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Efficient *in vitro* Culture Protocols for Propagating *Phalaenopsis* 'Cool Breeze'

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Key words: Phalaenopsis, Micropropagation, Plant regeneration, Hardening

Abstract

Phalaenopsis orchids have high economic value in the floriculture industry as cut flowers and potted plants throughout the world. Plant tissue culture technology is being widely used for large scale plant multiplication of *Phalaenopsis* to feed into this industry. In order to increase the efficiency of this technology, four experiments were undertaken: Plantlet regeneration from seeds, nodes or leaves, and hardening of the regenerated plants. In the first experiment, seed germination was examined in three media (half MS, Chen and Vacin-Went) of which the Chen medium had the best result (83.4%) in comparison to the other two media. In the second experiment, nodes on the flower stalk were studied for their shoot formation potential to different concentrations of BA and NAA, The highest frequency of shoot regeneration was achieved on MS containing 4 mg/l BA and 1 mg/l NAA while *in vitro* derived leaves formed clusters of somatic embryos directly when cultured on MS containing TDZ at different concentrations (0.5, 1, 2 and 3 mg/l). The embryos turned green and developed into protocorm-like bodies after 7 weeks of culture followed by plantlet regeneration. The highest plantlet regeneration from the leaf-derived embryos was obtained from MS supplemented with 3 mg/l TDZ. Finally, regenerated plants from (seeds, nodal explants and leaves) were compared in two medium for hardening, regenerated plant from nodal explants showed the highest survival rate (100%) on the medium containing cocopeat, coal, industrial cartridge and the bites of yonolit (1 : 1 : 2 : 4).

Introduction

Orchidaceae is one of the largest families and most diversified among the angiosperms, comprising 30,000 - 35,000 species of terrestrial plants (Chase et al. 2003, Singh et al. 2007). Some orchid genera, such as *Phalaenopsis*, *Dendrobium*,

Oncidium, *Paphiopedilum*, and *Cymbidium*, have become economically important as potted plants and cut flowers throughout the world. *Phalaenopsis*is one of the few genera within the Orchidaceae whose plants bloom every six months, with long lasting inflorescences. It is a monopodial genus and does not normally form offshoots and occasionally produce plantlets (keikis) (Paul and Starosta 1988) on their flower stalks which is used for in vitro culture. Tissue culture has been widely used for mass propagation of *Phalaenopsis*. The first clonal propagation *in vitro* of orchids was achieved by Gavino Rotor (Rotor 1949). Several methods for direct micropropagation of *Phalaenopsis* were developed (Tanaka and Sakanishi 1977), including the culture of flower stalks with auxiliary buds, meristems, flower stalks explants and internodal segments of flower stalks (Arditti and Ernst 1993). Some of these methods gave a lot of protocorms-like-bodies (PLBs), but these may develop slowly or poorly to vital plants (Košir et al. 2004).

Orchid seeds are minute and have no endosperm. In nature, they are symbiotic with some kinds of fungi in order to germinate (Bhadra 1999). Moreover, orchid seeds may have poor germination capacity that may be lost very quickly in nature by unfavorable environments (Segaw and Kunisaki 1982, Stenberg and Kane 1998). *In vitro* micropropagation is now being suggested for quick propagation of commercially important orchid species such as *Phalaenopsis* and conservation of wild orchids (Kauth et al. 2006, Stewart and Kane 2006, Baskar and Narmatha Bai 2006). It has been reported that explants showed different responses towards tissue culture depending on their source, physiological state and nutrient environment (Vij et al. 1983). Information on how best to micropropagate is lacking (Dohling et al. 2008). In this paper, we rectified shortcoming by reporting a rapid method for the large-scale multiplication of *Phalaenopsis* orchids cv. Cool Breeze.

