In vitro Seed Germination and Shoot Multiplication of Seven Endemic Subalpine and Alpine Plant Species Grown on Mount Jaya, Papua, Indonesia

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Abstract

Exploitation on plant population may put the endemic plants into an endangered state, hence, these plants will need to be conserved. In order to pursue conservation on endemic plants, we conducted *in vitro* seed germination and shoot multiplication of seven alpine and sub-alpine species endemic to Mount (Mt.) Jaya, in Papua, Indonesia, i.e. *Tetramolopium klossii, Deschampsia klossii, Papuacalia cartenszensis, Epilobium hooglandii, Gaultheria novoguinensis, Rhododendron correoides* and *Rhododendron culminicolum*. These species are categorized as slow-growth plants found in higher altitude (over 3700 m above sea level) and low temperature of Mt. Jaya. Seeds were surface-sterilized using Na-hypochloride and germinated aseptically on Murashige and Skoog (MS) medium. Dytikinin benzyl adenine (BA) was used for shoot multiplication. Seedling cultures were maintained in a controlled environment with continuous low light intensity (800 lux) and at temperature 26-27°C. Results showed that most species had more than 80% of germination rate on MS medium after a week in culture. BA was required to enhance shoots multiplication. Woody Plant (WP) (Lloyd & McCown, 1981) medium gave better shoot multiplication for *R. culminicolum.*

Keywords: *Tetramolopium klossii, Deschampsia klossii, Papuacalia cartenszensis, Epilobium hooglandii, Gaultheria novoguinensis, Rhododendron correoides* and *Rhododendron culminicolum*, *in vitro* germination, shoot multiplication, endemic alpine and sub-alpine Papua plants

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Introduction

Plant biodiversity in Mount Jaya, Papua, Indonesia is unique, especially in high altitude areas where typically alpine and sub-alpine vegetation, the endemic vegetation, are found. The sub-alpine and alpine plants and vegetation of highland New Guinea are of great scientific interests and important for plant conservation because they are vulnerable to the climate changes (Johns *et al.*, 2006). Furthermore, many plants grown in Mt. Jaya are very important for local communities, such as Amungme people. The plant species have been subjected to ethnobotanic studies. Grasberg area of Mt. Jaya was important to local people before the mining activities, such as for animal hunting, harvesting vegetables and traditional medicines, and for spiritual or traditional ceremonies (Shea *et al.*, 2006). The mining activities disturbed the surround environment, therefore, conservation and re-vegetation are very much important to preserve the endemic species growing in their original ecosystem.

Seven species endemic to alpine and sub-alpine area grown at Mt Jaya, Papua, i.e. *Tetramolopium klossii* (Asteraceae), *Deschampsia klossii* (Poaceae), *Papuacalia cartenszensis* (Asteraceae), *Epilobium hooglandii* (Onagraceae), *Gaultheria novoguinensis* (Ericaceae), *Rhododendron correoides* (Ericaceae) and *Rhododendron culminicolum* (Ericaceae) are important for re-vegetation in the mining area of Grasberg and maintaining the biodiversity of endemic plants in their own habitat (Shea *et al.*, 2006). Of these species, some species are very useful for Amungme people. Stems of *D. klossii*, an extensive grass often dominates in open area

after mining, is used for traditional house walls, for bed and for a cord or rope. Leaves of *P. cartenszensis* are useful for cigarettes and antimalaria drugs. *G. novoguinensis* is used in the traditional worship ceremony of Amungme people. Women of Amungme use *Rhododendron* flowers and leaves to attract one particular man (Shea *et al*., 2006).

Plant tissue culture technique is one of alternative techniques for plant genetic conservation, which requires only small part of plants for plant multiplication and regeneration (George & Deberg, 2008). The environment for growing the plant can be easily adjusted for different species and explants in order to reach better plant development (Kozai & Xiao, 2006). In *in vitro* germination, seeds are germinated in glass or plastic jars or dishes containing an agar-based medium with important sugars and minerals for seed germination and plant growth. *In vitro* germination is the starting point and the crucial stage for plant micropropagation through multiple shoot formation. Therefore, it is important to find the nutritional composition of culture medium and the environmental conditions for optimum germination rates, multiple shoot development and plantlets performances (George & Deberg, 2008).

