Propagation of an Indonesian native species *Tetrastigma glabratum* (Blume) Planch, a medicinal plant from Mount Prau, Central Java, Indonesia

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Abstract

A study was conducted to determine the growth and the best method of cutting propagation of *Tetrastigma glabratum* (Blume) Planch, a host of *Rafflesiaceae*. *Rafflesia* is the largest and magnificent flower in the world that requires growing parasitically on the host plant. *T. glabratum* was found to grow in the naturally protected rainforests at Mount Prau, Candiroto, North Kedu, Central Java, Indonesia. *T. glabratum* existence is threatened due to illegal harvesting by the local people who use liquid exudates from the mature stems as an energy drink and to cure various diseases. The population of *T. glabratum* is increasingly hard to find, particularly the mature plants. Ex situ conservation and cultivation is very important to protect *T. glabratum*. Propagation studies were conducted using stem cuttings and treated with IBA at 500, 1000, 2000, and 4000 ppm, and a commercially available rooting hormone containing naphthalene acetic acid (NAA) versus non-treated as a control, and through air layering. In the second experiment, stem explants were cultured in vitro using MS media. IBA and NAA increased the percentage of survived and rooted cuttings over control, and IBA at 2000 ppm significantly improved the percentage of rooted cuttings (50%) compared to 5% without IBA. Air layering techniques had more success than cuttings, with tree fern media giving 70% success rates with the highest number and longest roots. In vitro methods have been developed to obtain sterilized explants from the mature plants growing in situ but have not been successful to induce proliferation even though the explants had started showing enlargement around the nodal section.

Keywords: vegetative propagation, *Rafflesia* host plant, Central Java

INTRODUCTION

Tetrastigma glabratum (Blume) Planch is a climbing perennial vine belonging to the *Vitaceae* family. Specimens are stored in the Herbarium Bogorensis at the Research Center for Biology-Indonesian Institute of Sciences, Cibinong Science Centre, Indonesia. It can grow to a height of about 20 m; the young stems are reddish in color and the stems have tendrils. *T. glabratum* appears to be endemic to Mount Prau, Central Java, it is found to grow in areas with an elevation >1,300 m a.s.l. (Lianah, 2013). The local names of *T. glabratum* include walikadep (Lianah, 2013), akar darik-darik, bantengan, gang putih, oyod epek and oyon waliran (Heyne, 1987). *T. glabratum* leaves consist of five leaflets, serrated and arranged palmately compound.

The liquid exudates from the stems of *T. glabratum* have been used by the local people in the villages at Mt. Prau for generations (Lianah, 2014). The stem exudate is believed to have health benefits including increasing children's appetites, have refreshing and stimulating effects, and has been used as a natural medicine in that area. The research in Indonesia on the culture and medicinal properties of *T. glabratum* is still at the early stages, in contrast to *T. hemsleyanum*, another species of *Tetrastigma*, that has been studied for many years in China.

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T. hemsleyanum is effective against inflammatory disorders and has been used as an analgesic (Dong et al., 2002). Extracts of total flavonoids from *T. hemsleyanum* have antibacterial, antiviral, antitumor, and antipyretic activities (Peng et al., 2019).

Tetrastigma is the sole host species of the *Rafflesia* (*Rafflesiaceae*), a genus of parasitic plants that have the largest flower of all flowering plants (Mursidawati and Irawati, 2014; Nair, 2001). *Rafflesia* plants live inside the roots and stems of its host because *Rafflesiaceae* entirely lack leaves, stems, roots, therefore they are completely dependent on their host plants for nutrients and water. *T. glabratum* is one of the hosts of *Rafflesiaceae* (Zuhud, 1998).

Extensive harvests of *T. glabratum* from the natural habitat for natural medical purposes is a threat to the population of this species. Lianah et al. (2015) recorded that the population of *T. glabratum* in this area between 2009 to 2014 and found that the population has significantly decreased, particularly of the old tree with a diameter of >30 mm.

Micropropagation in vitro allows the mass and rapid production of plants using relatively small amounts of space, supplies and time. In vitro propagation of woody species is relatively difficult, particularly if the plant materials are sourced from the natural habitat as opposed to, for example, growing the mother plants in the controlled glasshouse. Procedure of micropropagation has several steps: explant selection, establishment of aseptic culture including explant sterilization, multiplication, rooting and acclimatization of the plants. Sterilization of explants is the important starting step, as micropropagation relies on clean, healthy and disease-free explants, i.e., the explants have to be free of exogenous contaminating microorganisms. To eliminate contamination during in vitro propagation different methods have been developed (Barrett and Cassells, 1994; Cassells and O'Herlihy, 2003).

