

ISSN 1682-296X (Print)

ISSN 1682-2978 (Online)



# Bio Technology



**ANSI***net*

Asian Network for Scientific Information  
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

## Effects of Carbohydrates on Callus Growth and Callus Derived Plantlet Regeneration in *Doritaenopsis* Orchid

Abu Reza Md. Mahfuzur Rahman, M. Obaidul Islam,  
A.K.M. Azad-ud-doula Prodhhan and <sup>1</sup>Syoichi Ichihashi  
Department of Crop Botany, Faculty of Agriculture,  
Bangladesh Agricultural University, Mymensingh, Bangladesh  
<sup>1</sup>Department of Life Science, Aichi University of Education,  
Hirosawa, Igaya-cho, Kariya, Aichi 448-8542, Japan

**Abstract:** *Doritaenopsis* callus utilized sucrose, maltose and sorbitol as energy sources for the growth and development, where each carbohydrate elicited a different response. Calli on sucrose supplemented New Phalaenopsis (NP) medium grew well and remained friable, bright yellow and translucent, similar to source calli, but turned green and produced Protocorm Like Bodies (PLBs) on maltose and sorbitol supplemented media. Sucrose supplemented medium was the best for callus growth over maltose and sorbitol. The initiation of PLBs from calli on maltose medium was the highest followed by sorbitol medium while the sucrose medium did not produce PLB. Sorbitol affected most intensively plantlet regeneration from PLBs over maltose and sucrose. However, maltose supplemented medium markedly enhanced the subsequent growth and development of regenerated mini plantlets over sorbitol and sucrose media.

**Key words:** *Doritaenopsis*, callus growth, plantlet regeneration, carbohydrates

### INTRODUCTION

Orchids, an interesting group of plants, are known for their intricately fabricated and long lasting colourful flowers, which account for nearly 7% of the total flowering plant species and have been increasing immensely to the international trade in floriculture. *Doritaenopsis* is one of the most popular orchid genera which is an intergeneric hybrid of *Phalaenopsis* (*Phalaenopsis* X *Doritis*). Beauty of flower, rapid growth, easiness in plantlet regeneration, year round flower production in control temperature and long lasting of flower stalk are the advantages in *Doritaenopsis* production.

*Doritaenopsis* is a monopodial orchid, which is difficult to propagate vegetatively and propagation through tissue culture has been desired. However, *in vitro* culture of *Doritaenopsis* using shoot tip, flower stalk buds and axillary buds may lead to kill or severely damage the mother plants. To overcome these problems, many researchers utilized leaves, root tips, lateral buds from young flower stalks and internodal section of flower stalks. However, none of these methods proved effective commercially in producing lots of plantlets in a short

period because of poor and low rate of PLB formation and different responses among hybrids<sup>[1]</sup>.

A feasible propagation method for year round, rapid and economical production of orchids is through callus derived plantlet regeneration<sup>[2]</sup>. *Doritaenopsis* callus has the potential for rapid mass clonal production<sup>[3,4]</sup>. Selective carbohydrates could induce PLB from this callus and subsequently produce plantlet from PLB<sup>[5]</sup>. If callus could be induced readily, remains stable genetically, grows quickly without changing its nature, produces PLBs easily and finally develops to plantlets, it could be an useful method for commercial micropropagation of *Doritaenopsis*. Callus derived plantlet regeneration in *Doritaenopsis* has been reported<sup>[6]</sup>. However, the effects of carbohydrates on growth and development of callus are not studied in detail. Therefore, the present experiment was conducted to investigate the effect of different carbohydrates like sucrose, maltose and sorbitol on callus growth and development.

