Acclimatization of *in vitro* Germinated Seedlings of Tiger Orchid (*Grammatophyllum speciosum* Blume.) in Hydroponic Culture Using Dynamic Root Floating Technique (DRFT) with Chitosan Spraying

P. Sutthinon*, K. Pan-aon, U. Meesawat and A. Jantasilp

Department of Biology, Faculty of Science, Prince of Songkla University, Songkhla, 90112, Thailand

**Corresponding author, Email: clipy.pornsawan@gmail.com*

Abstract

In vitro seedlings of *Grammatophyllum speciosum* Blume. were transplanted to *ex vitro* acclimatization by growing in dynamic root floating technique (DRFT) hydroponic culture with two levels of nutrient ionic strength of KMITL (King Mongkut's Institute of Technology Ladkrabang) formula. During acclimatization period of 125 days, plantlets were sprayed weekly with chitosan solution of 0, 10 and 15 ppm. The experiment was conducted under a glass-roof greenhouse during monsoon period (September-January) with high relative humidity, relatively low temperature, and low light intensity. Throughout the experiment, leaf number and leaf length were recorded for growth comparison of two nutrient strengths and different chitosan concentrations. Plant growth increased equally for all treatment combinations. The acclimatized plantlets were able to survive in 10-fold-diluted nutrient solution (75-87.5%) slightly better than in 5-fold dilution (66.7-75.0%) while chitosan treatment had no clear effect on plant survival. After harvesting, growth data of the orchid plantlets in two levels of nutrient strength with various concentrations of chitosan application were collected and analyzed for statistical differences. All leaf number, leaf length, root number and plant fresh weight were not significantly different.

Keywords: acclimatization, hydroponic culture, chitosan, Tiger Orchid (*Grammatophyllum speciosum* Blume.)

Introduction

Plantlet acclimatization, the final stage of *in vitro* micropropagation for plantlet adaptation to ex vitro conditions, is as important as the prior stages. Since the ex vitro climates are different from *in vitro* ones, i.e., lower relative humidity, variable air temperature, and high light intensity, these cause high rate of water loss in transplanted plants because the structures and functions of *in vitro* developed plants are different from greenhouse-grown plants, e.g. thin, soft leaves with poorly developed wax (cuticle) on leaf surface and inactive stomatal closure mechanism (Pierik, 1987; Zimmerman, 1988). Thus, various techniques have been employed to improve acclimatization efficiency of micropropagated plants (Pierik, 1987; Zimmerman, 1988).

Hydroponic system is possibly the potential method for hardening acclimatized plantlets since nutrient solution in hydroponic culture can compensate water loss by transpiration and also provides nutrients for plant growth. Thus, hydroponic culture is partially similar to *in vitro* culture in liquid medium. However, there have been few reports on the acclimatization of ex vitro orchid plantlets in hydroponic system, except some reports on growth of potted mature orchid plants. In the production of potted *Phalaenopsis* plants, several hydroponic systems were investigated for the improvement of this orchid growth and quality (Lee and Lee, 2004). Nutrient film technique (NFT) and

ebb-and-flow (EBB) system have been reported to provide higher vegetative growth than other irrigation systems. In addition, flower production with good quality was the highest in the EBB culture (Hwang et al., 2004).

In commercial orchid farms for cut flowers, the introduction of hydroponic technology in orchid cultivation has not been widely acceptable because of several problems, especially root rot of epiphytic orchids, that still remains to be solved. Thus, Temasek Polytechnic in Singapore developed a hydroponic technique called "Precise Influx Hydroponic Growth System" (PIHGS) which could successfully eliminate root rot problem and for the most important goal of orchid farming, pseudobulb formation and flower production were increased significantly when compared to traditional practices (Leow and Tan, 2007).

Chitosan, exhibiting a role as plant growth promoter, was investigated for its growth-stimulating activities in *in vitro* micropropagation of orchids, as well as greenhouse-grown plants. In the *in vitro* culture of *Dendrobium* 'Eiskul' orchid, six chitosan types, polymeric and oligomeric forms with three different degrees of deacetylation (DD), were tested for the stimulation efficiency in protocorm-like body multiplication, shoot regeneration, plantlet development, and finally plantlet acclimatization (Pornpienpakdee et al., 2010).