The aim of the research was to investigate the protocol for optimum *in vitro* germination and shoot multiplication of seven above-mentioned species endemic to alpine and sub-alpine area at Mt. Jaya, Papua by measuring germination rates and shoot multiplication. Reports on *in vitro* tissue culture of these species are very limited and the establishment of tissue culture has been done only for *T. klossii,* and *D. klossii* (Ermayanti *et al*., 2011 and 2012; Ermayanti & Mukhsia, 2012). Therefore, the protocol for *in vitro* germination and shoot multiplication gained from this research will be very useful for massive propagation of these species.

Materials and Methods

Plant materials. Seeds of seven sub-alpine and alpine species (*T. klossii, D. klossii, P. cartenszensis, E. hooglandii, G. novoguinensis, R. correoides* and *R. culminicolum*) were used in this experiment. Mature seeds were collected from Grasberg mining area in Mt. Jaya, Papua, Indonesia. All seeds were harvested from plants grown at 4,000-4,300 m above see level, collected in plastic bags, and dried in the open air. Seeds were sorted, cleaned and prepared for surface sterilization before being germinated on MS (Murashige & Skoog, 1962) medium.

Surface sterilization of seeds. Seeds from seven species were placed in plastic screen bags prior to surface sterilization. Seeds were washed with 25 ml of tap water containing 3 drops of liquid detergent and kept in the running water for at least 30 min. Seeds were, then, dipped in ethanol 70% for 1 min followed by soaking them in 1% of fungicide for about 10 min. Seeds were soaked in 1% of sodium hypochlorite for 10 min, and rinsed with sterile distilled water 3 times.

***In vitro* seed germination and shoot multiplication**. Seed germination was done on MS medium, containing 30 g/l of sucrose (Merck, USA) (for those species having high contamination problems, sucrose concentration was reduced to 20 g/l). The medium had no addition of any growth regulators, solidified with 8 g/l of agar (Caisson, USA) or 3 g/l of phytagel (Sigma, USA). The pH medium was adjusted to 5.8. Medium was sterilized using autoclave at temperature of 120°C, and pressure of 1 atm for 20 min. Aseptic seeds were removed from the screen bag and cultivated in petri dishes containing MS medium. Each petri dish contained 25-30 seeds. Each species had 6 replicates. All cultures were incubated in a culture room at temperature of 26-28°C provided with continuous light with light intensity of 800-1,000 lux. Percentage of germination and days when germination started were recorded.

Two to three weeks after germination, 40 seedlings of each species were transferred to a fresh MS medium and Woody Plant medium (WP; Lloyd & McCown, 1981), except for *P. cartenszensis* and *R. culminicolum*. Benzyl Adenine (BA) at 0.5 and 1.0 mg/l were used for shoot multiplication treatment. In *R. culminicolum*, callus was used as explants for shoot multiplication, since no shoots were obtained after seeds germination. All cultures were maintained in a culture room at the same condition as for that of seed germination. Multiple shoot formation was recorded at week 7 after seedling transfer.

Results and Discussion

In vitro seed germination

Seeds of *T. klossii* were started to germinate after 5-10 days in culture and showed high rate of germination up to 85.71% (Table 1; Figure 1). After germination, seedlings were developed into a single shoot and root after 2 weeks in culture. These results showed that medium and environmental temperature were suitable to induce *T. klossii* germination.

In MS medium solidified with agar, seeds of *D. klossii* had lower germination rate and needed longer period to germinate than seeds germinated on medium with phytagel (Table 1; Figure 2), and grew further (Figure 3). Besides type of gelling agent, different composition of the culture medium also affects germination rate as shown in *Zeyhera montana* (de Sousa *et al*., 1999).

P. carstenszensis seeds had low rate of germination (29.09%; Table 1; Figure 4). In nature, this plant is propagated from seeds. The seed availability in the originated area was scarce, because it is easily blown by the wind when they are mature due to its light weight and only a few of them remained on the seed stalks.

