Micropropagation of *Tetramolopium klossii* (Moore) Mattfeld, endemic subalpine and alpine plant species to New Guinea

Tri Muji Ermayanti[1*], Erwin Al Hafiizh[1], Andri Fadillah Martin[1], Ary Mandessy[2], Arief Hermono[2] and Andi Mukhsia[2]

[1]Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI), Indonesia
[2]PT Freeport Indonesia, Indonesia

Abstract

The protocol of micropropagation of *Tetramolopium klossii* (Moore) Mattfeld through adventitious shoot proliferation was established. This is the first report of *T. klossii* tissue culture. Seeds were surface-sterilized by dipping them in 10% of ethanol for 1 min followed by soaking in 1% of sodium hypochlorite for 10 min. The sterile seeds were germinated on MS medium solidified with 8 g/l of agar, supplemented with 20 g/l of sucrose. Two-month old seedlings formed some shoot clumps on MS solid medium without addition of any plant growth regulators. Small clumps containing 4-6 adventitious shoots were used for shoot proliferation on MS solid medium containing 0; 0.1; 0.5; 1.0 and 2.0 mg/L of BAP (benzylaminopurine) or kinetin. The results showed that medium MS without cytokinins (control treatment), and the addition of 0.1 or 0.5 mg/L of BAP was more effective for shoot proliferation compared to the medium with addition of BAP at higher concentration and the addition of kinetin. In further subcultures, multiple shoots grown on MS medium without cytokinins was more vigorous than that of shoots grown on other media, so that the shoots were ready for rooting. Medium containing 0.5 mg/L of BAP was the best for maintaining multiple shoot formation. Shoots formed roots on MS medium containing half strength of macro nutrients.

Keywords: *Tetramolopium klossii*, micropropagation, Murashige and Skoog medium, shoot multiplication

---

[*]Corresponding author
Jl. Raya Bogor Km. 46, Cibinong 16911, Indonesia
Tel. +62-21-8754587, Fax. +62-21-8754588
E-mail. tmermayanti@hotmail.com

Introduction

Tetramolopium is a genus of shrubby to suffrutescent perennial Compositae (Asteraceae) known originally from New Guinea, the Cook Islands and Hawaiian Islands (Lowrey & Crawford, 1985). The genus of *Tetramolopium* consists of 36 described species occurring in these three regions (Lowrey, 1990), and *T. klossii* is endemic to Mount Jaya in Papua on subalpine area (Shea *et al*., 2000; John *et al.*, 2006). The Hawaiian taxa only consists of 11 species, and some of them are rare, extinct, and listed as endangered species (Laven *et al.* 1991; Falkner *et al*., 1997).

Research on this genus is very limited. First report on taxonomy of genus *Tetramolopium* was published by Sherff (1934), and rediscovery of *T. arenarium* was reported by Douglas & Shaw (1989). Genetic biodiversity of Hawaian *Tetramolopium rockii*, *T. humile* and some other species has been reported by Whitkus (1998), Whitkus *et al*. (2000), and Okada *et al*. (1997; 2000). An experiment on germination of *T. arenarium*, *T. consanguineum* and *T. lepidotum* was reported by Falkner *et al.* (1997) and a new species endemic to Mitiaro, Cook Islands, *T. mitiaroense* was described by Lowrey *et al.* (2005).

Tetramolopium klossii is a shrub or shrublet growing to a maximum height of 14 cm, with small hairy leaves along its stems, with white flower. The plant is found to grow at Mount Jaya in Papua and Papua New Guinea at sub-alpine and alpine zones (Shea *et al*., 2000). At Grasberg, this species are found to grow at open shrub-land communities, grasslands, edges of river banks, on rocks, on rocky slopes and other area at the altitude of 2800-4200 m above the sea level (John *et al.*, 2006). In nature, this plant is propagated by seeds, which are usually dispersed by the

wind. The seed is tiny, light, easily to disperse right after the florets are dry. Local people in Papua use this plant for bedding when they travel of hunt at high elevation area, and use as a fuel for fire.