During acclimatization, when transplanting plantlets were sprayed with chitosan, all types of polymeric form, except at the highest concentration (80 mg L^{-1}), could support 100% survival. The positive effect on plant survival is possibly due, partially, to chitosan activity on the reduction of stomatal aperture (Lee et al., 1999) which consequently decreases water loss by transpiration through stomata. In addition to survival enhancement, an appropriate type and concentration of chitosan could promote vegetative growth of ex vitro acclimatized plants (Pornpienpakdee et al., 2010).

The application of chitosan also affected greenhouse-grown plants. Willow cuttings rooted in nutrient solution added with chitosan were stimulated both root and shoot growth (Gryczka et al., 2008). In *Paphiopedilum niveum* orchid, chitosan from mushrooms induced both plant growth and resistance to fungal diseases (Samae et al., 2008). The spray of chitosan on *Dendrobium* 'Eiskul' plants, floral production was promoted while shoot growth was not significantly increased (Limpanavech et al., 2008). According to the enhancement of flower production, chitosan application was evaluated at farming level for orchid cut-flower production. Flower productivity of *Dendrobium* 'Sensational Purple' plants sprayed with chitosan were significantly increased with high quality of cut flowers (Chandrkrachang et al., 2005). On the other hand, chitosan application to *Dendrobium* Sonia 'No. 17' plants at early flowering stage was not effective in improving the inflorescence quality after harvest (Uthairatanakij et al., 2008). The orchid response in this investigation differed from the above is probably due to chitosan types, concentrations, applying method, and orchid cultivars.

Tiger Orchid (*Grammatophyllum speciosum* Blume.), the largest orchid species, is the only one species in genus *Grammatophyllum* that was found in Thailand. Due to the slow growing and the rare status of Tiger orchid, it has been studied on micropropagation of this species for rapid propagation. Chitosan stimulating on the growth of *Grammatophyllum speciosum* protocorm-like bodies (PLBs) was examined throughout the *in vitro* culture (Sopalun et al., 2010). The results indicated that 15 mg L^{-1} chitosan supplemented in liquid medium was the optimal concentration giving the highest relative growth rate of PLBs. Therefore, the suitable method of acclimatization is still needed for plantlet survival of this species.

The aim of this research was to evaluate growth and survival of transplanted orchid seedlings acclimatized in hydroponic culture with chitosan spraying. The *in vitro* germinated seedlings of Tiger orchid (*Grammatophyllum speciosum* Blume.), the largest tropical orchid species, was employed as the experimental plant material.

Materials and Methods

The 9-month-old *Grammatophyllum speciosum* Blume. seedlings from *in vitro* seed germination were provided by the Agricultural Occupation Promotion and Development Center (Tissue Culture) in Trang province, Thailand. Plantlets were cleaned and soaked in fungicide solution

(Eulaxyl^{TM}) for 15 mimutes. Uniform plantlets (Figure 1) were selected and divided into 3 groups of control plants and chitosan-treated plants. Chitosan O-80 (45,000 MW, >90% DD) with concentration of 10,000 ppm was purchased from Center for Chitin-Chitosan Biomaterials, Chulalongkorn University, Bangkok (Limpanavech et al., 2008). The treated plantlets were immersed in chitosan solution of 10 ppm and 15 ppm for 30 minutes while distilled water was used for the control plantlets. Following chitosan pretreatment, individual plantlets were planted in a plastic pots (5.5 cm height and 5.5 cm diameter) filled with expanded-clay beads of 0.5 cm diameter.

The recirculating hydroponic system used in this study was DRFT or Dynamic Root Floating Technique which is able to adjust nutrient solution depth depending on the length of plant roots. Two rectangular basins of 102×140×10 cm (W× L× H) were filled with nutrient solution of KMITL (King Mongkut's Institute of Technology Ladkrabang) (Table 1) at 2 levels of 5-fold and 10-fold dilutions. The diluted nutrient solution was circulated in the system by pumping from the reservoir tank to the opposite end of the basin. Pots with orchid plantlets were inserted into the holes (5×5 cm spacing) on foam sheets covering the top of the basin (Figure 2).