Material and Methods

In this experiment pollination by hands was carried out under glasshouse conditions in 8 month of the year. After 150 days, five mature and green capsules were collected randomly for the seed germination experiment. Pre-sterilization was done with 20% v/v commercial bleach (1% sodium hypochlorite) and one drop of Tween 20 for 10 min followed by 70% ethanol for 30 second then 15% v/v commercial bleach for 15 min; the capsules were then rinsed with sterile distilled water thrice for 15 min. Immature seed were cultured on basal ½-strength MS, Vacin-Went (Vacin and Went 1949) and Chen media (Chen et al. 1999) (Table 1). Seed cultures were placed in growth chamber at 25 ± 2^0C and 70-80% relative humidity under 16 hrs light and 8 hrs dark condition with a light intensity of 20 - 50 μmol/m^2/s provided by cool white fluorescent lamps for 150 days. A

completely randomized design was used for this experiment. 15 replicates were used for each treatment and the effect of basal medium on seed germination was investigated. Percentage of germination was calculated for each treatment by dividing the number of germinated seeds by the total number of seeds.

Table 1. Media composition.

Medium	½ MS	Chen	Vacin and Went
Macro element (mg/l)			
NH_4NO_3	825	825	-
KNO_3	950	950	525
$(NH_4)SO_4$	-	-	500
$CaCl_2.2H_2O$	220	220	-
$Ca_3(PO_4)_2$	-	-	200
$MgSO_4.7H_2O$	185	185	250
KH_2PO_4	85	85	250
NH_2PO_4	-	170	-
Micro element (mg/l)			
$MnSO_4.4H_2O$	11.15	11.5	7.5
$ZnSO_4.7H_2O$	4.3	4.3	-
H_3BO_3	3.1	3.1	-
KI	0.415	0.415	-
$Na_2MoO_4.5H_2O$	0.125	0.125	-
$CoCl_2.6H_2O$	0.0125	0.0125	-
$CuSO_4.5H_2O$	0.0125	0.0125	-
Na_2EDTA	18.65	18.65	37.3
$FeSO_4.7H_2O$	13.9	13.9	27.8
Vitamins (mg/l)			
Glycine	2	2	-
Nicotinic acid	0.5	0.5	-
Pyrodoxine	0.5	0.5	-
Thiamine	0.1	0.1	-
Myo-inositol	100	100	-
Gellingagent(g/l)			
Gelrite	-	2.2	-
Agar	8	-	8
Carbohydrate, Organic compounds (g/l)			
Sucrose	30	20	20
Peptone	-	1	-
pH			
pH	5.7	5.7	5.7

Two-year-old *Phalaenopsis amabilis* cv. Cool Breeze orchids were purchased from Anthura Company of Netherlands. Flower stalk were collected from plants

which had three opened buds. After transferring to the laboratory, the flower stalk nodes were cut into 5 - 6 cm lengths and pre-sterilized by using a solution of 1% benomyl fungicide and one drop of Tween 20 for 10 min. After pre-sterilization the explants were sterilized using 70% ethanol for 30 second followed by 35% v/v commercial bleach for 15 min and finally rinsing the explants with sterile distilled water thrice for 15 min. Before transferring to medium, two sides of the nodes which were damaged due to sterilization were cut and the bracts on the bud were removed and finally cultured on MS supplemented with different concentration of BA and NAA (Table 2) for shoot regeneration. The explants were sub-cultured onto the same medium every 14 days and kept at 16 hrs light and 8 hrs dark condition and 25 ± 1°C. Regenerated plantlets were cultured on MS supplemented with different concentrations of NAA and IAA for root induction (Table 3). A completely randomized design was used for this experiment, 20 replicates were used for each treatment and the effects of hormonal treatments on shoot formation and root induction were investigated, respectively.

Table 2. Treatments applied for shoot regeneration.

Treatments	Growth regulators (BA + NAA mg/l)
1	Without growth regulators
2	2 + 0.5
3	3 + 0.75
4	4 + 1
5	5 + 2

Table 3. Treatments applied for root induction.

Treatments	Growth regulators (mg/l)
1	0.5 IAA
2	1 IAA
3	0.5 NAA
4	1 NAA

For plantlet regeneration, *in vitro* leaves (1 × 0.5 cm) which were obtained from nodes explants were cultured on Chen medium supplemented with different concentrations of NAA, BA and TDZ (Table 4). The explants were sub-cultured every 14 days. Data were recorded every week. A completely randomized design was used for this experiment, 30 replicates were used per treatment and the effects of hormonal treatments on plantlet regeneration were investigated.