### MATERIALS AND METHODS

**Preparation of callus:** Embryogenic calli of *Doritaenopsis* which were developed earlier by Ichihashi and Hiraiwa<sup>[3]</sup>

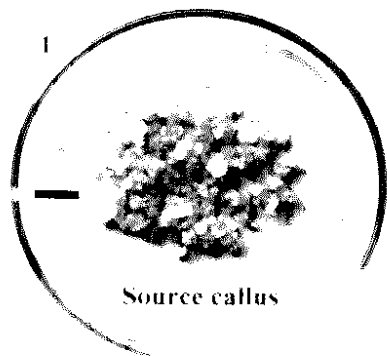


Fig. 1: Showing source callus of *Doritaenopsis* used as plant material. Scale = 1.0 cm

culturing flower stalk were maintained by subculturing monthly on NP medium supplemented with  $20 \text{ g L}^{-1}$  of sucrose. At the beginning, 1.0 g of source callus was subcultured twice on 20 mL of NP medium supplemented with  $20 \text{ g L}^{-1}$  of sucrose in 50 mL flasks at monthly intervals to obtain homogeneous callus. Uniform, friable, translucent, yellowish callus was used as plant material (Fig. 1).

**Callus culture:** Two cultures were performed in callus culture, the first culture (1st 8 weeks) and second culture (2nd 8 weeks) have been indicated as 8 weeks culture and 8 weeks  $\times$  2 culture, respectively which have been used in the subsequent results and discussion. In the first culture, 0.2 g of homogeneous callus was cultured on 20 mL of NP medium in 100 mL flask, supplemented with  $20 \text{ g L}^{-1}$  sucrose or maltose or  $10 \text{ g L}^{-1}$  sorbitol as treatments for 8 weeks to evaluate their effects on callus proliferation and PLB initiation. For the second culture, 0.2 g of proliferated callus obtained from the first culture was cultured again on the same fresh medium with same treatments for another 8 weeks.

**PLB culture:** PLBs obtained from second 8 weeks culture of callus were cultured on NP medium with same sugars for 10 weeks to investigate their effects on the growth and development of PLB. Larger than 2 mm sized uniform 8 PLBs were used in PLB culture in each flask.

**Plantlet culture:** Mini plantlets regenerated from PLB were cultured on NP medium with same supplements for 12 weeks to evaluate their effects on the subsequent growth of plantlets.

**Media and culture condition:** NP medium<sup>[6]</sup> was used as the basal medium (BM). Sucrose, maltose and sorbitol at the rate of 20, 20 and  $10 \text{ g L}^{-1}$ , respectively (equimolar

concentration of 0.056 M) were supplemented to BM separately and each treatment was replicated six times. The pH of the medium was adjusted 5.6 to 5.7 and gelrite  $3.0 \text{ g L}^{-1}$  (Merck and Co., Inc.) was added to solidify the medium. The mixture was then gently heated with continuous stirring till complete dissolution of gelrite powder. After dissolving the solidifier, 20 mL of hot medium was dispensed into 100 mL conical flasks. The conical flasks containing the medium were autoclaved with  $1.16 \text{ kg cm}^{-2}$  of pressure at  $121^\circ\text{C}$  for 21 min. After autoclaving the flasks containing the medium were allowed to cool and the callus was cultured. Cultures were maintained in a growth room and allowed to grow at  $25 \pm 1^\circ\text{C}$  under 16 h photoperiod of  $50 \mu \text{ mol m}^{-2} \text{ s}^{-1}$  illuminated with fluorescent tubes.

**Culture evaluation:** After each culture period, proliferated callus and PLBs were weighed and the number of callus derived PLBs (larger than 2 mm) per flask was counted. Different growth contributing characters of plantlets were also recorded.

**Chemical analysis:** After 8 weeks culture, the amount of total nitrogen of proliferated callus was estimated by micro-kjeldahl method. Organic carbon, P, K and S were also estimated by following the methods of Piper<sup>[7]</sup>, Olsen *et al.*<sup>[8]</sup>, Black<sup>[9]</sup> and Page *et al.*<sup>[10]</sup>, respectively.

## RESULTS AND DISCUSSION

**Effects on growth of callus:** Depending on the types of carbohydrates supplemented into the BM the growth and development of callus was different.