The basins with 2 levels of nutrient concentrations were main plots and plantlets in each basin were divided into 3 groups of chitosan treatment. The plantlets previously pretreated with chitosan (0, 10, and 15 ppm) were sprayed with chitosan of similar concentrations as pretreated once a week in the morning throughout the experiment. The experiment was a 2×3 factorial design with 3 replications and 10 plants per experimental unit and it was carried out in an open sided greenhouse with a glass roof.

Physical conditions during the experimental period, the climatic conditions fluctuated because it was monsoon season (September-January). The daily accumulation of phytosynthetic photon flux (PPF) varied between 348-5330 mmole m^{-2} and maximum temperature was 26-34°C and minimum was 22-28°C. The humidity was relatively high with RH 62-91 %. The climatic conditions of relatively low light intensity, relatively low

Figure 1 Seedlings of *Grammatophyllum speciosum* Blume. 9-month-old germinated from *in vitro* seed culture.

Figure 2 Ex vitro transplanted seedlings of *Grammatophyllum speciosum* Blume. in a hydroponic system of the dynamic root floating technique (DRFT).

Table 1 Composition of King Mongkut's Institute of Technology Ladkrabang (KMITL) nutrient solution for hydroponic culture.

Salt constituent	Amount (g) in 10 L	Preparation of nutrient solution at full strength
Stock solution A		
$Ca(NO_3)_2.4H_2O$	1797	1L stock A+1L stock B added to water, making 80L final volume.
Fe-EDTA	81.5	
Stock solution B		
KNO_3	1012	
KH_2PO_4	105	
$NH_4H_2PO_4$	190	
$MgSO_4.7H_2O$	495	
$MnSO_4.H_2O$	7.5	
$CuSO_4.5H_2O$	0.51	
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.17	
$ZnSO_4.7H_2O$	2.38	
H_3BO_3	6.23	

temperature and high RH were optimum for acclimatization of *in vitro* plantlets. The pH of recirculating nutrient solution was maintained at 6.0-6.5 while the EC of 5-fold-diluted solution was higher (0.82-1.09 mS cm^{-1}) than those of 10-fold-diluted one (0.48-0.67 mS cm^{-1}). To compare the difference of plant growth in various treatments, length of leaf and number of leaf per plant were also recorded weekly. At the end of the experiment (125 days), the percentage of survived plantlets and yield parameters was evaluated as number of leaf per plant, root numbers per plant, leaf length and fresh weight of the plants. The data were analyzed for statistically difference by two-way ANOVA at the significant level of 0.05.

Results and Discussion

The combination of ionic strength of nutrient solution in hydroponic culture and folia spray of chitosan solution had no effect on growth of acclimatized seedlings. Leaf length increased gradually during 125 days (Figure 3) while number of leaf per plant increased during the first 6 weeks but it decreased subsequently because of leaf abscission (Figure 4). When plant survival and plant growth were evaluated after the termination of the experiment (125 days) the survival percentage of plantlets cultured in 10-fold-diluted nutrient solution was higher than those in the 5-fold-diluted one (Figure 5). This indicated that the ionic strength of KMITL nutrient solution was too high for survival of the acclimatized seedlings of this orchid species. The culture of potted *Phalaenopsis* in an ebb and flood hydroponic system was also observed, in that, lower ionic strength of nutrient solution promoted its growth and quality (Hwang et al., 2004). On the other hand, chitosan ineffectively supported survival of this Tiger orchid seedlings, compared to the control treatment, while a 100% survival rate of acclimatized plantlets has been reported in *Dendrobium* 'Eiskul' orchid sprayed with polymeric chitosan (Pornpienpakdee et al., 2010). The survival difference of plantlets is possible because oligomeric chitosan used in our investigation was not effective, compared to the polymeric form. Plants grown in 10-fold-diluted solution without chitosan application had the highest survival percentage (87.5%) while the lowest percentage of 66.7% were in 5-fold-diluted solution with 10 ppm chitosan spraying.

