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# SURVIVAL RATE AND GROWTH PERFORMANCE OF *IN VITRO* RAISED PLANTLETS OF ORCHID (*DENDROBIUM* SP.) IN DIFFERENT HARDENING SUBSTRATES

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## ABSTRACT

Orchids are the most beautiful flowers and they are valued for cut flower production. They are known for their long lasting beautiful flowers which occupy a very high price in the market. Micropropagation plays an important role to meet growing market demand. The present study was carried out to standardize potting media for hardening of the *in vitro* raised plantlets of *Dendrobium* sp. under net house conditions. The potting media used for acclimatization contained coconut husk, brick pieces, charcoal and chip stones in different ratios. The observations were recorded on survival percentage, shoot length, number of leaves per plant, leaf width, number of root per plant, length of longest root after transplanting to *ex vitro* conditions. In this experiment, the treatment contained coconut husk: brick pieces: charcoal: chip stones at ratio 1:1:1:1 proved the best potting medium for higher survival rate (90%) at four weeks after transplanting to *ex vitro* conditions and for the subsequent development of plantlets under *ex vitro* conditions during acclimatization.

**Keywords:** Acclimatization, orchid, *in vitro* plantlets, potting medium,

## INTRODUCTION

Orchids are important floricultural plants which belong to the family Orchidaceae. They are widely distributed all over the world and considered as largest flowering plant with 20,000 to 35,000 species (Chugh *et al*., 2009). Although there are many varieties of orchid like *Dendrobium, Epidendordium, Phalaenopsis, Onicidium, Vanda, Cattleya etc. Dendrobium* orchid is the popular in global flower market (Puchooa, 2004). They are generally grown as an ornamental plant and are used as potted plants and cut flowers (Chugh *et al*., 2009). It can be propagated by seeds or propagated vegetatively through division, offshoots and cuttings. Seed propagation of orchid is greatly difficult due to very tiny seeds and their symbiotic association with mycorrhizal fungi for germination (Dressler, 1990). Hence, conventional propagation is mainly done by using vegetative tissues. But, it is limited due to slow growth rate and lack of plant materials for orchid production within a short period (Martin and Madassery, 2006). *In vitro* propagation of horticultural crops using seed culture is not generally advisable because plants produced by them are not genetically uniform and have the long juvenile period before flowering (Decruse *et al*., 2003). Several orchid species had been effectively propagated under *in vitro* conditions using different explants (Martin and Madassery, 2006).

There are developed protocols for the large scale propagation of orchid species through *in vitro* culture of various plant parts including shoot tips, flower stalk, nodes, buds, stems, root tips and rhizome segments (Chang and Chang, 2000; Chugh *et al*., 2009; Paudel and

Pant, 2012). Regeneration of multiple shoots without callus stage reduces the regeneration duration and the occurrence of somaclonal variation (Polonca *et al*., 2004). It is an essential to develop protocol to obtain clones from various vegetative parts of mother plants. The *in vitro* micropropagation technique is an alternative method to obtain sufficient, disease-free, uniform plant materials rapidly from vegetative parts of mother plants. Those clones can be used by orchid growers for its commercial cultivation. *In vitro* plantlets of orchid are successfully developed by using appropriate culture medium containing plant growth regulators (Aktar *et al*., 2008). *In vitro* culture techniques are widely used for plant multiplication and germplasm conservation (Engelmann, 2011).

Micropropagation has been largely used for the rapid multiplication of disease free plants under *in vitro* conditions. But there may be plant damaged during transferring of *in vitro* plantlets to *ex vitro* conditions. The percentage of plant loss depends on hardening substrates, environmental factors and plant species. Thus, acclimatization of *in vitro* plants to greenhouse environment is an important stage to obtain quality plants of orchids for the commercial cultivation. Plant mortality is high when transfer of *in vitro* microshoots to *ex vitro* conditions because the cultured plants under *in vitro* conditions have weak root system and poorly developed cuticle (Mathur *et al*., 2008). The plantlets at this stage are vulnerable to high percentage of damage and loss (Ortega-loeza *et al*., 2011). Hence, it is necessary to select suitable growing medium in the acclimatization stage and also provide optimal environmental conditions to obtain high survival rates of *in vitro* plantlets under *ex vitro* conditions.