Seeds of *E. hooglandii* had high germination rate after 4-6 days (Table 1) and the seedling formed several nodes in 3 weeks after germination. The seedling stems etiolated and contained few leaves. At this stage, the seedlings were transferred to fresh medium.

Seeds of *G. novoguinensis* started to germinate 7-9 days after sowing (Figure 5; Table 1), However, several days after germination, most seedlings turned to yellowish, brown then died, therefore, the remaining seedlings were transferred to medium containing cytokinins for shoot multiplication experiments.

Table 1. Germination rates and of seven species endemic alpine and subalpine area of Mt. Jaya on MS medium

No	Name of species	Solidifying agent	Sucrose (g/l)	Germination rate (%)	Days started to germinate after sowing
1	*T. klossii*	8 g/l agar	20	85.71	5-10
2	*D. klossii*	8 g/l agar	30	33.33	6-10
	D. klossii	3 g/l phytagel	30	91.67	4-8
3	*P. cartenszensis*	3 g/l phytagel	30	29.09	14-22
4	*E. hooglandii*	8 g/l agar	20	96.5	4-6
5	*G. novoguinensis*	8 g/l agar	20	94.44	7-9
6.	*R. correoides*	3 g/l phytagel	30	86.67	15-23
7.	*R. culminicolum*	3 g/l phytagel	30	33.33	16-24

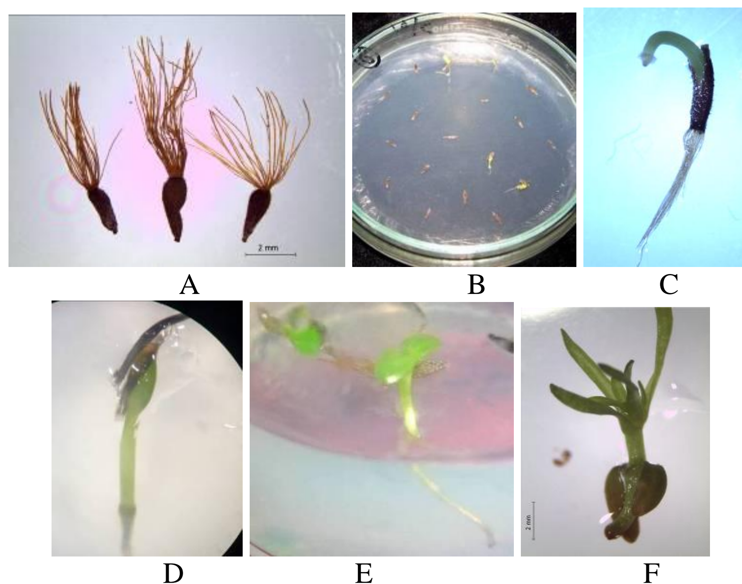
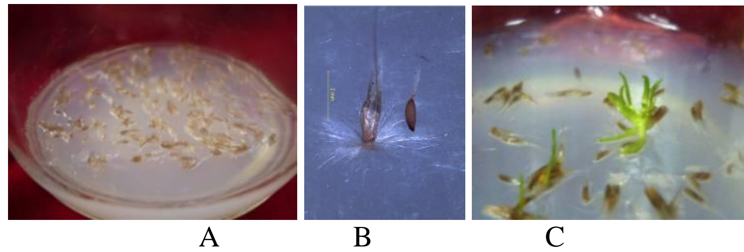
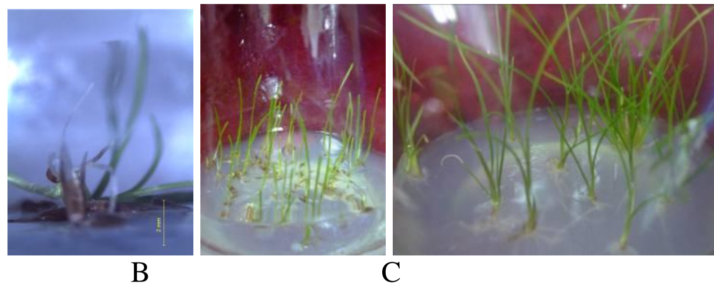


Figure 1. Seeds of *Tetramolopium klossii* (A) germinated on MS medium without plant growth regulators (B). Seeds started to germinate after 5-10 days in culture (C-F).



