Progress in Biological Sciences
Vol. 6, Number 2, Summer / Autumn 2016/213-221 - DOI: 10.22059/PBS.2016.590022

Effects of culture medium and supplementation on seed germination, protocorm formation and regeneration of some *Phalaenopsis* hybrids

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Received: August 28, 2016; Accepted: December 21, 2016

ABSTRACT

An efficient protocol is suggested for *in vitro* culture of five *Phalaenopsis* hybrids obtained by hand-cross-pollination of three commercial hybrids Calgary, Ankara, and Kendall. Four nutrient media- namely half-strength Murashige and Skoog, Knudson, Phytamax containing activated charcoal, and Mitra– once supplemented (with coconut water, peptone, or both), and once without any supplement were considered as the experimental and control groups of the study which were then compared and evaluated for seed germination and protocorm formation. All of the seeds of hybrids H and N were germinated on half-strength Murashige and Skoog medium supplemented with peptone. To evaluate plant regeneration rate, three different media including half-strength Murashige and Skoog, Viking-Ship containing 2.75gr/L NPK (10-20-30), and Hyponex containing [1gr/L NPK (20-20-20)+1gr/L NPK (6.5-6-19)] were compared. The maximum number of healthy plantlets, roots per plantlet, and leaves per plantlet were induced in the half-strength Murashige and Skoog medium. Around 93% of the plants produced *in vitro* were able to establish *ex vitro*. The obtained results showed that, the use of the half-strength Murashige and Skoog medium is well suited for the mass propagation of *Phalaenopsis*.

Keywords: *Phalaenopsis*; Micropropagation; Protocorm; Seed germination; *In vitro* culture

Introduction

Although the family Orchidaceae with around 26000 species is one of the largest families of flowering plants, the species within this family are considered as endangered species as they are rapidly decreasing in number due to careless collection (1). More than 100,000 commercial hybrids are currently known in this family (2).

The species of the genus *Phalaenopsis* are the most important cut flowers and potted plants of the family Orchidaceae. *Phalaenopsis* species are epiphytic and monopodial orchids native to southeastern Asia and

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are famous for their beautiful flowers. Extensive hybridization in *Phalaenopsis* has resulted in large variations in flower shapes, sizes and colors as well as color patternings (3).

In order to meet large-scale world market demands for *Phalaenopsis* orchids, and to prevent these orchids from extinction and loss of genetic diversity, plant producers need to develop a reliable regeneration protocol for commercial production of these orchids. In orchids, protocorm-like bodies (PLBs: the structures that resemble protocorms but are formed by tissue explants and/or callus *in vitro*) are equivalent to somatic embryos (4, 5). In this species, *in vitro* regeneration could be done through either callus or PLB formation (6). Although tissue culture is an effective mode of orchid multiplication, the indirect method can not be very useful due to lower growth rate and regular necrosis in culture (7). Moreover, direct regeneration without formation of intermediate callus fastens the process and reduces the occurrence of somaclonal variation (8). The rate of natural seed germination in orchids is very low and they frequently need to have mycorrhizal association for their germination (9). Although the techniques of *in vitro* propagation have been widely accepted and are being used for conservation of the endangered species of orchids, there is limited scientific research on *in vitro* seed germination of orchid seeds (10). Asymbiotic seed germination procedures have gained wide acceptance after Knudson found an easy and simple way for seed germination on a medium which contained minerals, and sugar (11, 12). After germination, the orchids seeds develop into a structure called the protocorm (the small spherical tuber-like bodies formed by germinating orchid seeds) (13). Among different ways of orchid micropropagation, the system *via* protocorm formation is used for the purpose of breeding, and for conserving endangered cases (14). *In vitro* seed germination (15, 16), growth and development (17), micropropagation using vegetative segments (8, 18-20), micropropagation using floral segments (21-23), and embryogenesis (24-31) have been successfully used for *Phalaenopsis* regeneration. Several studies have been performed to survey the acclimatization of *Phalaenopsis*. Another type of research is to design an automatic machine vision-guided grasping system for *Phalaenopsis* tissue culture plantlets (32).

The aim of this study is to develop an easier and more economical procedure to induce protocorms as well as better regeneration and acclimatization of *Phalaenopsis.* The new idea in this research is to compare the regeneration efficiency of some new hybrids of *Phalaenopsis*. The interaction between hybrids and the type of medium as well as supplements has also been investigated, and is discussed in more detail.