Only a few publications reported on *T. klossii* as its distribution is in very limited area, only at elevation of 2800-4200 m above sea level in Papua. Research on *T. klossii* at Grasberg Mine area on Mount Jaya showed that this species is often found at surrounding mining area, therefore, this species is reported to be potential for the rehabilitation of overburden deposits. Research on ecological characteristic, botanical description, distribution, its natural propagation, and its potential for the ecological rehabilitation of overburden deposits has been recorded (Shea *et al*., 2000). However, there is no report on their conservation and mass propagation yet.

Tissue culture technique offers a tool for a large scale production genetically identical plant for many species and for *in vitro* conservation of endemic and rare plant species. This technique has several advantages over the conventional propagation method. Using tissue culture technique, we can provide transplants all over the time, independent to seasonal conditions, and mass production will be done easier because it is carried out in a laboratory. This technique also provides transplants for *ex situ* conservation and maintenance of *in vitro* germplasm so that the bioprospection of targeted species may be investigated. This technique will be beneficial for *T. klossii* to maintain its biodiversity in Indonesia as well as to provide transplants by mass micropropagation for reclamation purposes.

The mass production of transplants is very important to support reclamation program at Grasberg mining area. Grasberg reclamation activities is currently implementing the plan period of 2009-2013. In 2010 the total area reclaimed was 62.34 ha (targeted was 50 ha). Planting this area required 152,950 clumps of transplants. Currently, seeds and seedlings are collected from DOM area as natural succession forest, subdivide and pot the collected plants, allow them to grow in the nursery area, then transplant them on the overburden stockpiles. The seed capacity will not be adequate for the reclamation program needs. Therefore, establishment of tissue culture for mass micropropagation will contributes significantly to the reclamation program. Tissue culture development will also become a means of conservation of the limited native grasses, shrubs including *T. klossii*, and ferns available within Grasberg and its surrounding areas. The objective of this research was to provide a protocol for micropropagation of *T. klossii* through adventitious shoot proliferation.

Materials and Methods

Surface sterilization of seeds. Seeds of *T. klossii* were collected from Grasberg mining area in Papua. In order to obtain the mature seeds, the open-dry florets containing mature seeds were covered by a plastic bag to prevent the dispersion of the seeds. Seeds were then isolated from their hairs, put inside plastic bags prior to surface sterilization. Screen bag containing seeds were washed with tap water containing 3 drops of liquid detergent and left them in the running water for at least 30 min. Seeds were, then, dipped in 70% of ethanol for 1 min followed by soaked them in 1% of fungicide for about 10 min.. Seeds were then soaked in 1% of sodium hypochlorite for 10 min, and rinsed with 3 changes of sterile distilled water.

***In vitro* seed germination.** Aseptic seeds were removed from the screen bag then placed in petri dishes containing MS medium (Murashige & Skoog, 1962) solidified with 8 g/L of agar, supplemented with 20 g/L of sucrose with no addition of plant growth regulators. The pH medium was adjusted to 5.8. Medium was sterilized using autoclave at temperature of 120^{o}C, pressure of 1 atm for 20 min. Each petri dish had 25-20 of seeds. All cultures were incubated in a culture room at temperature at 28^{o}C provided with continuous light.

Shoot proliferation. Shoot primordial formed on MS medium was transferred to MS solid medium containing 0.5 mg/L BAP to obtain stock culture for the source of shoot multiplication experiment. A clump of shoots containing 4-6 shoots with height of about 0.3-0.5 cm length of each shoot was used as explants for shoot proliferation. Explants were placed on MS containing 0.1; 0.5; 1.0 and 2.0 of benzylaminopurine (BAP) or kinetin. The

culture medium was solidified with 8 g/L of agar, with addition of 20 g/l sucrose. The pH of the medium was adjusted to 5.8 by the addition of 1 N KOH or 0.1 N HCl before autoclaving at 121°C for 15 min at 1 atm. Each treatment had 20 replicates. All cultures were incubated in an incubation room at 26-28ºC and under continuous light provided by cool white influorescent tubes. Subculture to the same medium composition was done every 4 weeks.

Root induction. Shoots at 2.0-2.5 cm length was grown on MS solid medium containing half strength of MS macro nutrients. Twenty shoots were subjected for root induction. Cultures were incubated at the same incubation room as that of shoots for proliferation.

