African Journal of Biotechnology Vol. 9(14), pp. 2062-2068, 5 April, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

In vitro multiplication of the rare and endangered slipper orchid, *Paphiopedilum rothschildianum* (Orchidaceae)

Chyuam-Yih Ng[1], Norihan Mohd. Saleh[1]* and Faridah Qamaruz Zaman[2]

[1]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.
[2]Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia.

Accepted 11 February, 2010

Paphiopedilum rothschildianum **is an endangered orchid species endemic to Mount Kinabalu, Sabah, and Malaysia. The vegetative propagation of this plant has always been restricted due to its slow growth and maturation rates. Thus, an *in vitro* tissue culture technique was explored in order to overcome this limitation. In this study, clonal propagation of *P. rothschildianum* was achieved through *in vitro* formation of multiple shoots from stem nodal and single shoot explants cultured onto half-strength Murashige and Skoog medium. The responses of the explants to the presence of different types of organic nitrogen additives viz. casein hydrolysate, peptone and tryptone-peptone (in amount of 0.5, 1.0 and 2.0 g/l) in the culture medium were also evaluated. The addition of these organic nitrogen additives into the basal medium slightly enhanced the number of multiple shoots formed on both types of explants when compared to additive-free MS medium. After 16 weeks of culture, an average of 2.9 shoots per stem nodal explant and 2.8 shoots per single shoot explant were obtained on half-strength MS medium supplemented with 1.0 g/l peptone and 2.0 g/l tryptone-peptone, respectively. All the newly-formed shoots were divided into single plantlets and subcultured onto similar respective medium. After an additional 12 weeks of culture on the same medium, plantlets with 3 - 4 roots were acclimatized and transferred to a glass house where they showed 90% survival rate. Thus, the method presented in this study had provided a promising strategy for the production of large numbers of phenotypically stable *P. rothschildianum*.**

Key words: Multiple shoot formation, organic nitrogen, *Paphiopedilum*, slipper orchids.

INTRODUCTION

The slipper orchid, *Paphiopedilum rothschildianum* (Orchidaceae), is among the most spectacular *Paphiopedilum* orchid in the genera (Cribb, 1998). This species is endemic to Mount Kinabalu, Sabah, Malaysia and is marketed as a high value pot plant because of its unique and exotic flowers. However, flower development in this species is extremely slow due to its slow growth and maturation rates (Catherine, 1993). This orchid has been classified as an endangered species in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) which means, it is under great threat. Wild populations of this plant are now considered to be one of the rarest in nature as a result of habitat destruction and commercial preference for its beautiful and unique flower (Cribb, 1998). Thus, it is very important that actions should be taken to conserve this plant before it becomes extinct.

Paphiopedilum orchids are generally propagated through the division of axillary buds from the mother plant. However, propagation through this method is very time consuming as only one new growth will be produced after

*Corresponding author. E-mail: norihanms@biotech.upm.edu.my. Tel: +60389468357. Fax: +60389467510.

Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; **BAP**, 6-benzylaminopurine; **MS**, murashige and skoog medium; **PGR**, plant growth regulator; **PLBs**, protocorm-like bodies; **TDZ**, thidiazuron.

a large mature plant had flowered. Although *in vitro* asymbiotic seed germination is an alternative for orchid growers to propagate *Paphiopedilum* species and hybrids, germination of these recalcitrant seeds are more difficult in comparison to other tropical orchids, since germination is affected by many unknown factors (Arditti, 2008). Furthermore, germinated seedlings are always not true-to-type. Due to these problems, orchid growers generally propagate *Paphiopedilum* orchids via vegetative divisions of the axillary bud.

In vitro propagation of orchids via multiple shoot formation provides an alternative means for the production of uniform clones of *Paphiopedilum* orchids. Up to now, several groups have reported *in vitro* propagation of *Paphiopedilum* orchids. Huang et al. (2001) established a clonal propagation protocol by multiplying shoots from geminated seeds of *Paphiopedilum* hybrids (*Paphiopedilum philippinense* × *Paphiopedilum* Susan Booth) in MS medium supplemented with 13 µM BAP. However, Chen et al. (2002, 2004) reported that induction of multiple shoot could be achieved from stem and leaf explants of *P. philippinense* (hybrids PH59 and PH60) cultured on MS medium supplemented with 4.52 µM 2,4-D and 4.54 µM TDZ, respectively. In addition, Hong et al. (2008) have also reestablished shoot multiplication from callus-derived plantlets of *Paphiopedilum* Alma Gavaert by culturing on MS medium supplemented with 4.65 µM kinetin. The progress made on *in vitro* propagation of *Paphiopedilum* orchids so far clearly indicates that genotypic difference between these orchids dictate the response of the *Paphiopedilum* explants on different media formulations. This further emphasizes the urgent need to establish an appropriate micropropagation system for *P. rothschildianum*, in order to prevent the plant from being extinct.