Materials and Methods

Seed source and sterilization procedure

The hybrids Calgary, Ankara, and Kendall were obtained from the orchid company of Anthura, Netherland. Five *Phalaenopsis* hybrids obtained by hand-cross pollination of three hybrids of *Phalaenopsis* were employed in the study. The five obtained hybrids were labeled as follows: hybrid F (pollen grain of Ankara with pistil of Calgary), hybrid B (pollen grain of Kendall with pistil of Calgary), hybrid H (pollen grain of Ankara with pistil of Kendall), hybrid J (pollen grain of Calgary with pistil of Kendall), and hybrid N (pollen grain of Kendall with pistil of Ankara).

The seeds collected 136 days after pollination (DAP) were used for *in vitro* culture to assess asymbiotic seed germination and formation of protocorms of *Phalaenopsis* hybrids. The capsules were disinfected in commercial bleach “Whitex” for 20 mins. Disinfection proceeded by dunking the capsules in alcohol and then lighting them on fire followed by rinsing them with sterile distilled water for three times. Seed germination started after 14 days. Seedlings were maintained in the primary medium for an additional 30 days period.

Plant material, culture medium and supplement

Four different culture media- namely, ½MS, Knudson's C (KC), Mitra (M), and Phytamax (PM) containing Activated Charcoal (AC)- were tested. In addition, two different supplements, that is 2 gr/L peptone and 15% CW, were also introduced into each medium, once separately and once in tandem, and their effects were investigated. After the induction of the protocorms, they were left on the primary medium for an extra four-month period.

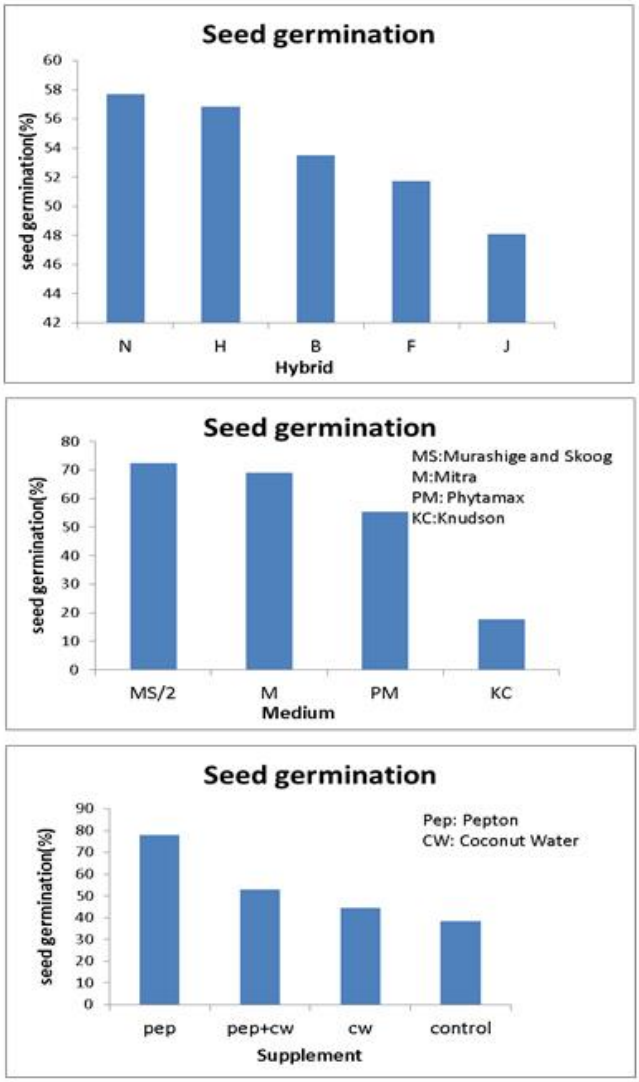

Figure 1. Comparison of seed germination in different hybrids of *Phalaenopsis*, supplements and media. For abbreviation of hybrids and their parental origin, see the text.