In vitro rooted plantlet (from seeds, nodal explants and *in vitro* leaves) were washed carefully with water to remove traces of agar and then transferred to the pots containing different potting mixtures *viz.* 1. Cocopeat, coal, industrial cartridge and the bites of yonolit (1 : 1 : 2 : 4), 2. Cocopeat, coal (5 : 1). The pots were covered with tight plastic cover to prevent desiccation and acclimatized in the mist house at 20 - 30ºC temperature and 14 hrs illumination. The plantlets were compared regarding hardening after one month acclimatization; 100 replicates were used for each treatment.

Table 4. Treatments tested for plantlet regeneration from in vitro leaves.

Treatments	Growth Regulators (mg/l)
1	Without growth regulators
2	0.5 TDZ
3	1 TDZ
4	2 TDZ
5	3 TDZ
6	1 BA + 0.3 NAA
7	2 BA + 0.5 NAA
8	3 BA + 0.75 NAA
9	4BA +1 NAA

Results and Discussion

Seeds from mature capsules (150 days after pollination) were germinated on half MS, Vacin-went and Chen media (Fig. 2), A significant variation was observed in germination of *Phalaenopsis* seeds cultured on different basal media (Fig. 2). Previous studies (Rahman et al. 2004) reported that the germination rate of orchid species *in vitro* is significantly influenced by basal medium used. In this study, the effects of three different basal media on seed germination of *Phalaenopsis* showed the highest (83.4%) and lowest germination rate (9.4%) on Chen and half MS medium, respectively (Fig. 1).

Coconut water, yeast extract, potatoes extract and peptone are among organic additives that have been commonly used in orchids for seed germination and protocorm growth and development (Lo et al. 2004, Ang and Yong 2005). Their effects, however, may vary among orchid species. In this study, addition of peptone to the Chen medium showed a significant effect on germination.

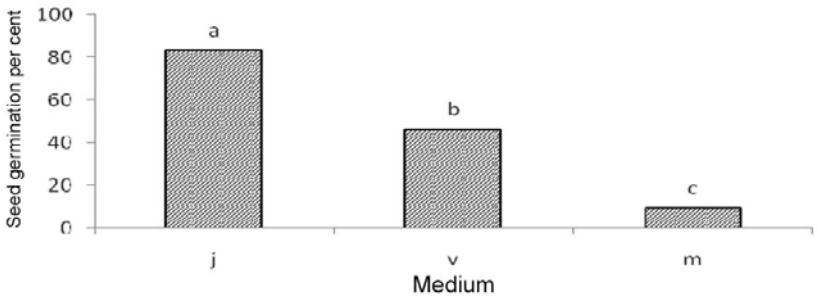

Fig. 1. Seed germination per cent on different media.