Currently no information regarding *in vitro* propagation of *P. rothschildianum* has been documented. Thus, the present study was initiated in order to induce formation of multiple shoots in *P. rothschildianum* from stem nodal and single shoot explants. However, as stated by Fay (1992), it is necessary to maintain the genetic integrity of the *in vitro* plant when working with plant of conservation importance. Since the addition of plant growth regulators into culture media is likely to induce somaclonal variation (Karp, 1995), the *in vitro* culture technique developed for conservation purposes should minimize the addition of PGR into the basal medium; instead further growth and development of the explants should be enhanced by manipulating the organic and inorganic nutrients in the basal medium.

Several reports have shown that the addition of organic nitrogen compounds into culture medium can significantly enhance shoot proliferation in almond (Ainsley et al., 2000), peanut (Vasanth et al., 2006), croton (Nasib et al., 2008) and avocado (Doung et al., 2008). These organic compounds (casein hydrolysate, peptone and tryptone-peptone) generally consist of low molecular weight proteins, amino acids, vitamins and plant growth substances, which are able to enhance plant growth by providing plant cells with readily available source of nitrogen (George et al., 2008). According to Persson et al. (2006), plant cell has higher capacity to uptake, metabolize and reallocate nitrogen from organic sources than from inorganic sources. Thus, in this study, the effects of organic nitrogen compounds on multiple shoot formation of *P. rothschildianum* were evaluated in the absence of PGR.

MATERIALS AND METHODS

Plant materials and explants preparation

Five-month-old green capsule of *P. rothschildianum* after self-pollination was collected and subjected to surface sterilization. The capsule was submersed in 95% ethanol and flamed for a few seconds under sterile conditions. The capsule was then split longitudinally with a sterile scalpel and the seeds were transferred onto solidified half-strength MS medium for seed germination. Germinated seedlings of *P. rothschildianum* propagated and maintained on half-strength MS medium were used as explants for multiple shoot induction. For single shoot explants, single shoots of 2 to 3 cm in height (2-month-old) with roots trimmed were sub-cultured onto multiple shoot induction media. For stem nodal explants, the leaves and roots were cut and 5 mm portions of shoots from the basal parts of plantlets were used as explants to induce multiple shoots.

Shoot multiplication

MS medium (Murashige and Skoog, 1962) containing half-strength of macro- and micro-elements of MS inorganic salts with additions of NaH_2PO_4 (170 mg/l) and sucrose (3 g/l) and solidified with 0.3% Gelrite was used as basal medium for induction of multiple shoots. The organic nitrogen compounds tested were casein hydrolysate (Fluka), peptone (Fisher Bio-Reagents) and tryptone-peptone (DIFCO) in amounts of 0.5, 1.0, and 2.0 g/l, respectively. All the organic nitrogen compounds were added into the basal medium prior to autoclaving. Half-strength MS medium without the addition of these organic nitrogen compounds was used as control. The pH of the media were adjusted to 5.5 - 5.7 with 1 M NaOH or 1 M HCl prior to autoclaving at 121°C for 15 min under 1.05 kg cm^{-2}. Stem nodal explants were placed onto 10 ml solidified media in 20 × 150 mm culture tubes and incubated in the dark at 25 ± 2°C. Single shoot explants were placed vertically on the medium in Magenta boxes containing 40 ml of solidified medium; and incubated under continuous light (40 µmol m^{-2} s^{-1}, provided by fluorescent lamps), at 25 ± 2°C.

Acclimatization

Plantlets with 3 to 4 leaves bearing 5 to 6 roots (approximately 2 cm in height) were taken out from the culture flask and washed thoroughly under running tap water to remove the remaining agar. The plantlets were then dipped in sucrose-free MS medium prior to being placed into pots containing sphagnum moss. The potted plantlets were then kept under shade in a glasshouse and sprayed with water twice a day to maintain the humidity. Sucrose-free MS medium was sprayed onto each plantlet weekly. The viability and growth of the plantlet were recorded when formation of new leaf was observed.