Observation. The parameters taken in this research were percentage of seed germination, formation of adventitious shoots, callus formation as well as the growth response at each treatment after 8 weeks in culture. Growth response was recorded qualitatively by looking at the formation and development of adventitious shoots at each medium treatment. Percentage of rooting was also recorded. Data were analyzed by Anova at 5% level, and advanced test used Duncan's Multiple Range Test (DMRT) at 5% level.

Results and Discussion

In vitro seed germination

After 5-10 days in culture, seeds of *T klossii* were germinated and developed into single shoot and root. After 2 weeks seedlings grew further to form more leaves. Figure 1 shows the *in vitro* germination of the seeds. More than 80% of mature seeds germinated on the culture medium. After a month, seedlings formed a number of little leaves, and they formed clumps of shoots. Each clump consisted many shoots with small leaves. The clumps were then subjected for shoot proliferation on medium with addition of benzylaminopurine (BAP) or kinetin at several concentrations. After several weeks clumps of shoots grew slowly and leaves started to change from green to yellow, so that clumps were transferred to MS medium containing 0.5 mg/L BAP to grow better.

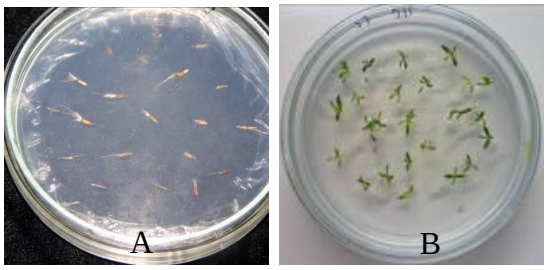

Figure 1. *In vitro* seeds germination of *Tetramolopium klossii*. A. Seeds cultured on MS medium with no addition of plant growth regulators. B. A 2-week old seedlings

Shoot proliferation

After 3-4 weeks shoot clumps showed distinctive increase in growth when they were cultured on MS medium supplemented with BAP after culturing from MS solid medium with no addition of plant growth regulators. Fig 2 shows shoot proliferation and multiplication stages of *T. klossii* on MS solid medium containing 0.5 mg/L of BAP. The shoots were maintained on this medium for source of explants to investigate the best medium for shoot proliferation. Cytokinin BAP at 0.5 mg/L was also induced multiple shoots of *Spilanthes acmella* (How & Keng, 2003). The addition of BAP to MS medium is also common to increase multiple shoot development for many plant species such as *Tacca chantrieri* (Charoensub *et al*., 2008) and *Typhonium flagelliforme* (Nabakht *et al.*, 2009). The role of cytokinin such as BAP in plant tissue culture is important l to induce cell division of plants, to break the lateral bud dormancy, to enhance the elongation of lateral buds and to increase the adventitious shoot formation (Gaspar *et al*., 1996, Gaspar *et al*., 2003).

Cytokinin BAP was more effective to enhance the *in vitro* growth of *T. klossii* compared to kinetin. Table 1 shows the ability of explants to induce shoot proliferation in response to the addition of different level of BAP or kinetin after 8 weeks on the culture. Table 2 describes the growth responses of multiple shoot development after treatment with BAP or kinetin at different levels. After 3 weeks in culture, all explants were developed to form adventitious shoots in all medium. At low concentration of BAP or kinetin multiple shoots formed roots. Roots were more on the control MS medium without addition of plant growth regulator. Callus was only found at the medium containing kinetin at 0.5 mg/L.