Rooting hormone indole butyric acid (IBA) and naphthalene acetic acid (NAA) has been applied to cuttings to induce adventitious root formation. IBA (Noor Camellia et al., 2009; Lianah, 2016) and NAA (Sanjaya et al., 2002) have been demonstrated to be effective to induce rooting of difficult-to-root woody species. Lianah (2016) also reported that growing medium selection is important for *T. glabratum* survival and growth; plants grown on tree fern medium had better growth than those grown on soil or soil + compost mixture. The development of a propagation protocol is important to protect *T. glabratum* from destruction and extinction, thereby contributing to the conservation of *T. glabratum* in the areas of Mount Prau, Central Java. This article presents propagation results of *T. glabratum* in situ, in vivo cutting propagation studies, and the development of sterilization protocol for micropropagation of *T. glabratum*. Morphology of *T. glabratum* can be seen in Figure 1.

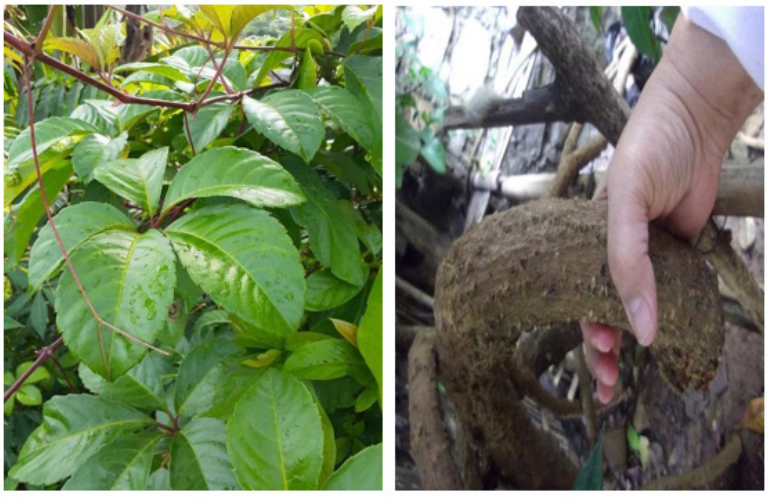

Figure 1. Morphology of leaves (A) and a mature stem of *Tetrastigma glabratum* (B) at Mt Prau, Central Java, Indonesia.

MATERIALS AND METHODS

Vegetative propagation of *T. glabratum* by air layering and cuttings

Propagation of *T. glabratum* was conducted by air layering from one old tree at the conservation area at Mount Prau, Candiroto, North Kedu, Central Java, Indonesia 7.3713900°S, 109.9866700°E in 2010-2011. Stems with a diameter of ±15 mm were selected for air layering. Rooted air layers were cut and maintained in a nursery located nearby to the conservation area. The study site is classified as humid with a relative humidity of 60-80%, soil pH of 6.9, slopes of 45-60°, day temperatures of 20-25°C and night temperatures of 16-20°C. Three types of media were tested for air layering, topsoil collected from the local area, burnt rice hulls, and tree fern media, each consists of 10 air layers.

Propagation of *T. glabratum* by cuttings was conducted twice, one at UIN Walisongo, Department of Biology nursery in Semarang, Central Java, and another one at the Nursery of Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University. In vitro propagation experiment was conducted at Tissue Culture Laboratory II, Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, Bogor, West Java.

Shoots were first washed thoroughly with running water for 5 min, then separated based on the diameter of the stems, i.e., <5 mm (apical sections), 5-10 mm (middle section), and >10 mm (basal section) (Figure 2). The shoots were cut into about 20 cm length cuttings containing 3 nodes. The cuttings were then immersed in bactericide (2 g L^{-1}) and fungicide (2 g L^{-1}) for 5 min, followed by dipping the cutting base in a commercial rooting powder Rootone F. The active ingredients of Rootone F are naphthalene acetamide (0.067%), 2-methyl-1-naphthalene acetamide (0.013%), 2-methyl-1-naphthalene-acetate (0.33%), indole butyric acid (0.057%), and thiram (4%). Indole-acetic acid (IAA), naphthalene-acetic acid (NAA) were technical grade compounds from Sigma. The apical part of the stem cuttings was covered with wet tissue to reduce evaporation. Each treatment consists of 10 cuttings. We could not use more cuttings to avoid too much damage to the parent trees.