Experimental design and data analysis

The experiment was performed in a complete randomized block design (CRBD). A total of 30 explants in three replicates per treatment for both stem nodal and single shoot explants were used in this study. The percentage of explants with shoots and the mean number of shoots formed per explant were determined for each experiment. The mean values of the various treatments were subjected to analysis of variance (ANOVA) using SPSS version 17. The differences between the means of each of the treatments were scored with Duncan's multiple range test (Duncan, 1955) at P value = 0.05.

RESULTS

Generally, the additions of organic nitrogen compounds into the culture medium not only promoted shoot and root development but also enhanced the formation of multiple shoot. The three organic nitrogen compounds (casein hydrolysate, peptone and tryptone-peptone) tested in this experiment significantly ($P < 0.05$) increased the mean number of multiple shoots formed from stem nodal and single shoot explants of *P. rothschildianum.* Leaves continued to grow and elongate from the stem nodal explants after two weeks of culture (Figure 1a) but the formation of new shoots was only observed after four weeks of culture. Tiny spike-like protrusions of shoots emerged from the basal parts of the stem nodal explants (Figure 1b) and continued to form normal shoots bearing two leaves after another four weeks of culture. The newly-developed shoots continued to form other new shoots when left for another four weeks on the same media without any subculture. More shoots continued to emerge from the basal parts of the original stem nodal explants after 16 weeks of culture (Figure 1c). All newly formed shoots turned green and continued to form two to three leaves after being subcultured onto the same fresh medium and kept under light. Similar morphological developments were also observed on single shoot explants. Newly-formed shoots sprouted from the basal part of the single shoot after four weeks of culture and eventually developed into normal plantlets bearing two to three leaves (Figure 1d). All newly formed shoots were separated into single plantlets when they reached 20 mm in height. After 12 weeks in culture (without any subculture), the plantlets with four to five roots (Figure 1e) were acclimatized and transferred to sphagnum moss and grown in a glasshouse with 90% survival rate (Figure 1f).

In this study, the formation of multiple shoots was clearly observed in *P. rothschildianum*. The formation of multiple shoots can be obtained from stem nodal explants cultured on half-strength MS medium without supplementation of organic nitrogen compound (control) with more than 80% of the explants showing an average of 2.4 shoots per explant (Figures 2a and 2b). However, the addition of peptone (as an organic nitrogen source) into the culture media was shown to increase the mean number of multiple shoots formed. The highest number of multiple shoots formed was scored on stem nodal explants cultured onto half-strength MS medium supplemented with 1.0 g/l peptone with an average of 2.9 shoots per explant, after 16 weeks of culture (Figure 2b). Increasing the amount of organic nitrogen to 2.0 g/l did not increased the mean number of shoots formed as shown in Figure 2b. In fact, the presence of a high amount (2.0 g/l) of organic nitrogen was shown to inhibit the formation of multiple shoot on the stem nodal explants. The number of shoots formed was significantly reduced in MS media supplemented with 2.0 g/l casein hydrolysate or 2.0 g/l tryptone-peptone when compared with the control medium.

In general, the percentage of multiple shoot formation on single shoot explants was slightly lower (approximately 65%) than that of stem nodal explants when cultured onto medium free of organic nitrogen supplement (Figure 3a). As shown in Figure 3b, only an average of 2.1 shoots were obtained from a single shoot explant cultured onto modified half-strength MS medium free of these organic nitrogen compounds (control). Unlike stem nodal explant, increasing the amount of tryptone-peptone (from 0.5 to 2.0g/l) was shown to promote the formation of multiple shoots. As shown in the Figure 3b, addition of 0.5g/l tryptone-peptone into culture medium has significantly ($P < 0.05$) increased the mean number of multiple shoot formed and the highest number of shoots formed was obtained on medium supplemented with 2.0g/l tryptone-peptone with an average of 2.8 shoots formed per single shoot explant. In contrast, peptone was not effective in the formation of multiple shoots when single shoot explants were used, except at a low amount (0.5 g/l). The addition of a high amount of peptone (1.0 and 2.0g/l) resulted in an inhibitory effect on the mean number of multiple shoots formed (Figure 3b). Besides that, the addition of casein hydrolysate (1.0g/l) was shown to promote the formation of multiple shoots and could increase the mean number of multiple shoots formed when compared to the control medium.