In first culture of 8 weeks, calli on sucrose containing medium remained friable, bright yellow and translucent, but turned green and dark green on maltose and sorbitol containing media, respectively (Fig. 2a-c). The growth of callus on sucrose medium was significantly higher than that on maltose or sorbitol supplemented media. Between maltose and sorbitol, callus growth was higher on maltose medium than that on sorbitol. The highest dry weight of callus was obtained on sucrose supplemented medium followed by maltose and sorbitol (Fig. 5). Rate of callus growth was also significantly higher on sucrose containing medium followed by maltose and sorbitol (Fig. 6). On maltose and sorbitol containing media, growth and development of callus was different to that on BM with sucrose. Callus cultured on maltose and sorbitol containing media developed very few number of PLBs in first 8 weeks culture (Fig. 3a and b).

The multiplication rate of fresh weight calli for 8 weeks on NP medium with sucrose was 13.0 folds

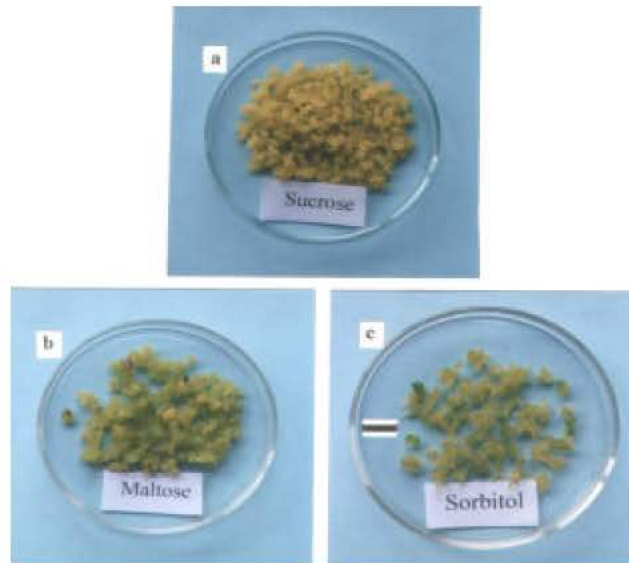


Fig. 2: Showing proliferated callus on the NP medium supplemented with sucrose (a), maltose (b) and sorbitol (c) after 8 weeks culture. Scale = 1.0 cm

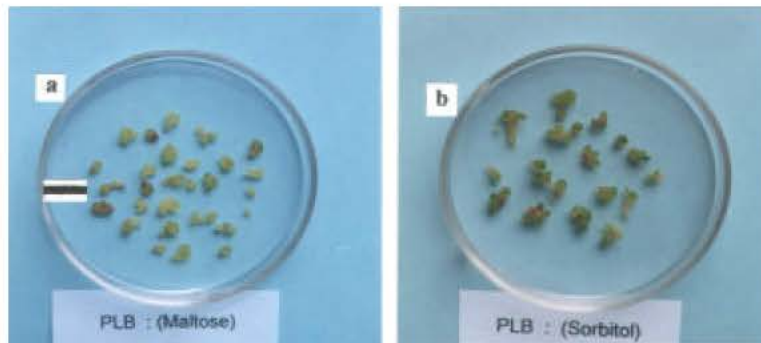


Fig. 3: Showing PLB regenerated from callus on NP medium supplemented with maltose (a) and sorbitol (b) after 8 weeks culture. Scale = 1.0 cm



Fig. 4: Showing PLB derived plantlets of *Doritaenopsis* developed on sorbitol supplemented NP medium after 8 weeks culture. Scale = 1.0 cm

Table 1: Content of C, N, P, K and S in proliferated callus after 8 weeks culture on NP medium supplemented with different carbohydrates

Treatment	%C	%N	C, N ratio	%P	%K	%S
Sucrose	42.00	0.560	75.00	0.087	1.768	0.324
Maltose	42.41	0.784	54.10	0.075	1.894	0.408
Sorbitol	41.16	0.980	42.00	0.082	2.727	0.867