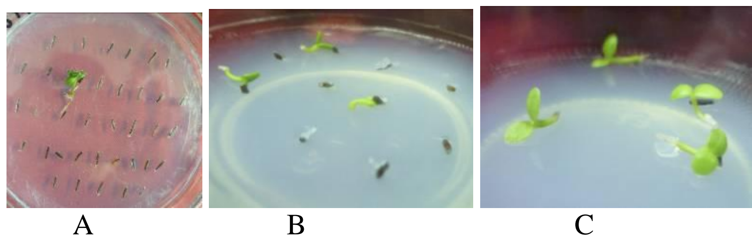
A B C

Figure 2. Seeds of *D. klossii* germinated on MS medium solidified with 8 g/l of agar without plant growth regulator, 1day (A and B) and 5 days (C) after germination.



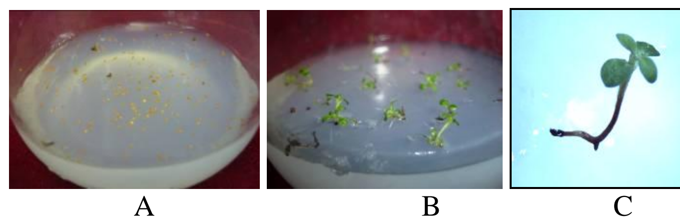
A B C

Figure 3. Seeds of *D. klossii* germinated on MS medium solidified with 3 g/l of phytagel without plant growth regulator, 5 days (A) and 7 days after germination.



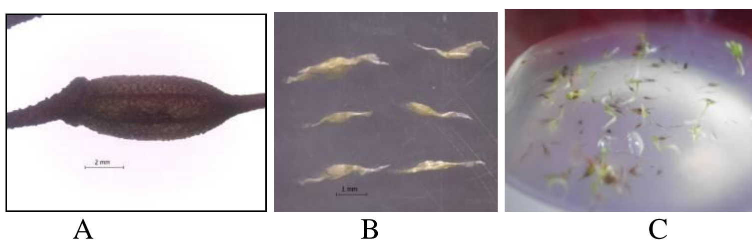
A B C

Figure 4. Seeds of *P. carstenszensis* germinated after 10 days (A and B) and after 12 days (C) cultured on MS medium.



A B C

Figure 5. *G. novaguineensis* seeds sown on MS medium (A) and germinated seeds after 10 days (B and C) in culture



A B C

Figure 6. *In vitro* seed germination of *R. correoides* on MS medium. A mature seed (A), a week (B) and 3 weeks (C) after germination.

Seeds of *R. correoides* started to germinate 2-3 weeks after sowing (Table 1 and Figure 6). Most seeds were germinated in good performances, however, the seedlings grew very slowly afterwards and some of them stopped to grow. Therefore the seedlings were transferred to a fresh medium containing cytokinins to induce the growth.

A B C D

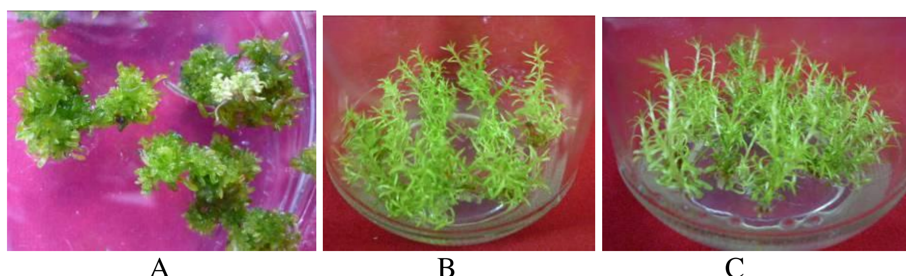
Figure 7. *In vitro* germination of *R. culminicolum* on MS medium on day 1 (A and B), and on day 7 (C and D)

After 2-3 weeks in culture, seeds of *R. culminicolum* started to germinate and the germination rate was low (Table 1). Some of them formed single shoot primordium then became brown at their tips, and died afterwards. Some others formed callus and germinated into small clumps (Figure 7). The growth of both callus and germinated seedlings were very slow. This may correlate to the nature of this species, which is a slow-growth plant. Further investigation to find the nutritional composition for accelerating the optimum growth is important.