DISCUSSION

Paphiopedilum orchids are one of the slowest growing orchids among the *Orchidaceae* family. There has been limited success in propagating *Paphiopedilum* orchids, mainly due to the difficulty in maintaining the explants in culture and that the plants are too valuable to sacrifice for experimental purposes (Arditti and Ernst, 1993; Arditti, 2008). Huang et al. (2001) reported that doubling of plant numbers occurred only after 12 weeks with a 3-shoot cluster in modified MS medium supplemented with BAP. Chen et al. (2002) reported that seven shoots can be obtained from a single stem nodal explant after 24 weeks of culture in modified half-strength MS medium supplemented with 2,4-D alone. However, only an average of

Figure 1. Formation of multiple shoots from stem nodal and single shoot explants of *Paphiopedilum rothschildianum*. **(a)** Development and elongation of leaf from stem explant after two weeks of culture (bar = 5 mm); **(b)** Formation of spike-like protrusions of shoot from stem explant after four weeks of culture (bar = 5 mm); **(c)** Formation of multiple shoots from stem explant after sixteen weeks of culture (bar = 10mm); **(d)** Formation of multiple shoots from single shoot explants after sixteen weeks of culture (bar = 10 mm); **(e)** Well-rooted plantlets ready for acclimatization (bar = 15 mm); **(f)** Acclimatized plantlets after eight weeks grown in glass house (bar = 50 mm).

one shoot was obtained from a stem nodal explant after 140 days of culture on medium free of PGR (Chen et al., 2002). Hong et al. (2008) reported that an average of three shoots were obtained from a single young shoot of *Paphiopedilum* Alma Gavaert after 60 days of culture on modified half-strength MS medium supplemented with