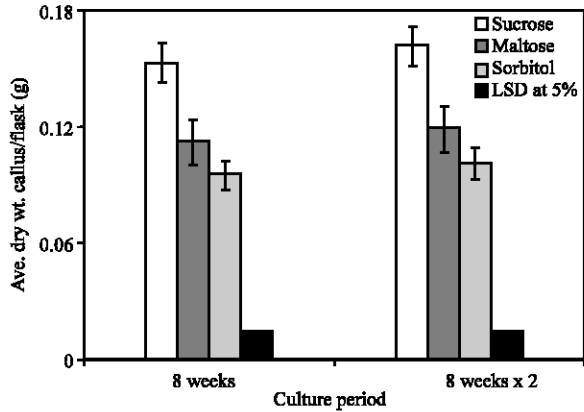


Fig. 5: Growth of *Doritaenopsis* callus cultured on sucrose, maltose and sorbitol supplemented NP medium. Linear bars indicate standard deviations

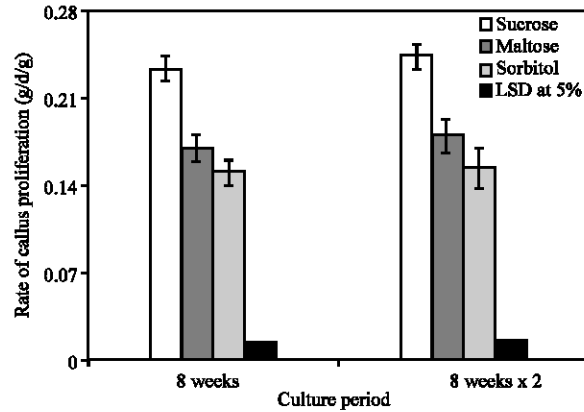


Fig. 6: Rate of *Doritaenopsis* callus proliferation cultured on sucrose, maltose and sorbitol supplemented NP medium. Linear bars indicate standard deviations

(2.607/0.2). One year is equivalent to  $6.52 \times 8$  weeks. Estimated multiplication rate per year was  $(13)^{6.52}$  or  $18.31 \times 10^6$  folds. This value is enough for mass, rapid clonal multiplication. The multiplication rate of calli for 8 weeks on NP medium with maltose and sorbitol was 9.53 and 8.42 folds and per year was  $24.19 \times 10^5$  and  $10.79 \times 10^5$  folds, respectively.

After 8 weeks  $\times$  2 culture, calli on sucrose medium also remained friable, bright yellow and translucent same as source callus. But green calli transplanted from maltose and sorbitol supplemented medium of 1st culture were

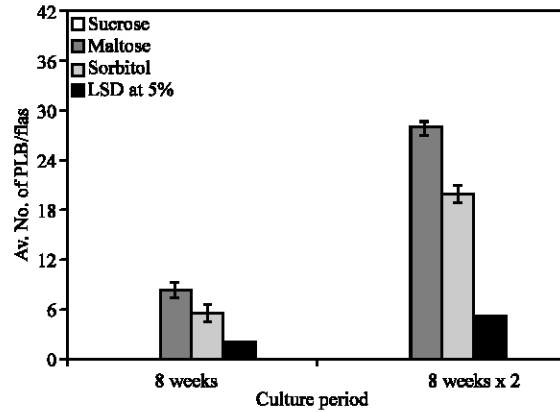


Fig. 7: PLB formation from *Doritaenopsis* callus cultured on sucrose, maltose and sorbitol supplemented NP medium. Linear bars indicate standard deviations

also remained green and developed more PLBs than first culture (Fig. 7). In second 8 weeks culture, growth trend of callus on the basis of weight was almost similar to that of first 8 weeks culture (Fig. 5 and 6).