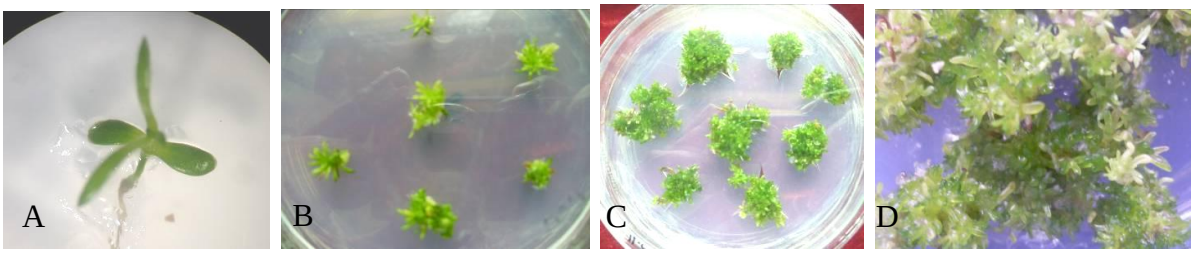

Figure 2. Development of shoot proliferation f *Tetramolopium klossii*. A. Single seedling after 2 weeks germination on MS medium without any plant growth regulators; B. Clumps of shoots after 4 weeks on MS medium without any plant growth regulators; C. Clumps of shoots after 4 weeks on MS medium containing 0.5 mg/L BAP; D. Clumps of shoots after 4 weeks on MS medium containing 0.5 mg/L BAP

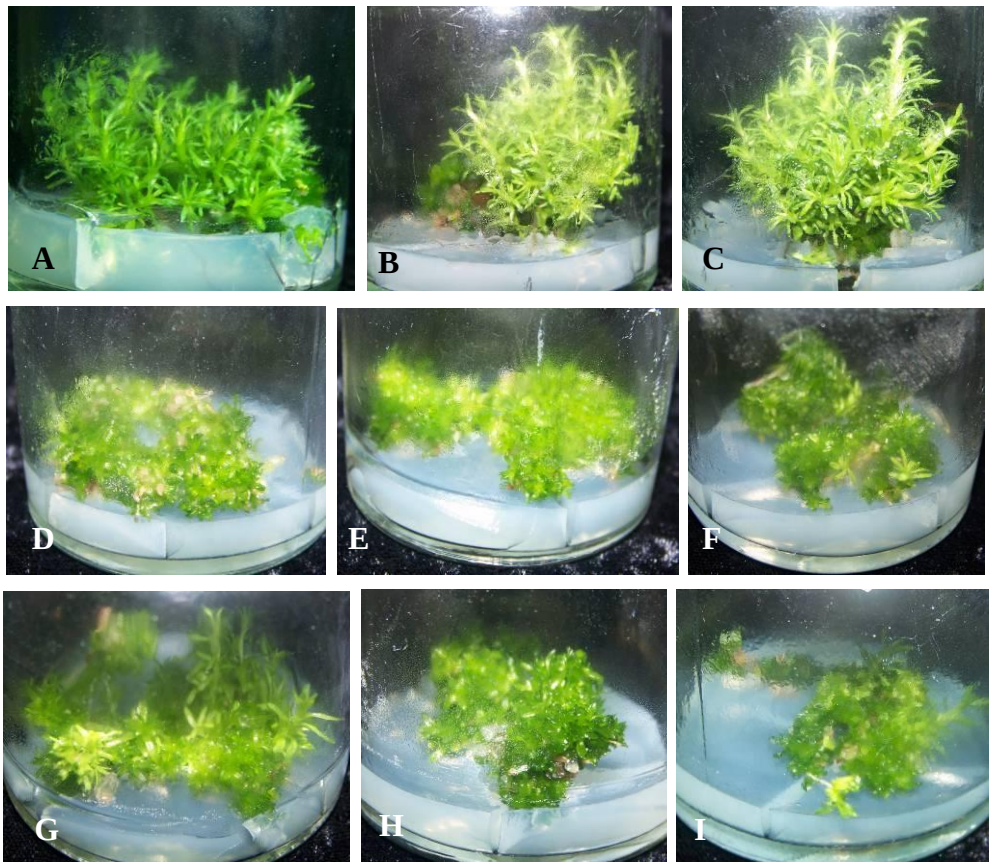

Figure 3. Shoot proliferation of *Tetramolopium klossii* after 8 weeks cultured on MS solid medium containing different levels of cytokinins. A. with no cytokinin; B. with 0.1 mg/L BAP; C. with 0.5 mg/L BAP; D. with 1.0 mg/L BAP; E. with 2.0 mg/L BAP; F. with 0.1 mg/L kinetin; G. with 0.5 mg/L kinetin; H. with 1.0 mg/L kinetin; I. with 2.0 mg/L kinetin