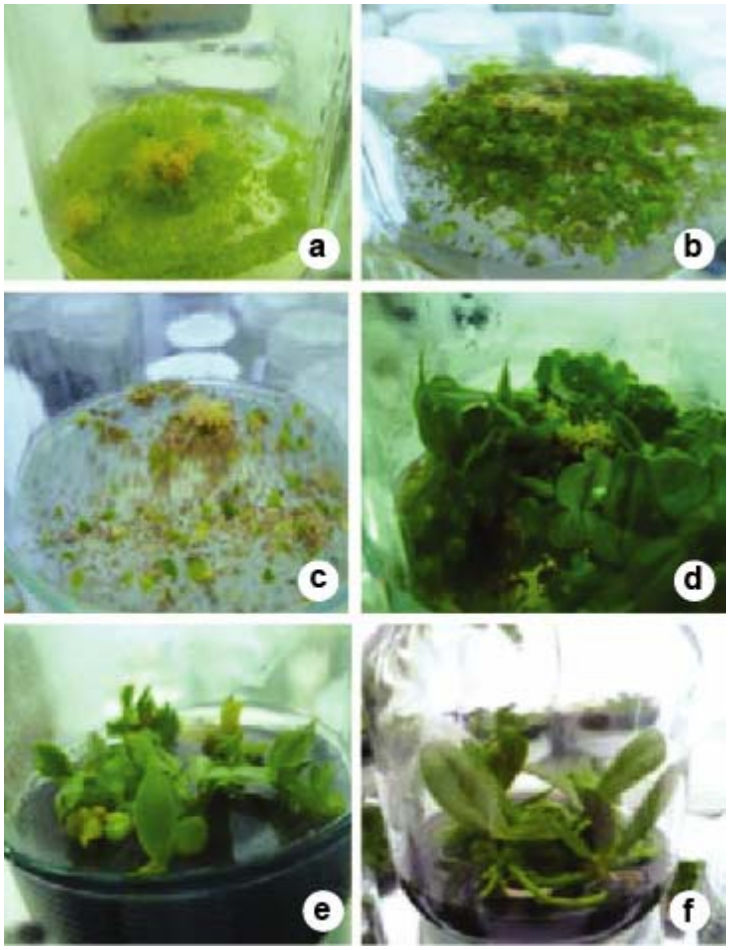

Fig. 2. Plant regeneration from seeds, germinated seedling on a. Vacin and Went medium b. Chen medium c. Half MS medium d. Leaf and root production on Chen medium e. Regenerated plant after one sub culturing on Chen medium f. Ready regenerated plant for hardening.

After two weeks of culture, the nodal explants showed two states: in some treatments buds were swollen and began to regrowth (Treatment 3 and 4) but in others the explants remained in a dormant state and did not show any sign of growth. Others have reported three different states if the nodes are used as explant: dormant or convert to the plantlet or transfer to reproductive stage and develop into flowers (Arditti 2008). Our results confirmed this report but none of the buds converted to flowers. The buds were swollen in the fourth week, but in the control treatment, the buds grew normally and converted to plantlets. In treatment 5, firstly the buds were vertically arisen which were similar to the peduncle after eight weeks. The numbers of regenerated shoots per node among treatments were different. The highest number of shoots (15.3) was obtained from MS supplemented with 4 mg/l BA and 1 mg/l NAA (Fig. 3) and minimum number of shoots (0.7) was obtained from control treatment (Fig. 6). The number of active buds on medium containing BAP and NAA (Fig. 5) in comparison with the control treatment showed the growth regulators have the ability to promote shooting, particularly BA because in two treatments (treatment 5 and 6) which contained 4 and 5 mg/l BA all the buds were active and none of them remained in the dormant state. This was in agreement with other experiments which reported that BA could activate the dormant buds on flower stalks (Arditti 2008). We obtained 15.3 plantlets per flower stalk, approximately twice the number that reported by Kosir and coworkers (8.35 plantlets per node) (Kosir et al. 2004).

Previous studies showed that wounding prior to culturing improves shoot formation (Tanaka and Sakanishi 1978, Tanaka et al. 1998) and TDZ and BAP had the best effects on shoot formation in *Phalaenopsis* (Tanaka et al. 1998). Another factor is explant age which influenced regeneration from young explants source showed a better response in orchids such as *Oncidium*, *Dendrobium* and *Phalaenopsis* (Intuwong and Sagawa 1973). We also used the young plants and obtained good results considering yield (Intuwong and Sagawa 1973).

One of the problems that has arisen was the lack of root induction in the produced plantlets. For this purpose, a new experiment was done to investigate root induction by culturing plantlets on half MS with different levels of IAA and NAA. The first symptom of root emergence after culturing was observed on MS with NAA while the treatments supplemented with IAA showed rooting at the fifth week. Considering the size and number of roots, the treatments had different effects on root induction. Among treatments, the medium containing 0.5 mg/l NAA produced the most roots (2.65 roots per plantlet) (Fig. 6) but the roots were small and thick (Fig. 4). In the medium supplemented with 1 mg/l IAA produced good number of roots (2.45 roots per plantlet) and also the size of roots was larger than treatment supplemented with 0.5 mg/l NAA.

