducing by *in vitro* the rooted callus on a cutting part of hypocotyl of *T. leucostaphylum*.

Based on the testing, it showed that the MS medium, added with 2 mg/L NAA, performed better in inducing the rooted callus formation compared to the MS medium, added with 2 mg/L 2,4-D (table 2). In the former process, it showed that the elongation process and callus formation already began 7 days after planting. Meanwhile, in the later process, it showed that the medium became brown (browning) and the tissue did nod not grow, but shrunk. The rooted callus formation noticeably began after 21 days after planting on the treatment MS Medium with 2 mg/L NAA. However, on the MS Medium with 2 mg/L 2,4-D, the callus was formed but no sign of root formation (Figure 2).

The two-weeks darkening period did not affect the time of callus formation because they showed similar responses for the two different treatments. The elongation and the initial callus formation occurred in the MS Medium with 2 mg/L NAA, meanwhile in the MS Medium with 2 mg/L 2,4-D, the hypocotyl became brown on its cutting print 60 days after planting (Figure 2).

Jaskani *et al* (2008) showed the rooted callus formation, which are potential for embryo formation on a cutting of grape leaf by adding 2 mg/L NAA as the promoting medium of callus formation on the MS basic medium. Formerly, Xu *et al*

(2005) added 1 mg/L 2,4-D to promote the callus formation which did not initiate the root formation , but to promote the somatic embrio. They also added 1 mg/L NAA and 0,25 mg/L BA which formed the callus without root formation of the grape. Akbas *et al* (2004) added 1 mg/L NAA on the medium to induce the shoot formation of grape.

Table 1. Range days of seed germination and percentage of germination after 60 days in lighting condition 12 HL/12HD

No	Tretament	Range days of germination (dap)	Percentage of germination(%)	Morphological Performance of shoot
1	MS	30-55	54	Root formed, cotyledons raised, hypocotyls elongated, medium became brown
2	MS + 0,5 mg/L kinetin	30-54	60	Root formed, cotyledons raised, hypocotyls elongated, medium became brown
3	MS + 1 g/L active carbon	30-52	56	Root formed, cotyledons raised, hypocotyls elongated.
4	MS + 1 g/L active carbon + 0,5 mg/L kinetin	30-52	56	Root formed, cotyledons raised, hypocotyls elongated.

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

Treatment	Period of callus formation (dap)	Period of rooted callus formation (dap)	Percentage of Callus Formation (%)	Percentage of 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	21-45	100	0

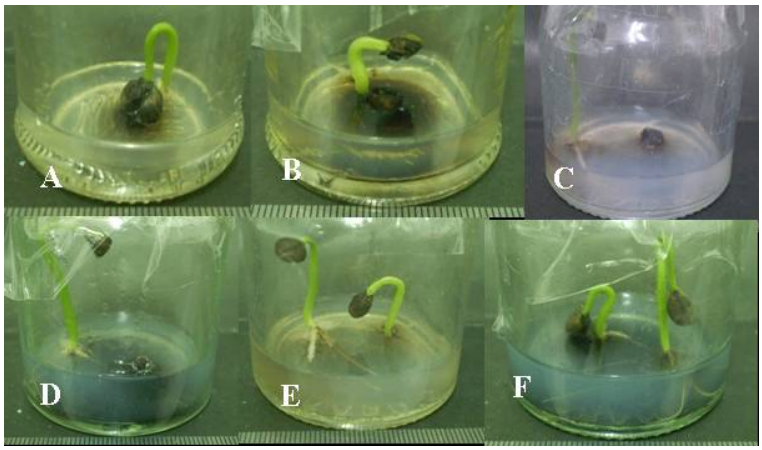

Figure 1. Seeds germination of T. *leucostaphylum* on two environmental conditions in MS basic medium after 8 weeks of treatment (A-B) and on 4 types media with 12 hours with light photoperiodic/12 hours without light after 60 days (C-F) A). On dark condition and B). On 12 hours light/12 hours dark condition, C). MS; D) MS + 1 g/L active carbon; E). MS + 0,5 mg/L kinetin and F). MS + 1 g/L active carbon + 0, 5 mg/L kinetin.

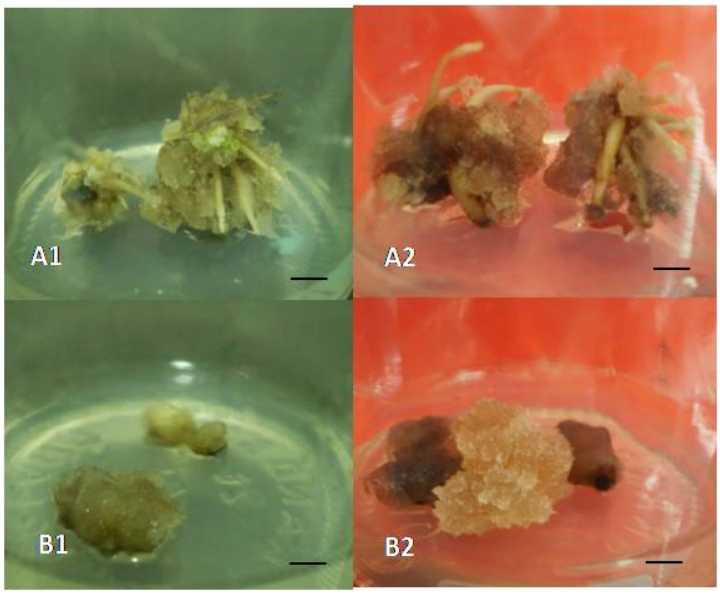

Figure 2. Formation of rooted callus on a cutting part of T. *leucostaphylum* hypocotyl. A) MS + 2 mg/L NAA + 0,5 mg/L kinetin and B) MS + 2 mg/L 2,4-D + 0,5 mg/L kinetin (symbol *1* means 30 days after planting, symbol *2* means 60 days after planting, bar = 0,5 cm)

The rooted callus formation can be a main medium to be used as infection sources of *Rafflesia* seed by *in vitro*. *Rafflesia* considerably depends on its host (plant) roots to grow. With the presence of rooted callus, it can be used as the main medium for germinating and growing the Rafflesia by *in vitro*. Zhou *et al* (2004) used *Brassica Napus* as the main sources to infect *Orobanche* by *in vitro*. Kusumoto *et al* (2007) used the root of *Trifolium pratense* as the source to *Orobanche minor* infection in studying the interaction between parasitic plant *Orobanche minor* and their host.

CONCLUSION

According to the research, some conclusions can be drawn:

1. The MS basic medium added with 0,5 mg/L kinetin worked as a better medium in promoting the seed germination of *Tetrastigma*.
2. The formation of rooted callus can be produced on the MS medium by adding 2 mg/L NAA + 0,5 mg/L kinetin. Meanwhile, the MS medium added with 2 mg/L 2,4-D + 0,5 mg/L kinetin performed better in promoting the callus without root.

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