At the end of the trial (125 days after transplanting), when the effect of ionic strength of nutrient solution in hydroponic culture on vegetative growth of *Grammatophyllum speciosum* Blume. was examined, all growth parameters, i.e. number of leaf per plant, number of root per plants, leaf length, and plant fresh weight were not significantly different (Table 2). On the contrary, the various ionic strengths of ebb and flood hydroponic culture caused significantly different

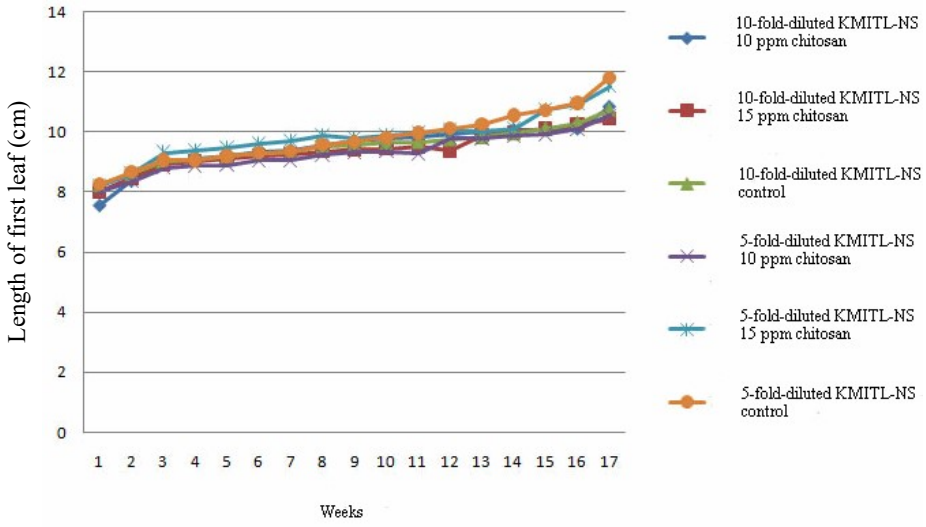

Figure 3 Growth of leaf length of *Grammatophyllum speciosum* Blume. as affected by KMITL (King Mongkut's Institute of Technology Ladkrabang) nutrient dilutions and chitosan concentrations during 125 day acclimatization.

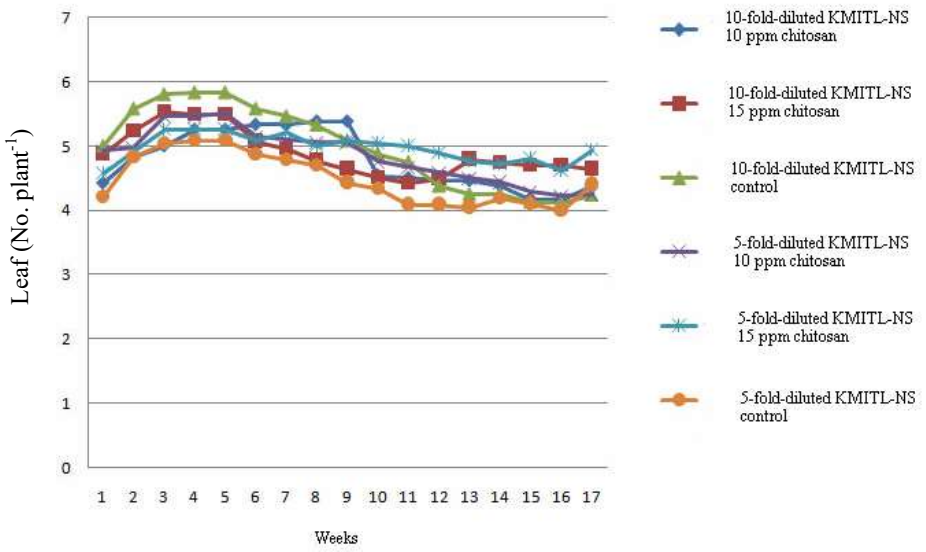

Figure 4 The increase of number of leaf of *Grammatophyllum speciosum* Blume. as affected by KMITL (King Mongkut's Institute of Technology Ladkrabang) nutrient dilutions and chitosan concentrations during 125 day acclimatization.