Table 2 indicates that growth response of shoot clumps was better when they were cultured on MS medium containing BAP compared to that of shoots cultured on the medium containing kinetin. Fig 3 represents the growth differences among the shoots after they were cultured for 8 weeks. Addition of BAP at low concentration (0.1 and 0.5 mg/L) gave the best growth in terms of having normal leaves, grew faster, with no or little vitrified leaves, compared to the use of BAP at higher concentrations (1.0 and 2.0 mg/L (Table 2). Therefore, MS solid medium containing 0.1 or 0.5 mg/L of BAP was suitable to maintain shoot multiplication for shoots production. At low concentration of BAP (0.1 and 0.5 mg/L) some shoots formed roots after 5-6 weeks in culture. Root formation also occurred on *Spilanthes acmella* at the same concentration (How & Keng, 2003). This may suggest that after several weeks, BAP at low concentrations may have depleted. With no BAP root formation may occur.

The addition of kinetin gave poor growth, abnormal as well as vitrified leaves. At low concentration of kinetin gave only few normal leaves, however, when the concentrations were increased, growth was slower and more leaves were abnormal. Fig 3 also shows that shoots had better performance when they were cultured on MS control medium as well as on the medium supplemented with BAP at concentration of 0.1 and 0.5 mg/L. Shoots had more vigorous performance and looked healthier. This performance is suitable for root induction. The number of multiple shoots could not be recorded quantitatively since abundant leaves and shoots were formed in the medium.

Table 1. Shoot proliferation of *T. klossii* after 8 weeks cultured on MS medium containing BAP or kinetin

BAP (mg/L)	Kinetin (mg/L)	Viability (%)	Callus (%)	Adventitious shoot formation (%)	Root (%)	Note
0		100	0a	100	40e	Number of adventitious shoot per explants could not be recorded due to high number of leaves formed per clumps as seen on Fig.3
0.1		100	0a	100	30d	
0.5		100	0a	100	20c	
1.0		100	0a	100	0a	
2.0		100	0a	100	0a	
	0.1	100	0a	100	10b	
	0.5	100	10b	100	10b	
	1.0	100	0a	100	0a	
	2.0	100	0a	100	0a	

Note: Value followed by the same letter on the same column is not sugnificantly different at P. values of 0.05 according to Duncan's Multiple Range test

Table 2. Growth response of *T. klossii* cultured on MS medium containing different type and concentration of cytokinins. Data were presented after 8 weeks growth on the treated medium

Cytokinin (mg/L)	Growth response
No cytokinin	Small shoot-clump developed bigger, formed more lateral shoots. After 4 weeks shoots started to show normal leaves, vigorous, no vitrified shoots were found. Some shoots formed roots after 5-6 weeks in culture.
BAP (0.1)	Shoots formed more multiple shoots, then started to form normal leaves after 4-5 weeks in culture. Only very little vitrified leaves were found. Roots were started to grow after 5-6 weeks in culture.
BAP (0.5)	Shoots formed multiple shoots and grew faster than that of other cultures grown on different media. This medium was the best for shoot multiplication. Only very little vitrified leaves were found. After 4 weeks in culture, shoot formed normal leaves and formed roots.
BAP (1.0)	Multiple shoots were formed, but shoots grew slowly and formed vitrified leaves.
BAP (2.0)	Same as growth on MS medium containing 1.0 mg/L of BAP. Multiple shoots were formed, but shoots grew slowly and formed vitrified leaves. Leaves were small and abnormal.
Kinetin (0.1)	Growth was very slow. Only few shoots formed normal leaves, some were vitrified. Normal shoots formed roots.
Kinetin (0.5)	The growth was similar to shoots cultured in the medium containing 0.1 mg/L of kinetin. Only few normal shoots with roots were found. The growth was slow. Some shoots were vitrified. Some calli were found at the base of clumps
Kinetin (1.0)	Growth was also very slow. No normal shoots were found, most of cultures were vitrified, leaves were small, abnormal with the leaf color was dark green.
Kinetin (2.0)	Growth response was similar to that of shoots grown on the medium containing 1.0 mg/L of kinetin but with more vitrified shoots.