## MATERIALS AND METHODS

This study was done during 2017-2018 at orchid nursery in Marawila, Nattandiya DS Division, Puttalam District, Sri Lanka to find out the suitable potting medium for *ex vitro* acclimatization of *in vitro* plantlets of orchid. Hardening substrates such as coconut husk, brick pieces, charcoal and chip stones were collected to prepare the different potting media for acclimatization of *in vitro* plantlets. The substrate pieces having 1.0 - 3.0 cm long were taken.

### Sterilization of potting materials

The charcoal and coconut husk were soaked in water for three days to remove acidic condition of those substrates. All substrate pieces were put separately into the clean cloth bags and they were then placed inside the water bath for 1 hr. After that, all the materials of potting media were treated by using 0.1% of captan for 30 min to reduce the contamination before making potting mixtures.

### Collection of *in vitro* plantlets

*In vitro* raised plantlets were purchased from Alo-Anthura nursery in Marawila, Nattandiya DS Division, Puttalam District for hardening of plantlets under *ex vitro* conditions. Tiny plantlets measuring 3.0-4.0 cm in height and having 3-4 leaves were taken out carefully from the *in vitro* culture vessels.

### Sterilization of *in vitro* plantlets

*In vitro* plantlets were taken outside carefully from the culture vessels and placed in a plastic tray. They were then washed in luke warm water for 10 min to remove any culture medium adhered to their roots. After that, they were washed with 0.01% captan for 1 hr and then carefully placed in a plastic tray. Subsequently the plantlets were kept on the tissue paper which was placed inside the growth chamber for three days before being planted on potting media.

**Preparation of potting media**

The different combinations of substrates (Table 1) were filled approximately three quarter of each clay pot (4.5 cm in height, 18 cm in perimeter and 5 cm in diameter).

**Table 1: The potting media used in this experiment**

| Treatments | Composition of potting media |
|---|---|
| T1 (Control) | Coconut husk |
| T2 | Brick pieces |
| T3 | Charcoal |
| T4 | Brick pieces: Charcoal (1:1) |
| T5 | Coconut husk: Brick pieces (1:1) |
| T6 | Coconut husk: Charcoal (1:1) |
| T7 | Coconut husk: Brick pieces: Charcoal (1:1:1) |
| T8 | Coconut husk: Brick pieces: Charcoal: Chip stones (1:1:1:1) |

**Acclimatization protocol**

The rooted plantlets of *Dendrobium* sp. (3.0-4.0 cm height) were taken for hardening in eight different potting media as indicated in Table 1. The healthy uniform plantlets were placed on the sterilized potting media and then properly labeled them. Ten plantlets were planted in each pot which contained different potting media. The plantlets were maintained for one week in a humid chamber, covered with transparent polythene sheet, at 25±1$^{o}$C and 16 hrs light and 8 hrs dark regimes with low light radiation inside the net house to preserve the moisture. During the second week, the polythene cover was slightly opened for 1 to 2 hrs every day to reduce relative humidity inside the humid chamber. During the third week, the polythene cover used was kept open for 5-6 hrs daily. Subsequently, the plantlets were completely uncovered at the end of fourth week of the transplantation. After four week of preacclimatization, only survival and well developed plantlets were maintained (three plants per pot) for acclimatization. The plants were hardened upto ten weeks inside the net house.

Once a week, captain solution was applied (1.0 g/l) to the plantlets. In other days, water and NPK fertilizer solutions (1.0 mg/l) were applied one by one in the morning that is watering for three days and fertilizer application for three days in a week. The plants were also watered every day in the evening of first week then alterative days according to the moistness of the substrates. Watering, fertilizer and fungicide application were done at the rate of 10 ml per pot by using sprayer. The observations were recorded on shoot length, number of leaves per plant, number of roots per plant, and length of longest root after ten weeks of hardening.