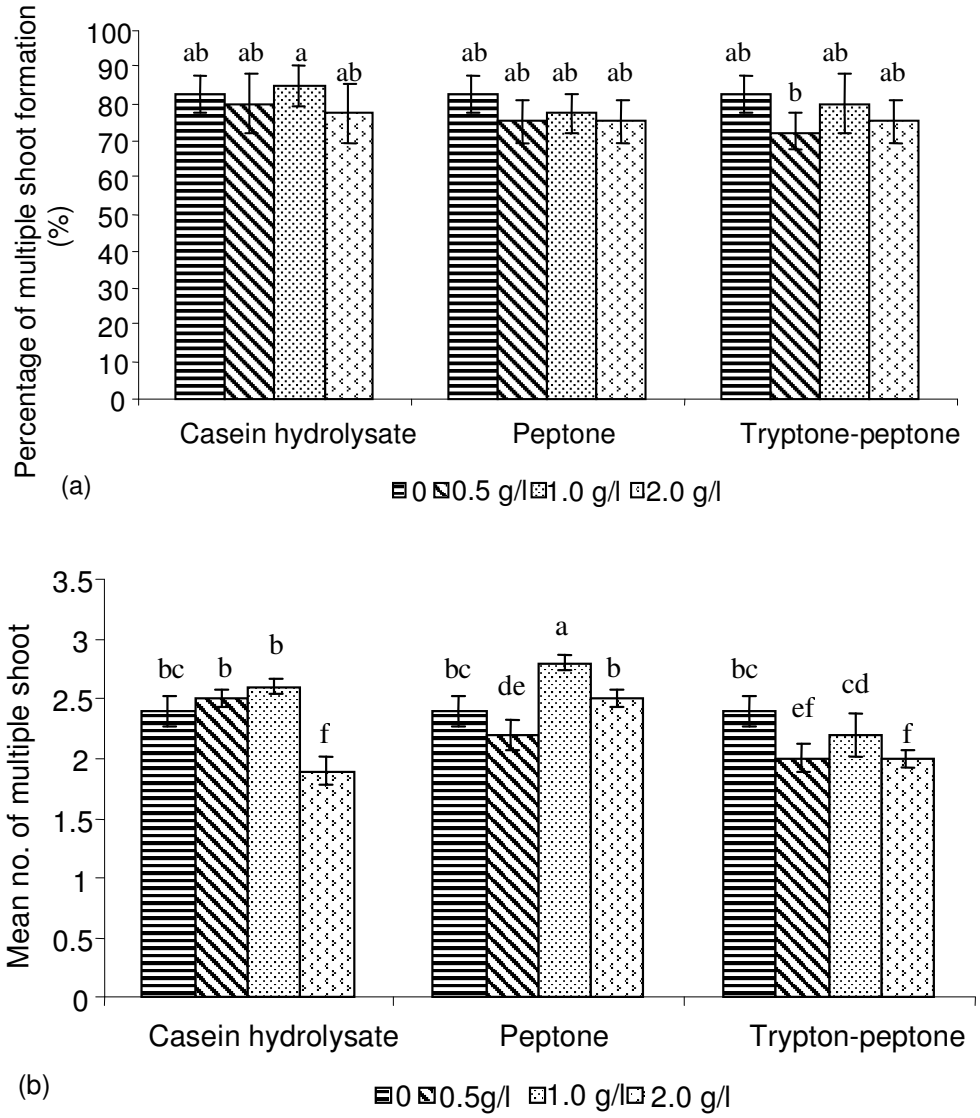

Figure 2. Effects of different types and amounts of organic nitrogen on multiple shoot formations from stem nodal explants of *P. rothschildianum*. **(a)** Percentage of multiple shoot formation on different organic nitrogen sources; **(b)** mean number of shoots formed per stem nodal explant, after 16 weeks of culture. Mean number represents a total of 30 explants in three replicates per treatment. Mean ± standard error followed by the same letter are not significantly different from each other according to Duncan's multiple range test at P = 0.05.

kinetin. However, the young shoots failed to multiply without the addition of exogenous PGR. In this experiment, an average of 2.9 and 2.8 shoots could be obtained from a stem nodal and a single shoot explant, respectively, after 16 weeks of culture on PGR-free media supplemented with either peptone or tryptone-peptone only. However, the response of the explants to different media formulation and the plant regeneration capability were very much dependent on the cultivars' genotypes (Chen et al., 2002).

The addition of organic nitrogen compounds into basal medium containing inorganic salts is widely used in *in vitro* propagation of orchids (Arditti and Ernst, 1993; Arditti, 2008, George et al., 2008). Organic nitrogen compounds such as peptone, casein hydrolysate and tryptone, have been reported as supplements of poly-peptides or free amino acids to enhance seed germination, growth of seedlings (Huang et al., 2001; Pierik et al., 1988), proliferation of callus (Chen and Chang, 2000), formation of protocorm-like bodies (PLBs) from callus as well as for the regeneration of PLBs (Shina and Roy, 2004) in orchids. Pierik et al. (1988) reported that tryptone and peptone (2.0 g/l) had beneficial effects on seed germination and further development of seedlings of *Paphiopedilum ciliolare*. Chen and Chang (2002) also reported that the addition of peptone into culture media