Table 2 Combined effects of KMITL (King Mongkut's Institute of Technology Ladkrabang) nutrient dilutions and chitosan concentrations on growth parameters of *Grammatophyllum speciosum* Blume. in DRFT dynamic root floating technique hydroponic culture for 125 days.

Nutrient concentrations	Chitosan concentrations (mg kg^{-1})	Leaves (No. plant^{-1})	Roots (No. plant^{-1})	Leaf length (cm)	Fresh weights (g)
5-fold-diluted KMITL	0	4.5±1.1	7.1±4.5	13.0±2.5	2.9±2.1
	10	4.4±1.0	7.1±2.4	11.4±2.5	2.8±1.3
	15	4.9±2.3	8.2±3.8	12.1±1.4	2.7±1.0
10-fold-diluted KMITL	0	4.3±1.2	5.7±1.4	11.6±1.9	2.5±0.9
	10	4.4±1.5	7.2±2.6	12.1±1.9	2.7±1.9
	15	4.7±1.1	6.4±1.1	11.6±1.8	2.8±1.1

Values are presented as means ± SD. Data analyzed according to two-way ANOVA was not significant different for both nutrient dilutions and chitosan concentrations treatment

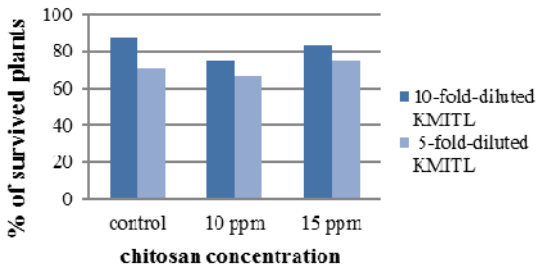

Figure 5 The survival percentage of plants cultured in 10-fold-diluted and 5-fold-diluted KMITL (King Mongkut's Institute of Technology Ladkrabang) nutrient solution with chitosan spraying after the termination of the experiment (125days)

vegetative growth of *Phalaenopsis* (Hwang et al., 2004). In half strength of nutrient solution, the greatest number of leaves, leaf length, leaf width, and fresh and dry weight were reported. The differences of the results are possibly due to different formula of nutrient solution and/or the orchid species used in both experiments. Nevertheless, employing the hydroponic system compared with conventional practices improved vegetative growth and quality of potted *Phalaenopsis* (Lee and Lee, 2004; Hwang et al., 2004).

The response of *Grammatophyllum speciosum* Blume. seedlings to chitosan treatments was not significantly different, corresponding to the effect of chitosan of the same type applied to *Dendrobium* 'Eiskul' plants (Limpanavech et al., 2003; Limpanavech et al., 2008). Though foliar application of chitosan could not promote vegetative growth of *Dendrobium* orchid plants, it was effective in inducing early flowering, more flower bud production, and good quality of flowers (Limpanavech et al., 2003; Chandrkrachang et al., 2005; Limpanavech et al., 2008). Moreover, chitosan extracted from various mushrooms was effective in improving vegetative growth of *Paphiopedilum niveum* plants (Samae et al., 2008). Therefore, the ineffective stimulation by chitosan in our experiment is possibly due to the tested chitosan produced from different sources and/or different orchid species responding differently.

Conclusions

During the experiment for 125 days, the *in vitro* germinated seedlings acclimatized by growing in hydroponic culture with chitosan foliar spraying, provided identical growth from all treatments. At the end of the experiment, survival percentage of the acclimatized plantlets in 10-fold-diluted nutrient solution was higher than those in 5-fold-diluted blend while chitosan application was ineffective to support plant survival. In addition, all of the examined growth parameters, i.e. leaf number, root number, leaf length, and fresh weight were not significantly different for both ionic strength levels and chitosan concentrations applied.

Acknowledgements

This research was financially supported and facilitated by Department of Biology, Prince of Songkla University. We are also grateful to Trang Agricultural Occupation Promotion and Development Center (Tissue Culture) for providing the *in vitro* plant materials. We also thank our members working on the botanical program, Ngernmuen A. and Cheadchim T. for their valuable help and suggestions for this study.