Since the number of samples in hybrid J was too small, this hybrid was excluded from the study after the seed germination phase of the study. The first leaves and roots that appeared after the four month period were transferred to three different media: ½MS, Viking-Ship containing 2.75 gr/L NPK (10-20-30) and Hyponex containing [1 gr/L NPK (20-20-20) + 1 gr/L NPK (6.5-6-19)]. After adding 2 g/L peptone and 30 g/L Potato Homogenate (PH), the effects of organic supplements on growth and development of plantlets were compared with those of the plantlets in the control group. The number of healthy plantlets, roots and shoots were counted. Based on the obtained results, the ½MS medium and the Hyponex medium containing 1gr/L NPK (20-20-20)/1 gr/L NPK (6.5-6-19) were selected. Healthy plantlets were transferred to these two media so that they could continue their next stages of growth and development. Plantlet length, number of leaves and roots per plantlet were recorded after the plantlets remained on the selected media for three months. Finally, the plantlets were transferred to soil for further growth, and their survival rate in pots was assessed.

Acclimatization

To remove residual gelling agent and nutrients from plant body, 30 regenerated plantlets ranging from 5 to 7 cm in length were rinsed thoroughly with tap water and were implanted on the following substrates: peat, perlite, pine bark mulch and charcoal at a ratio of 20-10-35-35. During acclimatization, the humidity of the growth chamber was maintained between 50-70%. The plants were acclimatized and let grow for additional four-month phase under greenhouse conditions.

Experimental design and data analysis

The following formula was used to calculate the percentage of seed germination:

$$\frac{\text{Number of seeds showing swelling of the embryo} \times 100}{\text{Total No. of seeds}}$$

The number of healthy plantlets, the number of roots and leaves per plantlet were calculated after two months of transfer to the secondary media with additional supplements. Plantlet length, number of roots and leaves per plantlet were recorded after three months of subculturing in the selected media. Data were obtained and analyzed using *SAS* software version 9; general linear model (GLM) and mean comparisons were performed using Duncan's multiple range test (DMRT) with a confidence level of $P<0.05$ (33). Multiple comparisons were also performed by GLM and Bonferoni post hoc using *SPSS* version 19.

Results

Seed germination

Seed germination initiated after 14 days. For hybrids H and N the maximum germination was obtained (100%) in ½MS medium supplemented with peptone which has been reported also previously (34). Hybrid N showed 99.1% germination in ½MS medium supplemented with peptone and CW. No germination was observed for any of the hybrids on the KC medium supplemented with CW (Supplementary Table 1). In respect to germination rate, hybrid N showed the best results (Figure 1). Multiple comparisons using Bonferoni post hoc test showed that hybrid J had a significant difference with other hybrids except for hybrid F ($p<0.05$). Moreover, hybrid F showed no significant difference with other hybrids except for hybrid H ($p<0.05$) (Supplementary Table 2). From the different media, only M and ½MS showed similar results (Supplementary Table 3 and Figure 1). All supplements showed significant differences (Supplementary Table 4). But, the most effective supplement for seed germination was peptone followed by peptone plus CW, CW, and control, respectively (Figure 1).

Plantlet regeneration

Seeds were maintained on the primary medium after germination. The protocorms formed, became green, and started to develop the first leaves and roots (Figure 2a-d). For hybrids B and N, the maximum number of healthy plantlets (=15) was obtained on the ½MS medium supplemented with peptone or PH. Hybrid N showed the highest number of roots per plantlet (=4.16) on ½MS medium supplemented with peptone. Nevertheless, hybrid H displayed the maximum number of leaves per plantlet (=4.37) on the ½MS medium without supplementation (Supplementary Table 5).

After subculturing the healthy plantlets of four selected hybrids in the ½MS medium and the Hyponex medium containing [1 gr/L NPK (20-20-20) + 1 gr/L NPK. (6.5-6-19)], plantlet length conserved in the two media did not differ significantly ($p<0.05$), but the maximum length (=3.35-3.37 cm) was obtained for hybrid B on the media supplemented with PH. For hybrid F, the highest number of roots per plant (=7.6) was reported on the ½MS medium supplemented with peptone. Likewise, hybrid B showed the highest number of leaves per plant (=5.88) on the ½MS medium supplemented with PH. Different stages of plantlet regeneration aer shown in Figures 2e-g.

Figure 2. The sequence of events of *in vitro* multiplication of *Phalaenopsis*. (a-c) Seed germination and induction of protocorms. (d) Formation of protocorms with first leaves and rhizoids. (e-f) *in vitro* rooted plants. (g) The average size of plantlets. (h) Plantlets after transfer to soil.

Acclimatization

After a period of four months, the survival rate of the plantlets which were planted in containers filled with peat, perlite, pine bark mulch, and charcoal at a ratio of 20-10-35-35, was 93%. The morphology of the regenerated plants did not show any significant difference (Figure 2h).