Shoot multiplication

Figure 8 shows the development of *T. klossii* from small clumps with abundant of shoots and small leaves to form bigger multiple shoots. The addition of BA gave faster multiple shoot formation (Table 2). On MS medium containing 0.5 mg/l of BA, within 2 months, the multiple shoots were developed rapidly. Increase in BA concentration from 0.5 to 1.0 mg/l resulted in shoots with vitrified leaves (Table 2). Similar result was reported showing that BA at high concentrations (1.0 and 2.0 mg/l) gave low performance of the multiple shoots, having needle-like leaves and shoots were shorter than those grown in MS medium with BAP (0.1 and 0.5 mg/l) (Ermayanti *et al*., 2011).

In *D. klossii*, BA is efective to stimulate shoot multiplication. Formation of lateral shoots was faster when a single shoot was cultured on MS containing BA (Table 2). High number of leaf formation was observed on MS medium containing 0.5 mg/l of BA after 3 weeks. In addition, root formation has been observed in the shoot cultured on the same concentration of BA after 2 weeks (Figure 9). The increase of BA concentration to 1.0 mg/l did not reduce multiple shoot formation, but the shoots were shorter (Table 2). Ermayanti *et al.* (2012) reported that the addition of 2,4-D (2,4-deoxyribonucleic acid) induced more number of leaves and shoots of *D. klossii* compared to those on medium containing BA or kinetin or without plant growth regulators.
The single shoot of *P. carstenszensis* grew very slow on MS medium with no addition of plant growth regulators (Table 2), *i.e.* it had only 3-5 leaves (Figure 10A) and roots (Figure 10B) after a month of germination. Formation of lateral shoots were initiated after 6 weeks with 2-4 lateral shoots (Figure 10C), which were increased gradually and reached up to 2-6 shoots after 9 weeks. (Figure 10D). To increase the growth and enhance lateral shoots formation, the single shoot was transferred to WP medium containing BA. WP medium containing 0.5 mg/l of BA slightly increased the formation of multiple shoots (Table 2).

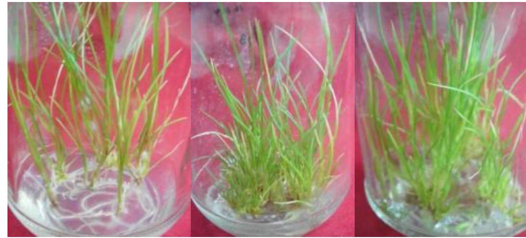


A B C

Figure 8. Multiple shoots of *T. klossii* after 4 (A) and 6 months (B and C) grown on MS medium containing 0.5 mg/l of BA.

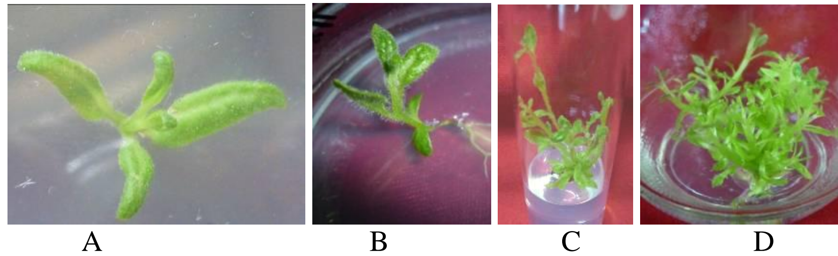
Table 2. Shoot growth of seven species endemic alpine and subalpine area of Mt. Jaya, Papua