**Statistical analysis**

The data collected were analyzed using analysis of variance with SAS software version 9. The differences between treatment means were separated by using Tukey's test at 5% probability level.

**RESULTS AND DISCUSSION**

*In vitro* culture technique is most effective for large scale plant production. Transfer of *in vitro* raised plantlets to *ex vitro* conditions is an important step in a successful micropropagation. Deb and Imchen (2010) stated that the broader use of micropropagation is mostly limited by high percentage of mortality or damage to plantlets during hardening process. Hardening of *in vitro* raised plantlets is a crucial prior to transplantation of the

plants to the field. The present study concerns the use of proper potting medium to increase the survival rate and sustain healthy growth of orchid plants.

**Survival of *in vitro* plantlets**

In the present study, healthy vigorous plantlets were transferred to the sterilized potting media. The result of the experiment showed that there was a significant difference (P<0.01) in the survival % among the different potting media (Table 2). Out of various potting media used for hardening, the highest survival percentage was found in T8 (90.0%) followed by T7 (83.3%), and T6 (66.6%) after four weeks of transplanting in *ex vitro* conditions. The results indicated that T8, T7 and T6 treatments were better than the control (T1). In T2, T3 and T4 treatments, survival rate ranged from 33 to36% after four weeks of transplanting. These results indicated the crucial impact of the potting mixture on successful transplantation process (Figure 1).

**Table 2: Survival % of *in vitro* plantlets in different potting media after four weeks under *ex vitro* conditions.**

| Treatments | Treatments code | Survival % |
|---|---|---|
| Coconut husk | T1 | 60.0 ± 1.29c |
| Brick pieces | T2 | 33.3 ± 0.99d |
| Charcoals | T3 | 36.3 ± 0.95d |
| Brick pieces: Charcoal | T4 | 36.3 ± 1.91d |
| Coconut husk: Brick pieces | T5 | 60.0 ± 1.29c |
| Coconut husk: Charcoal | T6 | 66.6 ± 0.70bc |
| Coconut husk: Brick pieces: Charcoal | T7 | 83.3 ± 0.63ab |
| Coconut husk: Brick pieces: Charcoal: Chip stones | T8 | 90.0 ± 0.00a |
| F value | | ** |

F test: ** - P<0.01. Data based on the availability of the survived plants. All the values are expressed as mean ± standard error of three replicates. Means followed by the same letter are not significantly different from each other at 5% significant level according to the Tukey's test.


**Figure 1: Survival plants in different potting media at four weeks after transplanting of *in vitro* plantlets to *ex vitro* conditions.**

The plantlets were grown under high humid conditions inside the culture vessels therefore high humidity and optimal temperatures around the plantlets were provided during initial days of transplantation. Sudden changes in environmental conditions especially temperature and humidity, produces wilting and increases plantlet mortality percentage (Posposilova *et al*., 1999). In the present study, relative humidity was maintained during the first week of transplantation by keeping the plantlets inside the completely closed humid chamber. However, the closing of humid chamber for a long time would increase the temperature and maximize the relative humidity which causes to damage the plantlets. Abul-Soad (2011) reported that relative humidity was gradually decreased after 3-7 days in order to reduce fungal infection and increase the adaptability of the date palm to the ambient conditions. In the present study, the humid chamber was regularly opened with increasing time duration after one week of transplantation to gradually reduce the relative humidity and temperature. The selection of a proper potting medium with high aeration, permeability and acidity is the major condition for initiation of *ex vitro* growth (Diaz *et al*., 2010).

The results showed that the potting medium having high water holding capacity and moderate aeration was the best for acclimatization of *in vitro* grown orchids. This explains why the treatment T8 and T7 showed better performance among the rest of the treatments as it contained coconut husk, brick pieces, charcoal and chip stones. Coconut husk is suitable to improve water holding capacity of the substrate and nutrient contents at the initial stage. Brick pieces and chip stones provide good aeration and mechanical support to plantlets. Charcoal is also useful to provide aeration and permeability to the potting medium.