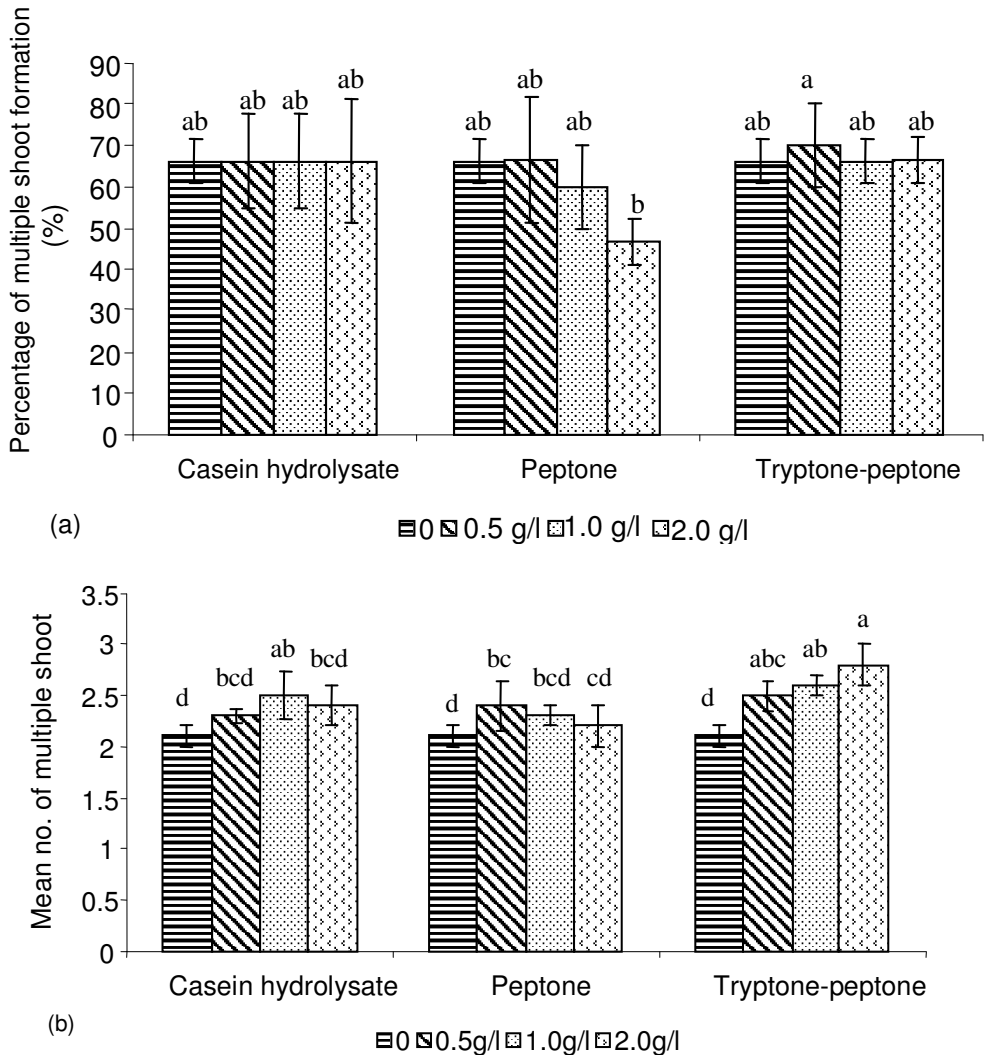

Figure 3. Effects of different types and amounts of organic nitrogen on multiple shoot formations from single shoot explants of *P. rothschildianum*. **(a)** Percentage of multiple shoot formations on different organic nitrogen sources; **(b)** mean number of shoots formed per single explant, after 16 weeks of culture. Mean number represents a total of 30 explants in three replicates per treatment. Mean ± standard error followed by the same letter are not significantly different from each other according to Duncan's multiple range test at P = 0.05.

significantly promoted direct embryo formation from leaf tips of *Oncidium*. These further supported our findings that the addition of trpytone-peptone and peptone was effective for multiple shoot formation in stem nodal and single shoot explants in *P. rothschildianum*. In avocado (Doung et al., 2008), the addition of peptone into culture media reported to increase survival rates of stem explants (up to 100%); whereas in croton, enhancement of axillary shoots formation (2 fold increase) was observed in the presence of peptone (Nasib et al., 2008). Our findings showed that the addition of peptone and tryptone-peptone could increase the mean number of multiple shoots formed from stem nodal and single shoot explants, respectively, when compared with the control medium. The ability of these organic nitrogen compounds to enhance shoot multiplication of *P. rothschildianum* may be due to the role of amino acids in regulating primary nitrogen assimilation, thus providing the plant cells with readily available nitrogen sources (Srivastava and Singh, 1999; George et al., 2008).

In this study, multiple shoots was successfully induced from stem nodal and single shoot explants of *P. roth-schildianum* cultured on half-strength MS media in the absence of PGR and organic nitrogen additives (control). The number of multiple shoots was further increased with the supplementation of organic nitrogen (peptone and tryptone-peptone) into the culture media. Somaclonal variations which resulted from the high multiplication rates of plant cells, could be influenced by the types and concentrations of PGRs used in culture media (Karp,

1995; Bairu et al., 2006). Since no exogenous PGR was involved in this study, it was expected that the occurrence of somaclonal variation would be relatively low in these cultures compared to multiple shoots induced in the presence of exogenous PGR. Although the addition of organic nitrogen into culture media resulted in an increase in the shoot multiplication rate, the multiplication rate was not high enough to cause somaclonal variations. This was also further supported by the morphological observation of the plantlets obtained, such as the height of plantlets, the leaf and root morphologies and the number of roots formed, which showed uniform characteristics among the plantlets derived from the multiple shoots. Therefore, there was a high possibility that uniform plantlets of *P. rothschildianum* were obtained through the PGR-free medium formulation.