A B C

Figure 2. (A) Stem cuttings of *T. glabratum*; each shoot from the same plant were separated into apical, middle, and basal sections. Each section was 15-20 cm in length; (B) *T. glabratum* stem cuttings treated with NAA at 21 days after planting; the cuttings had axillary bud initiation after about 14 days after planting. Only basal stem cuttings with a diameter of ±10 mm survived and grew an axillary shoot; (C) *T. glabratum* that had been replanted to the original habitat at Mt. Prau, Central Java, Indonesia.

Cutting propagation media consist of topsoil, burnt rice hulls, compost and cocopeat 2:1:1:0.5 (v/v). The media mix was thoroughly mixed before placing into pots. Cuttings were planted on the media with 1 node below the media surface and placed under shade with plastic cover. Humidity was maintained by watering daily and spraying water to the cuttings under the cover twice a day.

Development of sterilization protocol and in vitro micropropagation of *T. glabratum*

The experiment was conducted at plant tissue culture laboratory II, Department of Agronomy and Horticulture, Faculty of Agriculture, IPB University, between September to December 2019. The shoots were collected from the population of *T. glabratum* at Mt. Prau. Shoots were selected 1.5 m above the ground and separated with care to allow minimum disturbance to the mother plants. Explants were stem sections and leaf petiole. The stem section had one node which was cut about 30-40 mm above and below the node. The leaf petiole explants were cut into three sections, basal, middle, and top section.

Before sterilization, shoots were washed in running water for 5 min and kept in the laboratory for a few days prior to sterilization. Stem and leaf petioles were first immersed in bactericide (2 g L^{-1}) and fungicide solution (2 g L^{-1}) and put on a shaker for 60 min, followed by washing twice with sterilized water. Stem and leaf petioles were then immersed in 30% Clorox for 30 min, split into 3 smaller sections (top stem, middle, and basal; middle stem containing axillary buds), re-immersed in 15% Clorox for 20 min until the tips of each section changed color to white. The sterilized stem section explants were then planted in flasks containing 30 mL media and placed in the culture room. Each flask contains 3 stem sections or leaf petiole explants.

The culture medium is MS, 30 g L^{-1} sucrose, MS vitamins, agar (6 g L^{-1}). The pH of the media was adjusted to 5.8 prior to autoclaving the media at 121°C and 1.5 atm for 20 min. The cultures were kept in a 22-24°C with 16 h photoperiod and 3500 lx of light intensity. After 14 days the percentage of contaminated survived, and dead explants were recorded. The contaminated culture was rescued using the sterilization protocol described before and replanted in the newly prepared media. Ten explants were used in each sterilization treatment, and each treatment was conducted in three replications.

RESULTS

Vegetative propagation of *T. glabratum* by air layering and cuttings

Propagation with air layering from the large plant growing in situ resulted in 70% success rate. The large plants were only found in the elevation of >1300 m with a slope of <45%, making it very difficult to access. The roots were formed after about 4 weeks and the air layers were ready to be separated from the mother plants after 6-8 weeks. Air layering with tree fern medium had the highest rooted shoots of 70%, the highest number of roots and longest roots (Table 1; Figure 3).

Table 1. Success rate, root number, and root length of *T. glabratum* propagated by air layering.

Media	Success rate (%)	Root number	Root length (cm)
Soil	5	1-3	2-3
Burnt rice hulls	40	5-6	5-6
Tree fern	70	8-10	8-12

Propagation from stem cuttings had a lower percentage of rooting (Table 2) compared to those from air layering (Table 1). From the three different sources of stem cuttings, only lower basal stem cuttings, i.e., those with a stem diameter of 10 mm, survived and rooted. All tip cuttings wilted after 7-10 days after sticking the cuttings and leaves of all types of cuttings had dropped after 7 days. Percentage of the surviving cuttings declined from all treatments after two weeks; it was about 80% after one week to less than 50% after two weeks (Table 2). From the two experiments conducted at two different locations, IBA-treated cuttings had the

highest percentage of rooted cuttings of 20% (Table 2).

Table 2. Survival and percentage of rooted cuttings of *T. glabratum* treated with different types of PGR.

Treatment	Concentration (ppm)	Survival after 14 days	Rooting %
Trial 1			
Control (untreated)	0	10	2.5
IBA	500	38	11.0
IBA	1000	60*	10
IBA	2000	62*	19.5*
Trial 2			
Control (untreated)	0	20	0
IAA	10	30	5.0
NAA	10	40	7.5
Rootone-F		40	10

*Significantly different from control at α=0.05.