This research showed that BAP at high concentrations (1.0 and 2.0 mg/L) resulted in low performance of the multiple shoots. Shoots were shorter than that of shoots grown at MS medium with addition of lower concentration of BAP (0.1 and 0.5 mg/L).

Various studies had also showed that high concentration of cytokinin generally inhibit multiple shoot formation as shown at *Spilanthes acmella* (How & Keng, 2003); and *Helianthemum inaguae* (Lopez *et al*., 2006).

The effectiveness of type of cytokinins (BAP or kinetin) and their concentration is very much species dependant. Cytokinin BAP and kinetin alone or in combination with auxins (NAA or 2,4-D) added the culture medium gives different response to the formation of multiple shoots in different plant species. In *Gladiolus grandiflorus*, addition of BAP (at 1, 2 or 4 mg/L) alone or in combination with auxins (NAA or IAA) was more effective to enhance multiple shoot formation compared that of kinetin alone or in combination with auxins at the same concentrations (Priyakumari & Sheela, 2005). At *T. klossii*, higher concentration of BAP reduced growth and produced more vitrified shoots. In this study, BAP was more effective rather than kinetin. The similar result was also found for micropropagation of kiwi fruit, and BAP at 0.5 mg/L was also the best for shoot proliferation (Akbas *et al*., 2007).

Root formation

Shoots of *T. klossii* formed some roots in the shoots proliferation medium containing low level of both BAP and kinetin (at 0.1 and 0.5 mg/L) and on the medium with no addition of plant growth regulators (Table 1). However, roots grew slowly and did not produce lateral roots. Vigorous shoots formed normal roots on MS solid medium containing half strength of MS medium. More than 80% of shoots formed roots. The use of MS medium containing half strength of MS medium was also common for some species for root induction and root development. Reduction of the level of macronutrient is needed to support root initiation and cell elongation for root development. After rooting in this medium, *T. klossii* plantlets were ready for their acclimatization steps. Fig. 4 indicates the shoots cultured after 4 weeks before forming roots (Fig. 4A), and shoots formed roots after 7 weeks in culture (Fig. 4B).

In many species, the use of MS medium or reduction of half strength of its macronutrients without addition of auxins is effective for rooting. In *Rosa damacena*, half strength of MS medium was used for rooting (Nikbakht *et al*. 2007), ¼ strength of MS medium was the best for rooting of *Rosa hybrida* (Nak-Udom *et al.*, 2009). However, in some other species addition of auxins on MS medium enhance root formation. In *Gladiolus grandiflorus*, NAA at 1 mg/L or IBA at 2 mg/L increased root number of roots more than 20 times higher compared to the control medium treatment MS with no addition of auxins. IAA was less effective than NAA or IBA (Priyakumari & Sheela, 2005). Auxin IBA was also effective for *Madhuca latifolia* root induction (Bansal & Chibbar, 2000).

Micropropagation is very useful for *T. klossii* to maintain its biodiversity as this species is endemic to Papua grown only at alpine and subalpine area, and to provide technique for mass production of transplants for reclamation. *In vitro* plants could also be utilized for further study such as *in vitro* conservation, phytochemistry, secondary metabolites, and genetic engineering of this plant.

Acknowledgements

The authors would like to thank Prof. Dr. Ir Bambang Prasetya and Dr. Puspita Lisdiyanti for their support to this project, and Evan Maulana for the medium preparation. We also would like to thank PT Freeport Indonesia staff for their support to this work. This work was financially supported by PT Freeport Indonesia under Services Agreement TP 1000198-001 Date: 28 June 2010; Amendment: 24 March 2011, Job Code: Granal; CR 100589-001.

References

Akbaş, F. A., C. Işikalan, S. Namli, & D. Başaran. 2007. Micropropagation of Kiwi fruit (*Actinidia deliciosa)*. Intl J Agric Biol 9: 489-493.

Bansal, Y. K., & T. Chibbar. 2000. Micropropagation of *Madhuca latifolia* Macb. trough nodal culture. Plant Biotechnol 17: 17-20.

Charoensub, T., D. Thiantong, & S. Phansiri. 2008. Micropropagation of Bat Flower plant, *Tacca chantrieri* Andre. Kasesart J (Nat Sci) 42: 7-12.