The different potting media were in use for hardening of different *in vitro* propagated plants by various workers, Soilrite for *Carica papaya* (Agnihotri *et al*., 2004), soaked cotton for sugar cane (Gill *et al*., 2004) and charcoal chips, bricks and decayed wood or moss for epiphytic or terrestrial orchid plantlets (Deb and Imchen, 2010). The potting materials in the potting medium support plantlets and provide nutrients and water to roots of plantlets. In the present study, coconut husk in the medium was found beneficial for survival and growth of plantlets of *Dendrobium.* Sharma and Chauhan (1995) reported 100% survivability of transferred plantlets of *D. chrysanthum* on potting medium containing brick chips, charcoal, bark pieces, leaf mould, tree fern and dry sphagnum. Indhumati *et al*. (2003) reported that charcoal, brick and cocopeat in the ratio 1:1:1 was found suitable for establishment of plantlets of *Dendrobium* Hybrid Sonia.

The hardiest and vigorous plants have been found to be easier to transplant as they are less susceptible to diseases and mechanical injuries. The plantlets transferred to the pots had healthy and vigorously growing root systems, which ensured better establishment and growth. The survival rate was found to be higher when plantlets were passed through various hardening and acclimatization stages without direct transfer of the plantlets to the field. A similar response was recorded in the three different orchid species where transplants exhibited more than 80% survival in natural conditions when plantlets were passed through preacclimatization phase of nearly three months (Deb and Imchen, 2010). In other words, the survival percentage is determined by the hardening of the plantlets.

**Number of leaves per plant and shoot length**

Until four weeks after transplanting to *ex vitro* conditions, there was a successful establishment of plantlets in terms of survival rate but the rate of shoot and root development was very poor and insignificant. Therefore, plantlets were maintained further ten weeks to investigate the vegetative growth of *ex vitro* acclimatized plantlets on above eight different potting media and in order to ensure the subsequent development of the

acclimatized plants. For that well developed survival plants were maintained in the same potting media but three plants in a pot under the net house. The results showed that the number of leaves per plant, shoot length and leaf width of the plants in T8 were better than other treatments. Nevertheless, the plant height and number of leaves per plant in T6 were not significant compared to T7 but it was relatively better than all other remaining treatments. Leaf width ranged from 0.99 (T3) to 1.87 (T8). The treatment T1, T2, T3, and T4 revealed very poor growth in terms of number of leaves per plant and shoot length than the other treatments (Table 3).

**Table 3: Standardization of potting media for hardening of *in vitro* plantlets in terms of leaf performance of *Dendrobium* sp.**

| Treatments | Shoot length (cm) | Number of leaves per plant | Leaf width (cm) |
|---|---|---|---|
| T1 | 2.10 ± 0.75d | 4.43 ± 0.10c | 1.73 ± 0.31ab |
| T2 | 2.06 ± 0.03d | 3.80 ± 0.08cd | 1.14 ± 0.11bc |
| T3 | 2.06 ± 0.07d | 3.53 ± 0.21cd | 0.99 ± 0.01c |
| T4 | 2.53 ± 0.09d | 3.43 ± 0.12d | 1.24 ± 0.12bc |
| T5 | 3.23 ± 0.13c | 6.33 ± 0.13b | 1.13 ± 0.04bc |
| T6 | 3.40 ± 0.09c | 7.01 ± 0.15b | 1.46 ± 0.09abc |
| T7 | 3.93 ± 0.07b | 7.06 ± 0.15b | 1.56 ± 0.28abc |
| T8 | 4.46 ± 0.11a | 9.00 ± 0.10a | 1.87 ± 0.26a |
| F value | ** | ** | * |

F test * - P<0.05, ** - P<0.01. Data based on the availability of the survived plants. All the values are expressed as mean ± standard error of three replicates. Means followed by the same letter are not significantly different from each other at 5% significant level according to the Tukey's test.