Overall, the results significantly differed when different factors– that is hybrid, medium and supplementation –were analyzed separately (Figure 3-5).

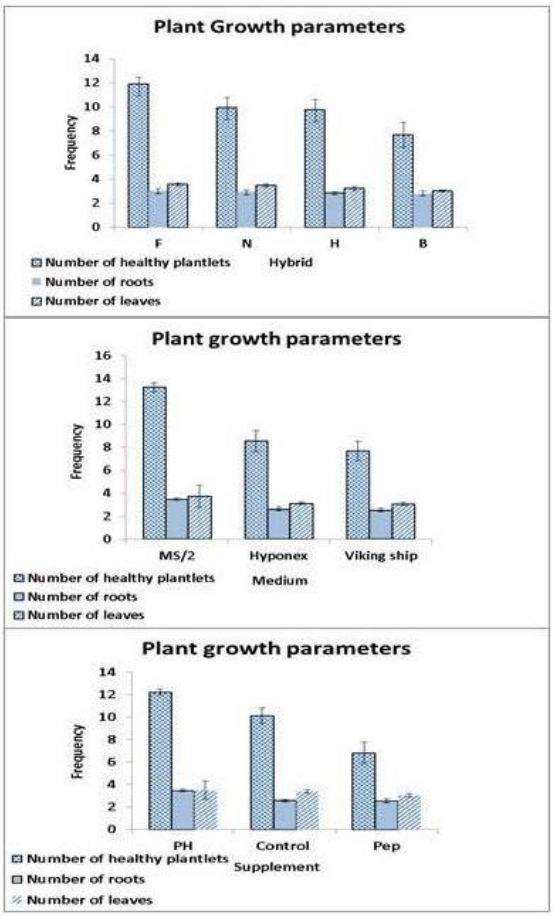

Figure 3. Number of healthy plantlets, roots and leaves in different hybrids, media and supplements. For abbreviation of hybrids ant their parental origin, see the text. Abbreviations for culture media: PH, Potato Homogenate; Pep, Peptone; Ms, Murashige and Skoog.

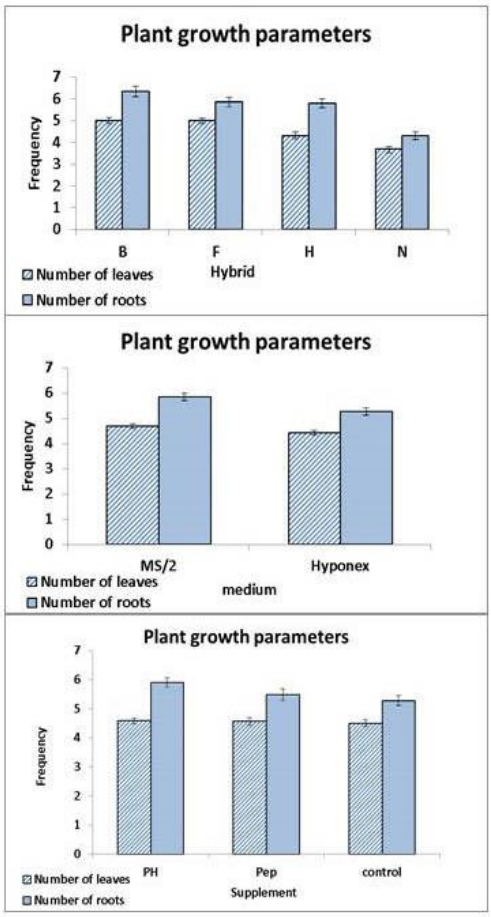

Figure 4. Number of leaves and roots in final subculture in different hybrids, media and supplements. For abbreviation of hybrids and their parental origin, see the text. Abbreviations for culture media: PH, Potato Homogenate; Pep, Peptone; Ms, Murashige and Skoog.

Discussion

In vitro seed germination and plant regeneration of orchid species including *Phalaenopsis* sp. is a suitable technique many for their clonal propagation. As stated above, studies have been conducted on the micropropagation of *Phalaenopsis*, but the novelty of the present study compared to previous ones is that it has simultaneously taken three factors into account: hormone, medium and hybrid. The results of this study confirm that there is some interaction between the factors, and that it significantly affects the success of *in vitro* plant regeneration. Furthermore, in the current research, we studied the direct regeneration of *Phalaenopsis* through seed germination, while in earlier studies micropropagation in *Phalaenopsis* was studied through somatic embryogenesis or indirect regeneration and this is another important strength of the present study.