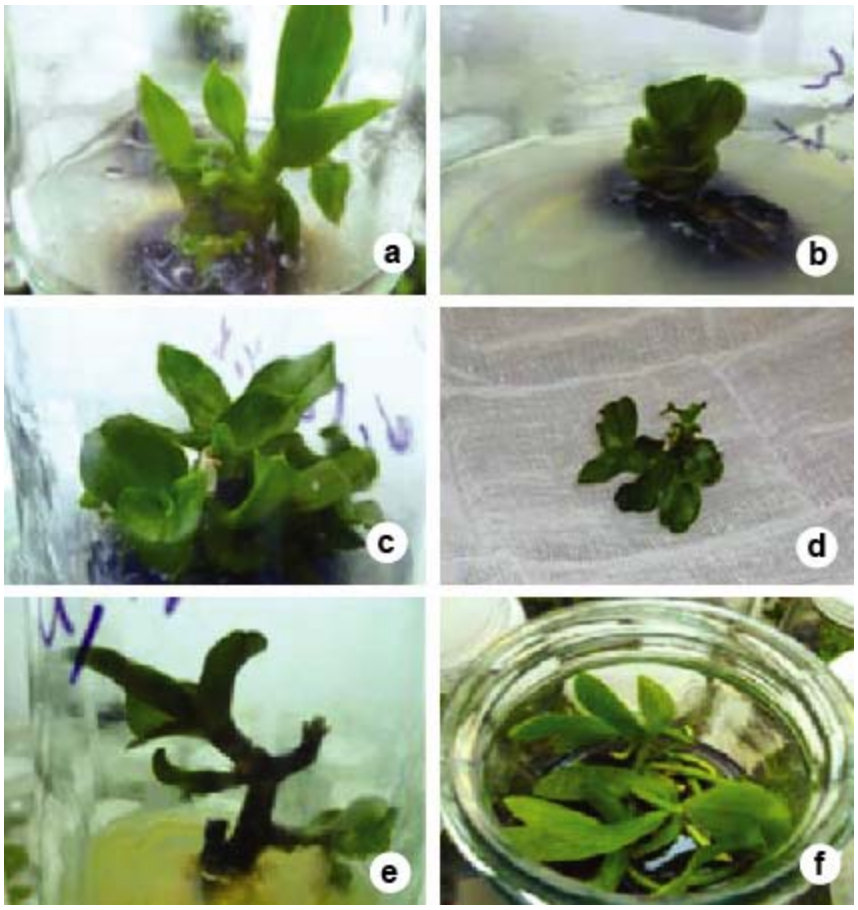

Fig. 3. Shoot regeneration from node cultures. a. Regenerated shoot in medium supplemented with 3 mg/l BA + 0.75 mg/l NAA. b. Swelling buds. c. Regenerated shoot in medium supplemented with 4 mg/l BA + 1 mg/l NAA. d. Regenerated shoot in medium supplemented with 2mg/L BA + 0.5 mg/l NAA. e. Regenerated shoot in medium supplemented with 5 mg/l BA + 2 mg/l NAA. f. rooted plantlet ready for hardening

PLBs emerged gradually from *in vitro* leaf surfaces at the sixth week of culture. The first observation was seen from Chen medium containing 3 mg/l TDZ. After seven weeks of culture PLBs only emerged on media supplemented with TDZ (Fig. 8). There was no sign of PLB growth on media supplemented with BAP and NAA (Fig. 8) and by the end of the experiment (week 12) the leaves had gradually yellowed and died. Chen and Chang (2006) suggested TDZ seems to be suitable for somatic embryogenesis. They suggested that only the application of TDZ can affect PLB production from cultivated leaves in *Phalenopsis* orchids. In the TDZ treatment, some of the leaves died without even one PLB production, commonly this included leaves which had greater size.

Smaller leaves showed greater tendency toward PLB production. The highest PLB production was observed at the proximal end of the leaves where it attached to the stem. Gow et al. (2008) analyzed some factors which affected somatic embryogenesis in *Phalanopsis* leaves such as explant size and it was reported that the best size for leaves was 1 cm and also the best part for PLB formation was the end of leaves which agrees with our result. Chen and Chang (2006) reported the best concentration (3 mg/l TDZ) for plantlet regeneration (after 12 weeks of culture). In our study plantlet regeneration was observed on half MS without hormone when supplemented with 2 gm activated charcoal for 8 weeks and finally rooted plantlets were transferred to the greenhouse for adaptation. Successful micropropagation of orchids via leaves depend on many factors such as the nutrient composition of culture medium, plant growth regulators, source of leaves (*in vitro* or *in situ*), the part of leaves, the position of leaves on the plant and plant age (Chugh et al. 2009).