No	Name of species	Basal medium	BA (mg/l)	Multiple shoot formation (%)	Growth response
1	*T. klossii*	MS	0	95.0	In 5 weeks the single shoot formed 3-4 multiple shoots having small needle-like leaves
		MS	0.5	100.0	In 3 weeks multiple shoots were formed, 2 weeks latter shoots reached 4-6 cm in height. Most shoots formed roots after 4 weeks in culture
		MS	1.0	100.0	In 4 weeks abundant of small shoots with vitrified leaves were formed
2.	*D. klossii*	MS	0	100.0	In 5 weeks the single shoot formed 3-7 shoots
		MS	0.5	100.0	In 3 weeks the single shoots started to form multiple shoots, and after 5 weeks each clump had 4-18 shoots
		MS	1.0	97.5	In 3 weeks the single shoot started to form new shoots, and in 6 weeks formed 3-7 shoots
3.	*P. cartenszensis*	MS	0	0	The single shoot was elongated, no multiple shoots formation
		MS	0.5	30.0	Shoots grew slow and in 6 weeks those shoots started to form multiple shoots
		MS	1.0	35.0	Shoot elongation and multiple shoot formation started 3-4 weeks after culture
		WP	0	40.0	In 3 weeks the single shoot started to form multiple shoots
		WP	0.5	40.0	Shoot elongation and multiple shoot formation started 3-4 weeks after culture
		WP	1.0	35.0	Shoot elongation and multiple shoot formation started 3-4 weeks after culture. In 9 weeks multiple shoots were formed 2-6
4.	*E. hooglandii*	MS	0	0	Shoot tips started to get brown in 2 weeks then died
		MS	0.5	0	In 3 weeks the singe shoot formed 5-9 nodes
		MS	1.0	5.0	In 3 weeks multiple shoots were formed, but died in 4 weeks
5.	*G. novoguinensis*	MS	0	0	No growth, shoots died in 3 weeks
		MS	0.5	0	No growth, shoots died in 3 weeks
		MS	1 .0	0	Shoots grew slow, in a month single shoots formed 3-4 leaves
6.	*R. correoides*	MS	0	0	No growth, shoots died in 3 weeks
		MS	0.5	0	In 3 weeks formed 2-3 leaves, then turned to brown gradually then died
		MS	1.0	0	No growth, shoots died in 3 weeks
7.	*R. culminicolum*	MS	0	0	No growth, callus died in 3 weeks
		MS	0.5	45.0	In 4 weeks proliferated callus developed into clumps of small shoots
		MS	1.0	55.0	In 4 weeks proliferated callus developed into clumps of small shoots
		WP	0	45.0	In 3 weeks proliferated callus developed into clumps of shoots, in 8 weeks multiple shoots were formed
		WP	0.5	80.0	In 3 weeks proliferated callus developed into clumps of shoots, in 8 weeks formed multiple shoots
		WP	1.0	82.5	In 3 weeks proliferated callus developed into clumps of shoots, in 8 weeks formed multiple shoots



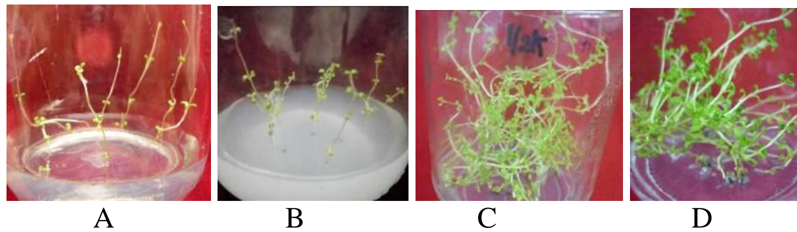
A B C

Figure 9. Multipe shoots of *D. klossii* grown for 2 weeks (A), 5 weeks (B), 7 weeks (C) on MS medium containing 0.5 mg/l of BA.