The method developed in this study provides a simple means to propagate complete *P. rothschildianum* plantlets, since multiplication and rooting occurred on the same medium without any exogenous PGR. The *in vitro* culture method presented was able to produce large numbers of uniform *P. rothschildianum* plantlets compared to the conventional propagation method. In an effort to protect this endemic endangered orchid from extinction, these tissue-cultured plantlets can be reintroduced into its natural habitats. The introduction of tissue-cultured plantlets into the horticulture market will also curb illegal collections of *P. rothschildianum* from the wild.

ACKNOWLEDGEMENT

The authors thank the School of Graduate Studies of Universiti Putra Malaysia for providing a Graduate Research Fellowship to the first author.

REFERENCES

Ainsley PJ, Collins GG, Sedgley M (2000). Adventitious shoot regeneration from leaf explants Almond (*Prunus dulcis* Mill.). In Vitro Cell. Dev. Biol. Plant. 36: 470-474

Arditti J, Ernst R (1993). Micropropagation of orchids. John Wiley and Sons, New York

Arditti J (2008). Micropropagation of orchids 2nd Edition. Blackwell Publishing Ltd.

Bairu MW, Fennell CW, Van Staden J (2006). The effect of plant growth regulators on somaclonal variation in Cavendish banana (Musa AAA cv. 'Zelig'). Scientia Horticulturae 108: 347-351

Catherine C (1993). The slipper orchids. Timber Press, Inc. Portland, Oregon

Chen JT, Chang WC (2000). Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae). Plant Sci. 160: 87-93

Chen JT, Chang WC (2002). Effects of tissue culture conditions and explant characteristic on direct somatic embryogenesis in *Oncidium* 'Grower Ramsey'. Plant Cell, Tissue Organ Cult. 69: 41-44

Chen TY, Chen JT, Chang WC (2002). Multiple shoot formation and plant regeneration from stem nodal explants of *Paphiopedilum* Orchids. In Vitro Cell. Dev. Biol. Plant. 38: 595-597

Chen TY, Chen JT, Chang WC (2004). Plant regeneration through direct shoot bud formation from leaf cultures of *Paphiopedilum* orchids. Plant Cell, Tissue Organ Cult. 76: 11-15

Cribb P (1998). The Genus *Paphiopedilum*, Second Edition. Natural History Publication (Borneo), Kota Kinabalu

Doung TN, Nguyen NT, Bui LTK, Vu QL (2008). Peptone stimulates *in vitro* shoot and root regeneration of avocado (*Persea americana* Mill.) Scientia Horticulturae. 115:124-128

Duncan DB (1995). Multiple range and multiple *F*-test. Biometrics. 11: 1-42

Fay MF (1992). Conservation of rare and endangered plant using in vitro methods. In Vitro Cell. Dev. Biol. 28:1-4

Hong PI, Chen JT, Chang WC (2008). Plant regeneration via protocorm-like body formation and shoot multiplication from seed-derived callus of maudiae type slipper orchid. Acta Physiol. Plant. 30: 755-759

Huang LC, Lin CJ, Kuo CI, Huang BL, Murashige T (2001). *Paphiopedilum* cloning in vitro. Scientia Horticulturae. 91: 111-121

George EF, Hall MA, Jan De Klerk G (2008). Plant propagation by tissue culture 3rd Edition. Springer, The Netherlands

Karp A (1995). Somaclonal variation as a tool for crop improvement. Euphytica. 85: 295-302

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

Nasib A, Ali K, Khan S (2008). In vitro propagation of Croton (*Codiaeum variegatum*). Pak. J. Bot. 40(1): 99-104

Persson J, Gardestrom P, Nasholm T (2006). Uptake, metabolism and distribution of organic and inorganic nitrogen sources by *Pinus sylvestris*. J. Exp. Bot. 57: 2651-2659

Pierik RLM, Sprenkels PA, Van Der Hask B, Van Der Meys QG (1988). Seed germination and further development of plantlets of *Paphiopedilum ciliorare* Pfitz. *in vitro*. Scientia Horticulturae. 34: 139-153.

Shina P, Roy SK (2004). Regeneration of an Indigenous orchid, *Vanda teres* (Roxb.) through *in vitro* culture. Plant Tissue Cult. 14(1): 55-61.

Srivastava HS, Singh RP (1999). Nitrogen nutrition and plant growth. Science Publishers, Inc., United States of America.

Vasanth K, Lakshimiprabhal A, Jayabalan N (2006). Amino acids enhancing plant regeneration from cotyledon and embryonal axis of peanut (*Arachis hypogaea* L.) Indian J. Crop Sci. 1(1-2): 79-83.