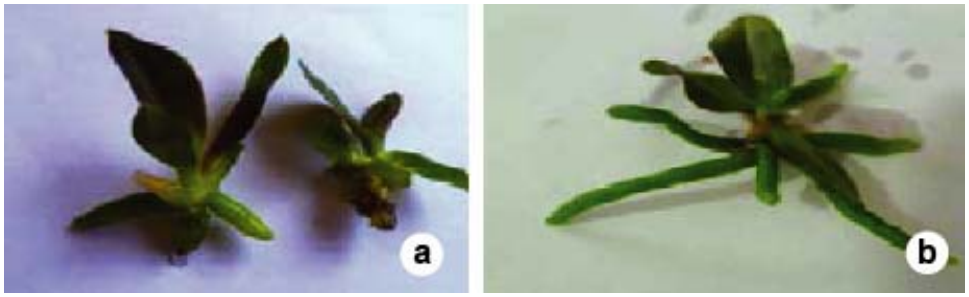

Fig. 4. Difference between IAA and NAA on root induction, root induction in medium supplemented with a. 0.5 mg/l NAA and b. 1 mg/l NAA

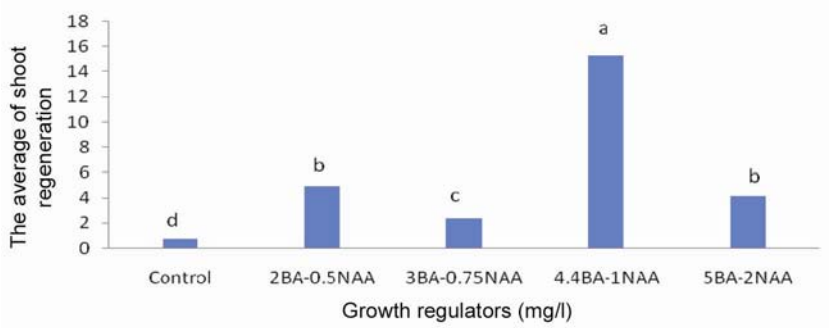

Fig. 5. Effect of different growth regulators combination on shoot regeneration.

Regenerated plantlets from nodular culture showed the best adaptation (100%) on the mix containing cocopeat, coal, industrial cartridge. The bits of yonolit (1 : 1 : 2 : 4) and 98.2% survival in the mix containing cocopeat, coal (5 : 1) were obtained. Regenerated plantlets from seeds and leaves showed 99, 93% in

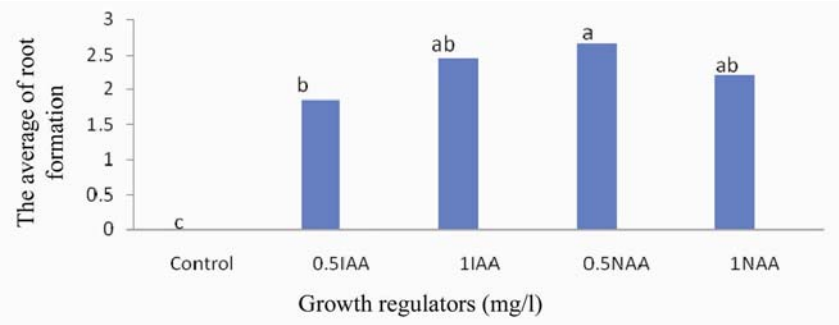

Fig. 6. Effect of different growth regulators combination on root formation.

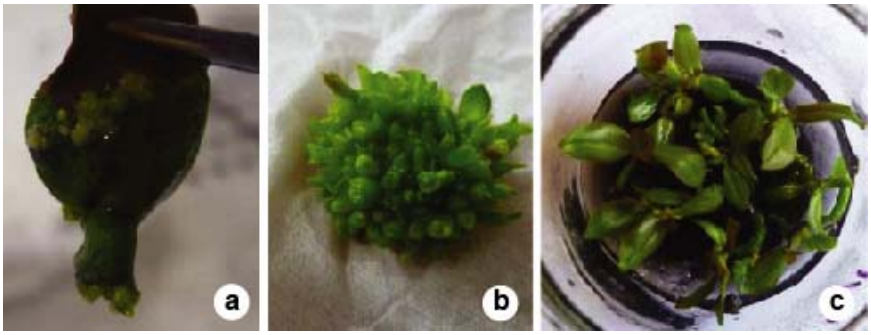

Fig. 7. Plant regeneration through direct somatic embryogenesis from leaf explants. a. Direct somatic embryogenesis. b. Shoot formation from PLBs. c. Regenerated plant on MS without any growth regulator.