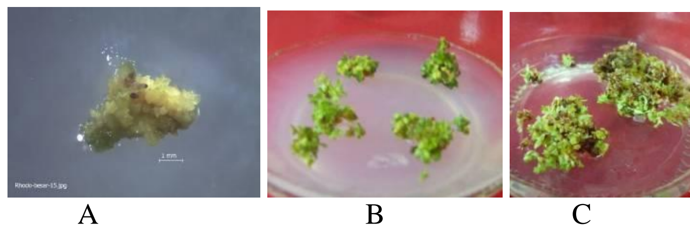
A B C D

Figure 10. Single shoot formed after germination on MS solid medium (A), *P. carstenszensis* formed roots in 1 month (B); then developed multiple shoots on MS medium containing 1.0 mg/l BA. Multiple shoots 6 weeks (C) and 10 weeks (D) after culture.



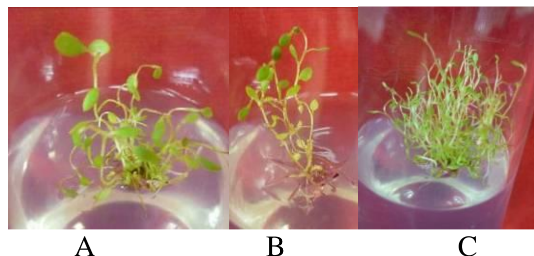
A B C D

Figure 11. Single shoots of *E. hooglandii* grown on MS medium after 7 days (A and B) and 21 days (C and D)



A B C

Figure 12. Callus of *Rhododendro culminicolum* developed into clumps of shoots on MS medium containing 1.0 mg/l of BA after 3 weeks (A), 4 weeks (B) and 5 weeks (C).



A B C

Figure 13. Multiple shoots of *Rhododendron culminicolum* on WP medium containing 0.5 mg/l of BA after 4 weeks (A and B) dan 5 weeks (C).

Multiple shoot formation of *E. hooglandii* was not observed on both MS medium without BA and with 0.5 mg/l of BA. The presence of 1.0 mg/l BA could induce multiple shoots

formation, however, those shoots did not continue to develop (Table 2). Those shoots could elongate with small stems and leaves after 3 weeks in culture (Figure 11), then, showing gradual symptoms of yellowing and browning on shoot tips before they died 2 weeks afterwards. On the other hand, the presence of BA stimulated callus formation at the end-cut of *E. hooglandii* stems, although these turned brown after 3 weeks in culture. These indicated that *E. hooglandii* may require different nutritional composition for each step of the development. Such a different nutritional requirement in each developmental stage has been observed as those in *Paphiopedilum* after the germination (Tay *et al*., 1988).

The young seedlings of *G. novaguineensis* and *R. correoides* were easily to get brown in MS medium without plant growth regulators. Addition of BA at concentrations of 0.5 and 1.0 mg/l showed no induction on shoot development and multiple shoots formation (Table 2).

The development of *R. culminicolum* callus was better when they were cultured on WP medium compared to MS medium. In addition, the formation of multiple shoots was faster and higher on WP medium than that on MS medium. The addition of BA on WP medium induced higher multiple shoots formation than those on MS medium containing similar BA concentration (Table 2; Figure 12 and 13).

Further investigation to find the basal medium and plant growth regulators which could enhance *R. culminicolum* optimum growth should be performed. In other species of *Rhododendron*, *R. catawbiense*, different combination of growth regulators at different concentration were required to induce the growth of explants and to promote their growth to the next developmental stages (Tomsone & Gertnere, 2003).

In conclusion, seven sub-alpine and alpine plant species namely *T. klossii, D. klossii, P. cartenszensis, E. hooglandii, G. novoguinensis, R. correoides* and *R. culminicolum* could be germinated *in vitro* on MS medium, although they were germinated under different temperature as in their original ecosystem, 26-27°C. Most of these species could develop into multiple shoots in MS medium suplemented with BA. WP medium is more suitable for *R. culminicolum* shoot development. However, the nutritional composition contained in the germination medium is dependent to the species, therefore, our research could be useful as the basic information for designing the protocol for *in vitro* propagation of seven endemic species of Grasberg area of Mt. Jaya, thus, will support their conservation.