Park et al. tested ½MS, VW, KC, LM and Hyponex media for PLB induction from leaf explants (23) and found the optimal results in ½MS. Thongpukdee et al. have reported the maximum percentage of *Phalaenopsis* seed germination in modified stationary-liquid Hyponex medium supplemented with 2 g/L peptone, 100 g/L potato juice and 1 g/L AC (35). The results of the current study also suggest that the ½MS medium is the most effective medium in all stages of seed germination and plant regeneration. Nitrogen source is a key factor that affects seed germination in *Phalaenopsis* hybrids. In the ½MS medium, nitrogen is in the form of ammonium nitrate while it is in the form of ammonium sulphate in Mitra and KC. The ½MS is also rich in macro- and micro-elements which are necessary for successful germination of seeds that could in turn enhance the seed germination in this medium.

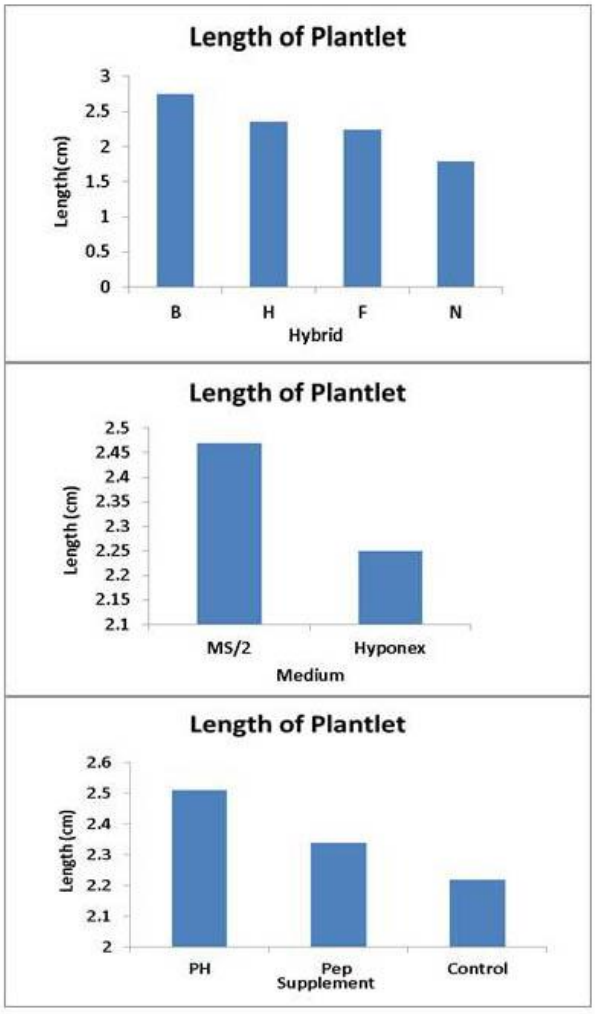

Figure 5. Length of *Phalaenopsis* plantlets in final subculture in different hybrids, media and supplements. For abbreviation of hybrids and their parental origin, see the text. Abbreviations for culture media: PH, Potato Homogenate; Pep, Peptone; Ms, Murashige and Skoog.

The addition of peptone or tryptone to the culture medium promoted the growth of PLBs derived from the seeds of *Phalaenopsis* Surfrider × (*Phalaenopsis* Joseph Hampton × *Doritaenopsis* Kaalan Gleam) (36). The enhancing effect of peptone on seed germination and the subsequent development of the protocorms of *Dendrobium aphyllum* has previously been shown (37). CW is also a supplement which has been used in micropropagation protocols of economically important species such as orchids (38). According to Yong et al., CW contains amino acids, organic acids, inorganic ions, vitamins, sugars, lipids, nitrogenous compounds, and hormones, and therefore can promote the growth of cells (39). The positive effects of 20% CW in VW medium (40) on PLB multiplication has been reported in *Phalaenopsis* plants (24). In the present research, peptone (used in isolation or in tandem with CW) significantly enhanced the germination of all hybrids in all media except for KC. Moreover, CW– especially in association with peptone– increased the rate of seed germination and protocorm induction in all media except for KC. The low rate of seed germination in KC has also been reported in another study on other species of orchids (41).