In order to utilize carbohydrate, the plant would have to produce and secrete hydrolyzing enzymes. Such enzymatic activity might be contributed differently in growth of callus in culture which might lead to a variation in callus growth. The type of carbohydrate supplied to cells and callus cultures affects morphological changes. Sugars affect sugar sensing systems that initiate changes in gene expression for photosynthesis, reserve mobilization and export process<sup>[11]</sup>. The greening of callus may be another expression of genes relating to photosynthesis via sugar sensing system, that's why callus may remain friable like source callus on sucrose supplemented medium but turns green on maltose and sorbitol. The highest proliferation of callus was with sucrose which might be due to easy utilization of sucrose in metabolism. It is observed that C, N ratio of callus is higher in sucrose (75:1) than maltose (54.1: 1) or sorbitol (42: 1). The amount of P in callus on sucrose medium is also higher than maltose and sorbitol (Table 1). These factors might be the cause for high callus proliferation on sucrose compared to that on maltose or sorbitol.

These findings support the reports of Ichihashi and Hiraiwa<sup>[3]</sup> in *Doritaenopsis* callus proliferation. But in *Phalaenopsis*, callus growth was better on maltose and sorbitol containing media than that on the sucrose medium<sup>[5]</sup>. Similar results were also reported in callus of *Neofinetia falcata*<sup>[12]</sup>. A wide variety of sugars may act as a carbon source for callus growth, where some species and hybrids showed preferences for specific sugar<sup>[13]</sup>.

Table 2: Effects of carbohydrates supplemented into NP medium on PLB (>2 mm) regeneration from *Doritaenopsis* calli after 8 weeks and 8 weeks × 2 culture

Treatments*	Weight of callus explanted (g)	After 8 weeks culture (average value)				After 8 weeks × 2 culture (average value)					
		Fresh wt. of callus (g)	No. of PLB	Fresh wt. of PLB (g)	Fresh wt. per PLB (g)	Callus PLB ratio	Fresh wt. of callus (g)	No. of PLB	Fresh wt. of PLB (g)	Fresh wt. per PLB (g)	Callus PLB ratio
Sucrose	0.2	2.5000	0.0000	0.0000	0.0000	0.000	2.6110	0.00	0.0000	0.0000	0.000
Maltose	0.2	1.8040	8.4000	0.1010	0.0120	17.860	1.6280	27.80	0.3890	0.0142	4.190
Sorbitol	0.2	1.6220	5.6000	0.0610	0.0109	26.590	1.4520	20.00	0.2680	0.0131	5.420
LSD at 5%		0.1840	0.0138	0.0138	0.0044	9.837	0.4287	11.59	0.1688	0.1380	2.839

Table 3: Effects of different carbohydrates on plantlet initiation from *Doritaenopsis* PLBs

Treatments*	No. of PLB cultured/flask	Ave. no. of regenerated plantlet/flask	% plantlet initiation
Sucrose	8	1.0000	12.500
Maltose	8	1.3300	16.630
Sorbitol	8	3.6600	45.750
LSD at 5%		0.1998	1.268

Table 4: Effects of different carbohydrates on the subsequent growth and development of initiated *Doritaenopsis* plantlets after 12 weeks culture.

Treatments*	No. of leaf/plantlet	Length of leaf (cm)	Width of leaf (cm)	Length of shoot (cm)	Dry wt. of shoot (g)	No. of root per plantlet.	Length of root (cm)	Diameter of root (mm)	Dry wt. of root (g)	Root, shoot ratio (fresh wt.)
Sucrose	1.0000	0.6500	0.4000	0.70	0.003	0.0000	0.0000	0.0000	0.0000	0.0000
Maltose	3.5000	1.4500	0.5500	2.25	0.006	3.0000	4.3000	2.5000	0.0130	2.1700
Sorbitol	5.0000	1.2500	0.5000	1.55	0.004	3.0000	1.3500	1.9000	0.0010	0.5140
LSD at 5%	0.8165	0.0893	0.0893	0.1413	0.0019	0.5756	0.9392	0.5893	0.0199	0.0893