**Figure 2: Acclimatized plants at ten weeks after transplanting in eight different potting media.**

Figure 2 shows the transplanted plants in different potting media. The growth of the plantlets of *Dendrobium* sp. studied was found to have reduced in a potting medium containing brick pieces and charcoal. This might be due to the reason that the medium containing brick pieces and charcoal could not supply enough nutrients required for the

growth of the transferred plantlets. Also, the medium was too porous and could have leached out the minimal of the nutrients available.

**Number of roots per plant and length of longest root**

This result showed that the number of roots per plant and length of longest root were differed with the medium compositions. Among all the media used in this study, the highest number of roots and longest root length were observed in the treatments T7 and T8 (Table 4). The development of the root system (root number and length) is a vital to anchor the plant and also to ensure water and nutrients absorption. This may probably be due to optimum water holding capacity, better aeration and drainage in the medium, which provides suitable condition for further growth and development of the plants (Griffis *et al.*, 1983). The present results indicated that the plants in the medium containing charcoal and coconut husk also had long root length. Charcoal medium has water holding capacity and holds fertilizer while pouring fertilizer solution to the growing medium and on subsequent watering it releases nutrient slowly to the growing plant. But medium containing brick pieces and charcoal (T4) has low number of roots and lower root length. This may be due to the poor water holding capacity of the medium, which made the medium dry and of high bulk density causing the compaction of roots.

**Table 4: Potting media for hardening of *in vitro* plantlets in terms of roots performance of *Dendrobium* sp.**

| Treatments | No. of roots per plant | Length of longest root (cm) |
|---|---|---|
| T1 | 10.13 ± 0.49bc | 4.36 ± 0.02d |
| T2 | 6.83 ± 0.07c | 3.83 ± 0.15d |
| T3 | 6.86 ± 0.15c | 4.16 ± 0.17d |
| T4 | 6.36 ± 0.22c | 3.96 ± 0.32d |
| T5 | 12.23 ± 0.40ab | 6.63 ± 0.36c |
| T6 | 11.93 ± 0.54ab | 8.56 ± 0.39b |
| T7 | 12.86 ± 0.64ab | 11.73 ± 0.21a |
| T8 | 14.86 ± 0.19a | 13.26 ± 0.04a |
| F value | ** | ** |

F test ** - P<0.01.All the values are expressed as mean ± standard error of three replicates. Means followed by the same letter are not significantly different from each other at 5% significant level according to the Tukey's test.

It showed that the transfer of plantlets to more aerated substrate after their adaptation to *ex vitro* conditions proved very significant for their subsequent rapid shoot formation and root development. These results were confirmed by Posposilova *et al*. (1999) who stated that during acclimatization of *Spathiphyllum floribundum* different stages were observed; an adaptation period with slow shoot growth and root formation, followed by a period of fast growth of roots and shoots.

## CONCLUSION

In the present study, a protocol was developed for an efficient acclimatization of *in vitro* grown plantlets to ensure subsequent growth of orchid under *ex vitro* conditions. Potting medium containing coconut husk: brick pieces: charcoal: chip stones at 1:1:1:1 ratio could be better to increase the rate of survival and the development of *in vitro* plantlets during acclimatization to *ex vitro* conditions.

**REFERENCES**

Abul-Soad, A.A. (2011). Micropropagation of Date palm using inflorescence explants. In: Jain, S.M., Al-Khayri, J.M., Johnson, D.V. (eds.). Date Palm Biotechnology. Springer, Dordrecht. 91–117.

Agnihotri, S., Singh, S.K., Jain, M., Sharma, M., Sharma, A.K. and Chaturvedi, H.C. (2004). *In vitro* cloning of female and male *Carcica papaya* through tips of shoots and inflorescence. Indian Journal of Biotechnology 3: 235-240.