PH also contains polyamine and biosynthetic enzymes that affect nucleic acid replication and cell division during mitosis, and therefore promotes plant cell growth and development. It also contains useful carbohydrates, sugars, proteins and vitamins required for plant growth (42). Arditti and Ernst reported that the addition of PH to orchid culture medium enhances seed germination and growth of seedlings (43). In the present study, PH, as a supplementation, had the maximum effect on some factors such as the number of healthy plantlets, the number of leaves per plantlet, and plantlet length.

Chen and Chen obtained the highest survival rate of 100% for *Phalaenopsis* grown on *Sphagnum* moss (27). Balilashaki et al. reported the highest survival rate of 99% for acclimatization on a combination of cocopeat, charcoal, industrial cartridge, and the bites of polystyrene (1-1-2-4) (44). Diaz et al. showed that the survival rate depends on the plant growth stage *in vitro* (45). In other words *Phalaenopsis* plants need to reach 2-4 cm in size under *in vitro* conditions to be able to endure external conditions in the greenhouse on moss, mesquite wood shavings, and perlite. By reaching this size, these plantlets get the proper number of sprouts, foliage area (leaf size and number) as well as root number and length and reach maximal survival rate (100%) compared to plantlets in the range of 1-2 cm which suffer from a smaller, survival rate of 44%. In the present study, plantlets in the range of 5-7 cm were implanted on substrate containing peat, perlite, pine bark mulch, and charcoal. The observed high survival rate of 93% might therefore be attributed to the size range of plantlets.

This paper is the first report describing the combined effects of the three factors of medium, supplementation, and hybrid on *in vitro* culture and

the regeneration of *Phalaenopsis* sp. The efficient seed germination, followed by convenient conversion to plantlets, provides a simple, easy and effective protocol for a large number of plants and for the mass propagation of this important ornamental orchid in a short period of time.

Conclusion

In summary, our results of the current study indicate that the ½MS is the best medium for seed germination and plantlet regeneration at different stages of growth and development. From the different supplements, peptone showed better results for seed germination, but PH was the best supplementation for the regeneration of plantlets. Nevertheless, each hybrid showed unique reaction to each experimental phase, and no single hybrid can be said to have had the best reaction to all of the experimental stages of this study simultaneously. Hybrid N, for instance, was best in seed germination but not in subsequent stages of growth and development.

Acknowledgement

The authors would like to thank the College of Sciences of the University of Tehran for providing plant physiology laboratory equipment, and the Young Researchers and Elite Club at the Tehran Medical Sciences Branch of the Islamic Azad University, for its supporting the financial part of this study (Grant number: 93131). The authors also to thank Dr. Vahid Ghahraman and Dr. Mohammad Ali Salmani Nodushan of the Iran Encyclopedia Compiling Foundation (IECF) and Mrs. Nardana Esmailie (PhD) of Texas University, USA, for critical reading of the manuscript.

REFERENCES

1. Bektaş, E., Cüce, M., and Sökmen, A. (2013) *In vitro* germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. *Turk. J. Bot.*, **37**, 336-342.
2. Poobathy, R., Nair, H. and Subramanian, S. (2009) Optimization of encapsulation-dehydration protocol for the orchid hybrid Ascocenda " Princess Mikasa". *Adv. Environ. Biol.*, **3**, 69-83.
3. Wang, Y.T., and Gregg, L.L. (1994) Medium and fertilizer affect the performance of *Phalaenopsis* orchids during two flowering cycles. *Hort. Sci.*, **29**, 269-271.
4. Soontornchainaksaeng, P., Chaicharoen, S., Sirijuntarut, M., and Kruatrachue, M. (2001) *In vitro* studies on the effect of light intensity on plant growth of *Phaius tankervilliae* (Banks ex L'Herit.) Bl. and *Vanda coerulea* Griff. *Sci. Asia*, **27**, 233-237.
5. Teixeira da Silva, J.A. (2012) Jasmonic acid, but not salicylic acid, improves PLB formation of hybrid *Cymbidium*. *Plant Tissue Cult. Biotechnol.*, **22**, 187-192.
6. Kishor, R., and Devi, H.S. (2009) Induction of multiple shoots in a monopodial orchid hybrid (*Aerides vandarum* Reichb.f.)×*Vanda stangeana* Reichb.f.) using thidiazuron and analysis of their genetic stability. *Plant Cell Tiss. Org.*, **97**, 121-129.
7. Zhao, P., Wu, F.M., Feng, F.S., and Wand, W.J. (2008) Protocorm-like body (PLB) formation and plant regeneration from the callus culture of *Dendrobium candidum* wall ex Lindl. *In Vitro Cell. Dev. Biol. Plant*, **44**, 178-185.
8. Košir, P., Škof, S., and Luthar, Z. (2004) Direct shoot regeneration from nodes of *Phalaenopsis* orchids. *Acta Agric. Slov.*, **83**, 233-242.
9. Swarts, N.D., and Dixon, K.W. (2009) Terrestrial orchid conservation in the age of extinction. *Ann. Bot.*, **104**, 543–556.
10. Suzuki, R.M., Moreira, V.C., Pescador, R. and Ferreira, W.D.M. (2012) Asymbiotic seed germination and *in vitro* seedling development of the threatened orchid *Hoffmannseggella cinnabarina*. *In Vitro Cell. Dev. Biol. Plant*, **48**, 500-511.