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References

Anderson, W. C. (1980). Tissue culture propagation of Red and Black Raspberries, *Rubus idaeus* and *R. occidentalus*. *Acta Hortuculturae*, 112-113.

de Sausa, P. B. L., de Santana, J. R. F., Crepaldi, I. C., & A. R. Lima. (1999). Germination *in vitro* of seeds of a threatened arboreal species in the Municipal district of Abaira (AB). *Sitientibus*, *20*: 89-99.

Ermayanti, T. M. & Mukhsia, A. (2012). Application of tissue culture for the production of native plant species to accelerate the alpine reclamation programme at PT Freeport Indonesia. *Proceedings of the 7th International Conference on Mine Closure*, Brisbane, 25-27 September 2012, pp. 507-516.

Ermayanti, T. M., Al Hafiizh, E., Martin, A. F., Mandessy, A., Hermono, A., & Mukhsia, A. (2012). Micropropagation of *Deschampsia klossii* Ridley-an endemic plant species grows in subalpine and alpine of New Guinea. *Proceedings of the 5th Indonesia Biotechnolgy Conference*, Mataram, 4-7 July 2012, pp. 252-262.

Ermayanti, T. M., Al Hafiizh, E., Martin, A. F., Mandessy, A., Hermono, A., & Mukhsia, A (2011). Micropropagation of *Tetramolopium klossii*, endemic alpine and sub-alpine plant species to New Guinea. *Annales Bogorienses*, *15*(2): 9-15.

George, E. F. & Deberg, P. C. (2008). *Micropropagation: Uses and methods*. In: George, E. F. *et al*., Plant Propagation by Tissue Culture. 3rd Edition. Vol. 1. The Background, Springer, The Netherlands, pp. 29-64.

Johns, R. J., Edwards, P. J., Utteridge, T. M., & Hopkins, H. C. F. (2006). A Guide to the Alpine and Subalpine Flora of Mount Jaya. *Kew Publishing*, Royal Botanic Garden, Kew, UK, 653 p.

Kozai, T. & Xiao, Y. (2006). *A commercialized photoautotrophic micropropagation system*. In: Gupta, S. D. & Ibaraki, Y. (Eds). Focus on Biotechnology. Vol. 6. Plant Tissue Culture Engineering, Springer, The Netherlands, pp. 355-370.

Lloyd, G. & McCown, M. (1981). Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *International Plant Propagator's Society. Proceedings*, *30***:** 421-427.

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia. Plantarum*, *15*: 473-497.

Preece, J. E. & Imel, R. M. (1991). Plant regeneration from leaf explants of *Rhododendron* 'PJM hybrids'. *Scientia Horticulturae*, *48*: 159-170.

Shea, G. A., Sarisa, W., & Uamang, P. (2006). *Ethnobotany of subalpine and alpine plants on Mt. Jaya*. PT. Hatfindo Prima. Indonesia, pp. 38.

Shea, G. A., Sarosa, W., & Zonggonau, D. (2000). Native trees and shrubs for the ecological rehabilitation and ecological restoration of overburden deposits and damaged ecosystem in the subalpine and alpine zones on Mt, Jaya in the PT Freeport Indonesia Contract of Work Mining Area, Mimika Regency, Papua, Indonesia. *Restoration Ecology Research Bulletin, 7*: 1-4. PT. Hatfindo Prima. Indonesia.

Shevade, A. & Preece, J. E. (1993). *In vitro* shoot and floral organogenesis from stamen explants from a *Rhododendron* RJM group clone. *Scientia Horticulturae*, *56*: 163-170.

Tay, L-J., Takeno, K., & Hori, Y. (1988). Culture conditions suitable for *in vitro* seed germination and development of seedlings in *Paphiopedilum*. *Journal of Japan Society for Horticulture Sciences*, *57*(2): 243-249.

Tomsone, S. & Gertnere, D. (2003). *In vitro* shoot regeneration from flower and leaf explants in *Rhododendron*. *Biologia Plantarum*, *46*(3): 463-465.

Tomsone, S., Gertnere, D., & Novikova, D. (2004). The influence of thidiazuron on shoot regeneration and proliferation of rhododendrons *in vitro*. *Acta Universitastis Latviensis, Biology*, *676*: 239-242.