Aktar, S., Nasiruddin, K.M. and Hossain, K. (2008). Effects of different media and organic additives interaction on *in vitro* regeneration of *Dendrobium* orchid. Journal of Agriculture and Rural Development 6:69-74.

Chang, C. and Chang, W.C. (2000). Effect of thidiazuron on bud development of *Cymbidium sinense* Willd *in vitro*. Plant Growth Regulation 30(2):171-175.

Chugh, S., Guha, S. and Rao, I.U. (2009). Micropropagation of orchids: a review on the potential of different explants. Scientia Horticulturae 122(4):507-520.

Decruse, S.W., Gangaprasad, A. Seeni, S. and Menon, V.S. (2003). Micropropagation and ecorestoration of *Vanda Spathulata*, an exquisite orchid. Plant Cell Tissue Organ Culture 72(2):199-202.

Deb, C.R. and Imchen, T. (2010). An efficient *in vitro* hardening technique of tissue culture raised plants. Biotechnology 9(1): 79-83.

Diaz, L. P., Namur, J. J., Bollati, S. A. and Arce, O. E. A. (2010). Acclimatization of *Phalaenopsis* and *Cattleya* obtained by micropropagation. Revista Colombiana de Biotecnologia 12(2): 27-40.

Dressler, R.L. (1990). The Orchids: Natural history and classification. Harvard University Press, USA

Engelmann, F. (2011). Use of biotechnologies for the conservation of plant biodiversity. *In vitro* Cellular and Development Biology Plant 47(1):5-16.

Gill, N.K., Gill, R. and Goshal, S.S. (2004). Factors enhancing somatic embryogenesis and plant regeneration in sugarcane (*Saccharum offinarum* L.). Indian Journal of Biotechnology 3:119-123.

Griffis, J.L. J., Hennen, G. and Oglesby, R.P. (1983). Establishing tissue cultured plants in soil. Proceedings of International Plant Propagators Society 33: 618-622.

Indhumati, K., Kannan M., Jawaharlal, M. and Amarnath V. (2003). Standardization of prehardening and hardening techniques for *in vitro* derived plantlets of *Dendrobium* Orchid Hybrid Sonia-17. Journal of Ornamental Horticulture 6(3)**:**212-216.

Martin, K.P. and Madassery, J. (2006). Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. Scientia Horticulturae 108(1):95-99.

Mathur, A., Mathur, A.K. Verma, *P.*, Yadav, S., Gupta, ML. and Darokar MP (2008). Biological hardening and genetic fidelity testing of the micro-cloned progeny of *Chlorophytum borivilianum* Sant. African Journal of Biotechnology 7(8): 1046–1053.

Ortega-Loeza, M. M., Salgado-Garciglia, R., Gomez-Alonso, C. and Avila-Diaz, I. (2011). Acclimatization of the endangered Mexican epiphytic orchid, *Laelia speciosa* (H.B.K.) Schltr. European Journal of Environmental Sciences 1 (2): 48–54.

Paudel, M.R. and Pant, B. (2012). *In vitro* plant regeneration of *Esmeralda clarke*i Rchb. f. via protocorm explant. African Journal of Biotechnology 11(54):11704-11708.

Polonca, K., Suzana, S. and Zalata, L. (2004). Direct shoot regeneration from nodes of *Phalaenopsis* orchids. Acta Agriculturae Slovenica 83(2):233–242.

Posposilova, J., Ticha, I., Kadlecek, P., Haisel, D. and Plzakova, S. (1999). Acclimatization of micropropagated plants to *ex vitro* conditions. Biologia Plantarum 42(4): 481-497.

Puchooa, D. (2004). Comparison of different culture media for the *in vitro* culture of *Dendrobium* (Orchidaceae). International Journal of Agriculture and Biology 6(5): 884-888.

Sharma, J. and Chauhan, Y.S. (1995). Establishment of *in vitro* raised seedlings of *Dendrobium chrysanthum* and *Paphiopedilum spicerianum*. Journal of Orchid Society India. 9: 37–41.