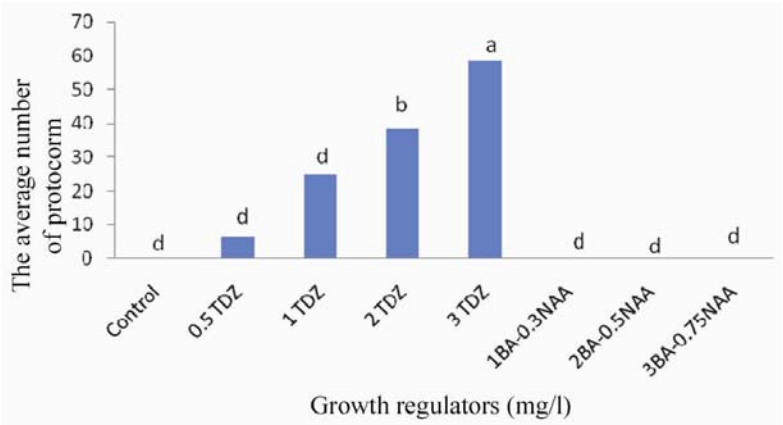

Fig. 8. Effect of different growth regulators combination on Plbs production.

medium 2 and 90, 96% in medium 1, respectively. One reason for this could be the large size of regenerated plantlets via nodes in comparison with other

regenerated plantlets (via seeds and leaves). But the important point between two medium was holding water in medium 1. The second medium because of the existence of industrial cartridge, coal and bits of yonolit was more porous, for this reason the cultivated plantlets at this medium had better growth and adaptation. One of the factors, which can affect the growth of regenerated plantlets, is its epiphyte nature, which showed better growth in medium such as medium 2. The time between adaptation to flowering from regenerated plants from seeds, nodes and leaves were different. Regenerated plants from nodes produced flower 16 months after adaptation but regenerated plantlets from seeds showed longer juvenility and produced flower after 21 months and regenerated plants from leaves produced flower 18 month after adaptation to environment. Several studies have shown that the seeds from immature capsules can germinate much earlier (Arditti et al. 1981, Stancato et al. 1998), the use of immature seed capsules as a seed source shortened the reproductive cycle by approximately 2 to 2.5 months. Also, the utilization of seeds from unripe capsules allows to avoid the negative influences of sterilized substances, in this experiment we used unripe capsule too which produced 5 months after pollination.

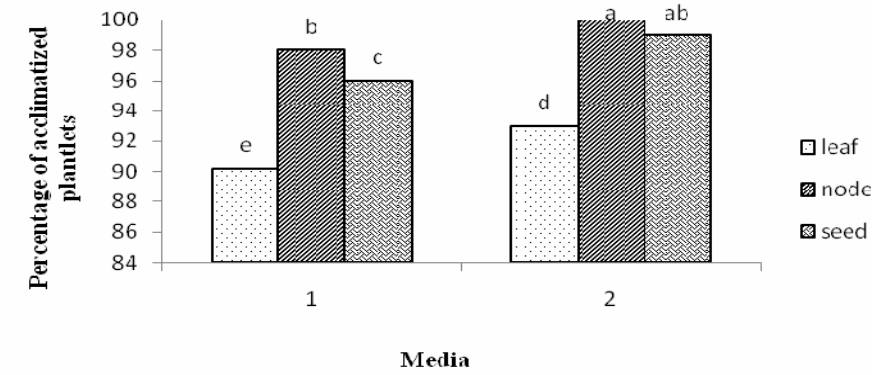

Fig. 9. Effect of two media on regenerated plant (from leaf, node and seed) adaptation.

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