\* Each treatment had 6 replications

**Effects on PLB initiation from callus:** After 8 weeks culture, calli on maltose and sorbitol containing medium produced few PLBs (Fig. 3a and b). PLBs were round, oval, elongated or irregular in shape with different sizes. PLB formation was significantly higher (8.4 PLBs/flask) on maltose than sorbitol (5.6 PLBs/flask) and no PLB was produced on sucrose (Fig. 7). Total fresh weight of PLB and fresh weight per PLB produced on maltose were significantly higher than that on sorbitol. Callus, PLB ratio was significantly higher on sorbitol than maltose (Table 2).

After 8 weeks × 2 culture, green callus explanted from previous culture developed more PLBs (>2 mm) than that of 1st culture on maltose and sorbitol supplemented media (Fig. 7). PLB formation was significantly higher (27.8 PLBs/flask) on maltose than sorbitol (20 PLBs/flask) and no PLB was formed on sucrose. Maltose containing medium showed higher fresh weight of PLBs and also weight per PLB than that on sorbitol supplemented medium (Table 2). Similar to 1st culture, callus, PLB ratio was significantly higher on sorbitol than maltose.

These results indicated that maltose followed by sorbitol were suitable for PLB production from calli and sucrose did not. These findings support the reports of Niimi *et al.*<sup>[12]</sup>. They reported that calli of *Phalaenopsis*, *Doritaenopsis* and *Neofinetia* proliferated without organogenesis and chlorophyll formation in a sucrose medium; these calli developed PLB and subsequently plantlets on maltose and/or sorbitol media. Chia *et al.*<sup>[14]</sup> reported that C/N ratio of cultured tissue determined the chlorophyll content of the tissues and the residual C/N

ratio of the medium affected protocorm formation in an orchid hybrid *Aranda* Tay Swee Eng.

Sucrose is added to a culture medium as energy source so that tissues require no photosynthetic activity for their growth. However, sucrose serves not only as energy source but it also controls other factors. Its elimination and replacement by maltose and sorbitol induced chlorophyll and PLB differentiation. N, K, S and other nutrients content of callus may be optimum in maltose in comparison to sorbitol which may responsible for better PLB production in maltose (Table 1).

**Effects on plantlet regeneration from PLB:** The subsequent growth and development of PLB initiated into plantlets (Fig. 4). On sucrose supplemented NP medium, only 12.5% of PLBs and on maltose medium 16.63% of PLBs initiated plantlets (Table 3). Sorbitol supported plantlet development from PLBs most intensively *in vitro*. Among transplanted PLBs, 45.75% initiated plantlets after 2 months. The percentage of plantlet production on sorbitol medium was significantly higher than those on sucrose and maltose supplemented media.

N content of proliferated green callus on sorbitol is higher (0.98%) than maltose (0.78%) and sucrose (0.56%) (Table 1). K and S content are also high in green callus on sorbitol which might have accelerated plantlet regeneration from PLB. Maltose and sucrose might be less effective to activate carbohydrate sensing gene which may responsible for low plantlet regeneration from PLB in maltose and sucrose. Which cause is accurately responsible is not clear from the present research.

**Effects on the subsequent growth and development of plantlets:** Sorbitol and maltose supplemented media significantly enhanced the subsequent growth and development of mini plantlets over sucrose (Table 4).

Number of leaf per plantlet was significantly higher on sorbitol containing medium. The length and width of leaf, length of shoot and dry weight of shoot were significantly higher on maltose containing medium than sorbitol and sucrose. Similarly, length of root, diameter of root, fresh and dry weight of root and root shoot ratio were significantly higher on maltose medium than sorbitol where sucrose supplemented medium did not produce any root (Table 4). Probably, maltose supplemented NP medium may contain the balance of the nutrients in the medium might be responsible for better growth of plantlets in comparison to sorbitol and sucrose.