11. Knudson, L. (1922) Non-symbiotic germination of orchid seeds. *Bot. Gaz.*, **73**, 1-25.
12. Knudson, L. (1946) A new nutrient solution for the germination of orchid seed. *Amer. Orchid Soc. Bull.*, **14**, 214-217.
13. Mayer, J.L.S., Stancato, G.C. and Glória, B.A.D. (2010) Direct regeneration of protocorm-like bodies (PLBs) from leaf apices of *Oncidium Flexuosum* Sims (Orchidaceae). *Plant Cell Tiss. Org. Cult*., **103**, 411-416.
14. Paek, K.Y., Hahn, E.J., and Park, S.Y. (2011) Micropropagation of *Phalaenopsis* orchids *via* protocorms and protocorm-like bodies. *Methods Mol. Biol.*, **710**, 293-306.
15. Lesar, H., Čeranič, N., Kastelec, D., and Luthar Z. (2012) Asymbiotic seed germination of *Phalaenopsis* Blume orchids after hand pollination. *Acta Agric. Slov*., **99**, 5-11.
16. Shekarriz, P., Kafi, M., Dianati Deilamy, Sh., and Mirmasoumi, M. (2014) Coconut water and peptone improve seed germination and protocorm like body formation of hybrid *Phalaenopsis*. *Agric. Sci. Dev.*, **3**, 317-322.
17. Murdad, R., Latip, M.A., Abdul Aziz, Z., and Ripin, R. (2010) Effect of carbon source and potato homogenate on *in vitro* growth and development of Sabah's Endangered orchid: *Phalaenopsis gigantean*. *Asia Pac. J. Mol. Biol. Biot.*, **18**, 199-202.
18. Tokuhara, K., and Mii, M. (1993) Micropropagation of *Phalaenopsis* and *Doritaenopsis* by shoot tips of flower stalk buds. *Plant Cell Rep.*, **13**, 7–11.
19. Duan, X.J., Chen, H., and Yazawa, S (1996) In vitro propagation of *Phalaenopsis via* culture of cytokinin-induced nodes. *J. Plant Growth Regul.*, **15**, 133-137.
20. Yifei, W., and Zhuping, Y.A. (1996) Study on *in vitro* propagation of *Phalaenopsis* hybrid and *Cattleya auranliace*. *Acta Agric*. *Shanghai*, **12**, 59-62.
21. Rotor, G. (1949) A method of vegetative propagation of *Phalaenopsis* species and hybrids. *Amer. Orchid Soc. Bull.*, **18**, 738-739.
22. Griesbach, R.J. (1983) The use of indoleacetylamino acid in the *in vitro* propagation of *Phalaenopsis* orchids. *Sci. Hort.*, **19**, 363–366.
23. Park, S.Y., Murthy, H.N., and Paek, K.Y. (2002) Rapid propagation of *Phalaenopsis* from floral stalk derived leaves. *In Vitro Cell Dev. Biol. Plant*, **38**, 168-172.
24. Ishii, Y., Takamura, T., Goi, M., and Tanaka, M. (1998) Callus induction and somatic embryogenesis of *Phalaenopsis*. *Plant Cell Rep.*, **17**, 446-450.
25. Tokuhara, K., and Mii, M. (2001) Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds in *Phalaenopsis* (Orchidaceae). *In Vitro Cell Dev. Biol. Plant*, **37**, 457-461.
26. Tokuhara, K., and Mii, M. (2003) Highly efficient somatic embryogenesis from cell suspension cultures of *Phalaenopsis* orchids by adjusting carbohydrate sources. *In Vitro Cell Dev. Biol. Plant*, **39**, 635-639.
27. Chen, J.T., and Chang, W.Ch. (2004) Induction of repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. *formosa* Shimadzu. *In Vitro Cell Dev. Biol. Plant*, **40**, 290-293.
28. Kuo, H.L., Chen, J.T., and Chang, W.C. (2005) Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. *In Vitro Cell Dev. Biol. Plant*, **41**, 453-456.
29. Chen, J.T., and Chang, W.Ch. (2006) Direct somatic embryogenesis and plant regeneration from leaf explants of *Phalaenopsis amabilis*. *Biol*. *Plant*., **50**, 169-173.
30. Gow, W.P., Chen, J.T., and Chang, W.Ch. (2009) Effects of genotype, light regime, explant position and orientation on direct somatic embryogenesis from leaf explants of *Phalaenopsis* orchids. *Acta Physiol*. *Plant.*, **31**, 363-369.
31. Gow, W.P., Chen, J.T., and Chang, W.Ch. (2010) Enhancement of direct somatic embryogenesis and plantlet growth from leaf explants of *Phalaenopsis* by adjusting culture period and explant length. *Acta Physiol*. *Plant*., **32**, 621-627.
32. Huang, Y.J., and Lee, F.F. (2010) An automatic machine vision-guided grasping system for *Phalaenopsis* tissue culture plantlets. *Comput*. *Electron. Agric.*, **70**, 42-51.
33 Duncan, D.B. (1955) Multiple range and multiple F test. *Biometr.*, **11**, 1-42.