A B C

Figure 3. Rooted air layers of *T. glabratum* (A); air layers with soil medium (B) had significantly fewer roots than those in tree fern medium (C).

Sterilization of *T. glabratum* for micropropagation in vitro

Sterilization techniques tested in this study were successful to obtain clean and sterilized explants (Figure 4), however, no further growth was observed apart from swelling on the stem sections around the nodal section. Sterilizing woody materials growing in the field has always been problematic. Surface sterilization is the most important step in the preparation of explants for micropropagation because controlling fungal and bacterial contamination of woody plants from field sources is very difficult as reported in Mihaljevic et al. (2013). More trials using different types and concentrations of PGR will be tested to optimize the media composition to propagate *T. glabratum* in vitro.

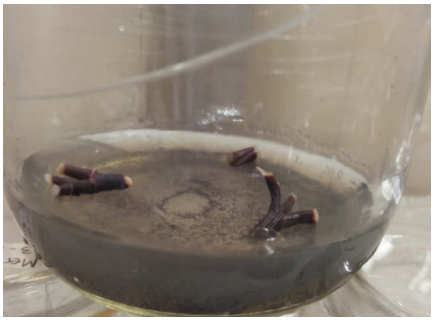

Figure 4. Sterilized nodal segment explants of *T. glabratum* at 4 weeks after culture.

DISCUSSION

Our study demonstrated that air layering propagation had a better success than cutting propagation. These results are expected; air layering has the advantage that the branch segment (the propagation portion) continues to receive water and nutrients from the parent plant while it is forming roots. This is important particularly for woody plants that form roots slowly. Another advantage is that the new plant will have a larger size than could be accomplished by taking cuttings.

Propagation by air layering; however, has disadvantages compared to cutting propagation. It requires experienced workers, takes a longer time to do, and is therefore expensive. Also, only a small number of air layers can be produced from a parent plant without significant damage to the parent plant, whereas vegetative propagation using another method like cuttings, buddings, or scion grafting, larger numbers of materials can be produced.

In our system propagation by cuttings had limited success (<20%), but those that survived can grow to maturity, and have been replanted in Mt Prau (Figure 2c). It appears that IBA is more effective than NAA and IAA to induce adventitious root formation of *T. glabratum* cuttings. A study on *Asparagus cochinchinensis* (Kim et al., 2021) confirmed that NAA is effective in inducing root formation in vitro. Our results agree with a study in *Pongamia pinnata*, that IBA was the most effective auxin to induce rooting compared to the other two auxins (NAA and IAA) (Kesari et al., 2009). They also reported that higher concentrations of auxin, in their study it was 7 mM of IBA, can inhibit adventitious root formation (Kesari et al., 2009). Štefančič et al. (2005) in their study comparing IAA and IBA on the 'GiseLA-5' cherry cultivar, found that cuttings treated with IAA produced callus, whereas those treated with IBA produced adventitious roots. However, in this study, we did not find any callus formation on cuttings treated with IAA.

Stem cuttings with a diameter of 5-10 mm survived longer than tip cuttings, perhaps due to more lignified cells in this section compared to those in the younger tip cuttings. A similar study was conducted in a biofuel crop, *Jatropha circus,* comparing different sources of cuttings (Severino et al., 2011). Stem cuttings characteristics affect the survival rate and early development of *J. curcas* cuttings, and basal stems produced more roots (25 roots per cutting) than apical cuttings (13 roots per cutting) (Severino et al., 2011). Length, basal area, and position on the branch were found to influence the survival rate and growth of the rooted cuttings (Severino et al., 2011). Our study did not include growth measurements of the rooted cuttings, but all rooted cuttings successfully grow and can be replanted in their natural habitat or cultivated by the local community in the area so they do not have to harvest the stems from the protected forests.

To date, there have not been measures from the local government to protect this species. We propose that the local government establish a protected area to let this species grow in their natural habitat and to set up a propagation program to plant it back. These efforts will not only protect *T. glabratum*, but also maintain the stability of the ecosystem of the Mt. Prau area.

CONCLUSIONS

T. glabratum propagation through cuttings with the application of auxin (IAA, NAA) or commercial rooting powder resulted in rooted cuttings, but the percentage was quite low (<20%). IBA seems to be more effective than IAA or NAA. Air layering techniques gave more success in propagating *T. glabratum*, but a limited number of air layers can be made to minimize disturbance of the plants in situ. In vitro propagation method was successful in obtaining sterile explants but has not been successful in producing viable explants. Future studies should explore the uses of different types and combinations of PGR in the propagation media in vitro and in vivo.

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results of propagation.

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