Douglas, P. P., & R. B. Shaw. 1989. Rediscovery of *Tetramolopium arenarium* subsp. *arenarium*

var. *arenarium* (Asteraceae: Asterae) on the Pohakuloa training area, Hawaii. Ann Miss Bot Gard 76: 1182-1185.

Falkner, M. R., R. D. Laven, & G. H. Aplet. 1997. Experiments on germination and early growth of three rare and endemic species of Hawaiian *Tetramolopium* (Asteraceae). Biol Conserv 80: 39-47.

Gaspar, T. H., C. Kevers, O. Faivre-Rampant, M. Crevecoeur, C. Penel, H. Greppin, & J. Dommes. 2003. Changing concepts inplant hormone action. *In vitro* Cellular and Developmental Biology Plant 39: 85-106.

Gaspar, T. H., C. Kevers, C. Penel, H. Greppin, D. M. Reid, & T. A. Thorpe. 1996. Plant hormones and plant growth regulators in plant tissue culture. *In vitro* Cellular and Developmental Biology Plant 32: 272-289.

How, A. B. 7 C. L. Keng. 2003. Micropropagation of *Spilanthes acmella* L., a bio-insecticide plant, through proliferation of multiple shoots. J Appl Hort. 5: 65-68.

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

Laven, R. D., R. B. Shaw, P. P. Douglas, & V. E. Diersing. 1991. Population structure of the recently rediscovered Hawaiian shrub *Tetramolopium arenarium* (Asteraceae). Ann Miss Bot Gard 78: 1073-1080.

López, I. S., F. V. González, & J. C. Luis. 2006. Micropropagation of *Helianthemum inaguae*, a rare and endangered species from the Canary Islands. Bot. Macaronesica. 26: 55-64.

Lowrey, T. K. 1990. *Tetramolopium* Nees. In: Wagner WL, Herbst DR, Sohmer SH (eds) Manual of the flowering plants of Hawaii. Bishop Museum Press, Honolulu. Pp 361-369.

Lowrey, T. K., & D. J. Crawford. 1985. Allozyme divergence and evolution in *Tetramolopium* (Compositae: Asteraceae) on Hawaiian Islands. Systematic Botany 10: 64-72.

Lowrey, T. K., R. Whitkus, & W. R. Sykes. 2005. A new species of *Tetramolopium* (Asteraceae) from Mitiaro, Cook Islands: Bibliography, phylogenetic relationships and dispersal. Systematic Botany 30: 448-455.

Murashige T, & F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Nak-Udom, N., K. Kanchanapoom, & K. Kanchanapoom. 2009. Micropropagation from cultured nodal explants of rose (*Rosa hybrida* L. cv. 'Perfume Delight'). Songklanakarin J. Sci. Technol. 31: 583-586.

Nikbakht, A., M. Kafi, M. Mirmasoumi, & M. Babalar. 2005. Micropropagation of Damask Rose (*Rosa damascena* Mill.) cvs. Azaran and Ghamsar. Intl J Agric Biol 7: 535-538.

Nobakhi, G. M., M. A. Kadir, & J.Stanias. 2009. *In vitro* mass propagation of *Typhonium flagelliforme* as affected by plant growth regulators. African J Biotech 8: 6840-6843.

Okada, M., T. K. Lowrey, & R. Whitkus. 2000. Quantitatively morphological variation in *Tetramolopium* (Asteraceae) in Hawaii and the Cook Islands. Plant Syst Evol 221: 1-13.

Okada, M., R. Whitkus, & T. K. Lowrey. 1997. Genetics of adaptive radiation in Hawaiian and Cook Islands species of *Tetramolopium* (Asteraceae: Astereae). I. Nuclear RFLP marker diversity. Amer J Bot 84 (1236-1246).

Priyakumari, I., & V. L. Sheela. 2005. Micropropagation of gladiolus cv. 'Peach Blossom' through enhanced release of axillary buds. J Trop Agric 43: 47-50.

Shea, G. A., W. Sarosa, & D. Zonggonau. 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. No. 7. PT Hatfindo Prima. Indonesia.

Sherff, E.E. 1934. A study in the genus *Tetramolopium* Nees (Family: Compositae). Bot Gaz 95: 498-502.