34. Sharifi, G., Mirmasoumi, M., Zahed, Z., and Entezari, M. (2014) *In vitro* seed culture and micropropagation of *Phalaenopsis* hybrid. 18th National and 6th International Congress of Biology, Iran. Kharazmi University, Karaj, Iran. P. 159.
35. Thongpukdee, A., Thepsithar, C., and Rojanawong, T. (2010) Optimum conditions for seed germination of *Phalaenopsis* Silky Moon. *Acta Hortic*., **878**, 237-242.
36. Amaki, W., and Higuchi, H. (1989) Effects of dividing on the growth and organogenesis of protocorm-like bodies in *Doritaenopsis*. *Sci. Hort*., **39**: 63–72.
37. Hossain, M.M., Sharma, M., and Pathak, P. (2012) *In vitro* propagation of *Dendrobium aphyllum* (Orchidaceae)- seed germination to flowering. *J. Plant Biochem. Biot*., **22**, 157-167.
38. Rangsayatorn, N. (2009) Micropropagation of *Dendrobium draconis* Rchb.f. from cross-section culture. *Sci. Hort.*, **122**, 662-665.
39. Yong, J.W., Ge, L., Ng, Y.F., and Tan, S.N. (2009) The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. *Molecules*, **14**, 5144-5164.
40. Vacin, E.F., Went, F.W. (1949) Some pH changes in nutrient solutions. *Bot Gaz*., **110**, 605-613.
41. Paul, S., Kumaria, S. and Tandon, P. (2012) An effective nutrient medium for asymbiotic seed germination and large-scale *in vitro* regeneration of *Dendrobium hookerianum*, a threatened orchid of northeast India. *AOB Plants*, **32**, 1-7.
42. Thepistar, C., Thongpukdee, A., and Kukieatdetsakul, K. (2009) Enhancement of organic supplements and local fertilizers in culture medium on growth and development of *Phalaenopsis* " Silky Moon" protocorm. *Afr. J. Biotechnol.*, **8**, 4433-4440.
43. Arditti, J., and Ernst, R. (1993) *Micropropagation of Orchid*. John Wiley & Sons, Inc., New York.
44. Balilashaki, K. (2015) Asymbiotic germination of *Phalaenopsis* cv." Dublin" in relation to pollination months and nutrient media. *Not. Sci. Biol.*, **7**, 330-333.
45. Diaz, L.P., Namur, J.J., Bollati, S.A., and Arce, O.E.A. (2010) Acclimatization of *Phalaenopsis* and *Cattleya* obtained by micropropagation. *Rev. Colomb. Biotecnol*., **12**, 27-40.