References

Chandrkrachang, S., P. Sompongchaiyakul and S. Sangtain. 2005. Profitable spin-off from using chitosan in orchid farming in Thailand. Journal of Metals, Materials and Minerals 15: 45-48.

Gryczka, U., H. Gawronska, W. Migdal, S.W. Gawronski and A.G. Chmielewski. 2008. Study on biological activity of chitosan after radiation processing, Nukleonika 53: S73-S76.

Hwang, S.J., M.R. Huh, J.I. Chung and B.R. Jeong. 2004. Growth of *Phalaenopsis* in a recirculating ebb and flood hydroponic system as affected by ionic strength of solution and medium composition. pp. 637-645. In P.J. Cantliffe, P.J. Stoffella and N. Shaw, eds., Proc. VII IS on Prot. Cult. Mild Winter Climates. Acta Hort 659.

Lee, Y.B. and D.S. Lee. 2004. Selection of optimal hydroponic system for growth of *Phalaenopsis*. J. Kor. Soc. Hort. Sci. 45: 109-114.

Lee, S., C. Shu, H.S. Doo, L.S. Oh, K.Y. Choi, E.J. Schroeder. A.T. Taylor, R.S. Low and Y. Lee. 1999. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis.* Plant Physiol. 121: 147-152.

Leow, A.C.T. and T.K.K. Tan. 2007.Versatile hydroponic technology for commercial orchid cultivation, pp. 75-83. In K.K. Chow, ed., Proc. Int. Cof. And Exhibition on Soilless Culture. Acta Horticulturae 742.

Limpanavech, P., S. Chaiyasuta, R. Vongpromek, R. Pichyangkura, C. Khunwasi, S. Chadchawan, P. Lotrakul, R. Bunjongrat, A. Chaidee and T. Bangyeekhun. 2008. Chitosan effects on floral production, gene expression, and anatomical changes in the *Dendrobium* orchid. Sci. Hort. 116: 65-72.

Limpanavech, P., R. Pichyangkura, C. Khunwasi, S. Chadchawan, P. Lotrakul, R. Bunjongrat, A. Chaidee and T. Akaraeakpanya. 2003. The effect of polymer type, concentration and %DD of bicatalyte modified chitosan on floral production of *Dendrobium* 'Eiskul', pp. 60-64. In National Chitin-Chitosan Conference. Chulalongkorn University. July 17-18, 2003. Bangkok, Thailand.

Pierik, R.L.M. 1987. *In vitro* Culture of Higher Plants. Martinus Nijhoff Publishers, Dordrecht.

Pornpienpakdee, P., R. Singhasurasak, P. Chaiyasap, R. Pichyangkura, R. Bunjongrat, S. Chadchawan, and P. Limpanavech. 2010. Improving the micropropagation efficiency of hybrid *Dendrobium* orchids with chitosan. Sci. Hort.124: 490-499.

Samae, A., R. Tamhoi, P. Asawatreratanakul and K. Asawatreratanakul. 2008. Effect of local mushroom chitosan on PR protein activation in Lady's Slipper orchid (*Paphiopedilum niveum*), pp.1-9. In 34th Congress on Science and Technology of Thailand. 31 October-2 November, 2008. Bangkok, Thailand.

Sopalun, K., K. Thammasiri and K. Ishikawa. 2010. Micropropagation of the Thai orchid

Grammatophyllum speciosum blume. Plant Cell Tiss Organ Culture. 101:143-15.

Uthairatanakij, A., P. Jitareerat and K. Obsuwan. 2008. Efficacy of chitosan spraying on improving quality of *Dendrobium* Sonia 'No.17' inflorescence, pp.291-298. In R.A. Criley, ed,. Proc. XXVII IHC-S5.

Zimmerman, R.H. 1988. Micropropagation of woody plants: Post tissue culture aspects, pp.489-499. In F. Loreti, ed., International Symposium on Vegetative Propagation of Woody Species. September 3-5, 1987. Pisa, Italy.

Manuscript received 25 September 2014, accepted 11 April 2016