In *in vitro* culture of orchids, sucrose is a common carbon source. However, some reports indicated that elimination of sucrose from a culture medium was beneficial and sometimes essential for some orchid species<sup>[15-17]</sup>. Present experiment indicated that a carbon source in the medium was indispensable for the growth of callus and regeneration of plantlets in *Doritaenopsis* and their growth and development were highly dependent on sugar type.

#### REFERENCES

1. Tokuhara, K. and M. Mii, 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Rep.*, 13: 7-11.
2. Sagawa, Y., 1990a. Orchids, Other Considerations. In: *Handbook of Plant Cell Culture*, Vol. 5 Eds. Ammirato, P.V., D.A. Evans, W.R. Sharp and Y.P.S. Bajaj, McGraw-Hill Pub. Co., New York, pp: 638-653.
3. Ichihashi, S. and H. Hiraiwa, 1996. Effect of solidifier, coconut water and carbohydrate source on growth of embryogenic callus in *Phalaenopsis* and allied genera. *J. Orchid Soc. India*, 10: 81-88.
4. Sagawa, Y., 1990b. Biotechnology in Orchids. In: *Proc. Nagoya Intr. Orchid Congress'90*. Kimura, T., S. Ichihashi and H. Nagata (Eds.), Nagoya, Japan, pp: 46-48.
5. Islam, M.O. and S. Ichihashi, 1999. Effects of sucrose, maltose and sorbitol on callus growth and plantlet regeneration in *Phalaenopsis*, *Doritaenopsis* and *Neofinetia*. *J. Japan. Soc. Hort. Sci.*, 68: 1124-1131.
6. Ichihashi, S., 1992. Micropropagation of *Phalaenopsis* through the culture of lateral buds from young flower stalks. *Lindleyana*, 7: 208-215.
7. Piper, C.S., 1950. *Soil and Plant Analysis*. Adelaide Univ. Hassel Press, Australia.
8. Olsen, S.R., C.V. Cole, F.S. Watanabe and L.A. Dean, 1954. Estimation of available phosphorus in soil by extraction with sodium bicarbonate. *U.S. Dept. Agric. Circ.*, pp: 929.
9. Black, C.A., 1965. *Methods of Soil Analysis*. Part I and II. Amer. Soc. Agron., Wisconsin, USA., pp: 160-165.
10. Page, A.L., R.H. Miller and D.R. Keeney, 1982. *Methods of Soil Analysis*. Part I, 2nd Edn. Amer. Soc. Agron., Wisconsin, USA.
11. Koch, K.E., 1996. Carbohydrate-modulated gene expression in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 47: 509-540.
12. Niimi, Y., C. Akaza and Y. Hayata, 1995. Organogenesis and Plant Regeneration from Callus Cultures in *Neofinetia falcata*. In: *Proc. Nagoya Intr. Orchid Congress'95*. Ichihashi, S. and H. Inoue (Eds.) Nagoya, Japan, pp: 81-90.
13. Arditti, J., 1967. Factors affecting the germination of orchid seeds. *Bot. Rev.*, 330: 1-97.
14. Chia, Y.K., C.S. Hew and C.S. Loh, 1988. Carbon/nitrogen ratio and greening and protocorm formation in orchid callus tissue. *Hort. Sci.*, 23: 599-601.
15. Kunisaki, J.T., K.K. Kim and Y. Sagawa, 1972. Shoot tip culture of Vanda. *Amer. Orchid Soc. Bull.*, 41: 435-439.
16. Teo, C.K.H. and C.H. Wong, 1978. Effects of sucrose on the growth of protocorms of *Halttumara Lock Tuck yip*. *The Orchid Rev.*, 86: 285-289.
17. Teo, C.K.H., J.T. Kunisaki and Y. Sagawa, 1973. Clonal propagation of *Stap-leveled vanda* by shoot tip culture. *Amer. Orchid Soc. Bull.*, 42: 402-405.