Journal of Tropical Biodiversity and Biotechnology
journal homepage: www.jtbb.or.id

Micropropagation of mini orchid hybrid *Phalaenopsis* "Sogo Vivien"

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ARTICLE INFO

Article history
Received 08 March 2016
Received in revised form 05 May 2016
Accepted 30 May 2016

Keywords
Micropropagation
Variegated leaves
Seed
Nodes of flower stalk
Phalaenopsis "Sogo Vivien"

Abbreviations
NP: New Phalaenopsis
VW: Vacin and Went
MS: Murashige and Skoog,
BA:Benzyl Adenine

ABSTRACT

Phalaenopsis"Sogo Vivien" is an orchid hybrid with mini size plant body, and exhibits numerous beautiful pink flowers, that is ideal as ornamental pot plant. Some plants of this orchid exhibit variegated leaves that improve the beauty of the plant, not only because of the flower but also as attracted leaves. This orchid has high economical value, but mass propagation of this orchid has not established yet. An effective method to propagate both the normal and variegated plants is worth to be generated. The objective of this research was to produce a large number of P. "Sogo Vivien" plants, including the variegated plants. The method used seeds from self pollinating variegated plant, and flower stalk nodes. The seeds were sown on three various medium: VW, NP and MS, and flower stalk nodes were planted on VW + BA 10 mg l^{-1} + active carbon. The results showed that the best medium for in vitro culture of P. "Sogo Vivien" was NP medium, in which all seeds could grew into plantlets. Most plantlets emerged from the seeds were non variegated, only one plantlet out of 1344 seeds was variegated (0.007%). Although all emerged plantlets from flower stalk exhibited variegated leaves. Particularly, the plantlets arised from the second and third basal nodes of flower stalk showed the highest growth rate than that from the other nodes. Histological analysis showed that at 11-13 days after shoot segment plantation on NP medium, the shape of apical cells in the nodes was changed, then followed by the change of cell shape in the basal part of the nodes, produced bipolar pattern, then gradually developed into shoot. These results suggest that mass propagation could be achieved using seed culture, but to get the variegated phenotypes, the second and third nodes of flower stalk from variegated plant were the best explants to be used.

1. Introduction

Orchid is one of horticultural commodities that has a significant role to the development of Indonesian agriculture. The orchid phenotype is characterized by the unique shape of flowers with long lasting time of flowering, and bright green leaves, therefore it has high potential economical values for commercial purposes (Rajanawong *et al*., 2006; Chugh *et al*., 2009; Khoddamzadeh *et al*., 2011). *Phalaenopsis "*Sogo Vivien"is one of the superior orchid hybrids that exhibits dwarf phenotype with numerous pink flowers. *P.*"Sogo Vivien"was obtained from cross pollination between *P."*Sogo Alice" and *P. "*Zuma's Pixie". According to the elders of *P.* Sogo Vivien when traced through the web of Royal Horticultural Society (The International Orchid Register), this hybrid orchid belongs to a group that consists of 10 parental of orchid species, 9 species of which were derived from genus *Phalaenopsis* and 1 species was derived from the genus *Doritis*. That the reason this orchid hybrid exhibits very attractive dwarf phenotype. Its mini size and numerous flowers cause this orchid fits the criteria of desired potted plants (Tang and Chen, 2007). Among the orchid hybrids, some plants show unusual variegated pattern of leaves that is green color in the center of the leaf and yellowish in the margin, here in after we term as variegated *P.* "Sogo Vivien". Some factors may cause the pattern of variegated leaf,including chimera (Marcotrigiano and Stewart 1984), genetic factor (Evenari 1989), virus (Saitoh and Terauchi 2002),

mutations (Sangsiri *et al*., 2007, Wu *et al*., 2011), and bacteria (Rossetti and De Negri 2011). Mutations in the nucleus gene, plastid gene, or mitochondrial gene cause the failure of plastids to accumulate photosynthetic pigments, either directly or indirectly, generating sector with cells containing white/ yellow plastids (abnormal) (Pandey and Blaydes, 1957; Chen *et al*., 1999). In general, inherited variegation is permanently defected and the inheritance is non Mendelian, maternal (common in Angiospermae) or biparental (Pandey and Blaydes 1957). However, the variegated phenotype significantly increase the beauty of this orchid that support the economic value. To meet the needs of flowers and ornamental plants industry, the mass propagation of *P.* "Sogo Vivien" plants should be prioritized to be developed

In vitro culture or micropropagation of orchids could be used for mass propagation either using seeds (embryo culture) or other parts of the plant as explants. Propagation by using explants of apical buds, lateral buds, nodes of flower stalks and pedicelus of *Dorietanopsis* Purple Gem 'Ching Hua' has been carried out with a combination of plant growth regulator NAA and TDZ, resulting a number of plants that morphologically identic to their parent (Vendrame and Maguire, 2007). Propagation could also be generated using leaves (Gow *et al*., 2009; Gow *et al*., 2010; Subramaniam and Rathinam, 2010; Khoddamzadeh *et al.*, 2011), roots (Park *et al*., 2003) and internodus of flower stalks (Lin, 1986; Tokuhara and Mii, 1993; Islam *et al*., 1998; Vendrame and Maquire, 2007) as explants, so it could produce uniform offsprings which identical to the parent (Arnold *et al.,* 2002; Piria *et al*., 2008). Since the transmission of variegated traits does not always follow the Mendelian law and not all of *P."*Sogo Vivien" exhibit variegated leaf, it is necessary to find an *in vitro* propagation method to maintain the variegated pattern of the leaf.

It is well known that most orchid seeds are microscopic in size and have no endosperm, therefore to induce embryo development during seed germination, additional nutrients are required (Arditti, 1992, Robinson *et al*., 2009). Therefore, the orchid seeds need appropriate medium as a source of nutrients (Mohanty*et al*., 2012). *In vitro* cultures of orchid seeds have shown that each species requiresspecific composition of culture medium for optimum germination and growth (Robinson *et al*., 2009). A balanced medium is required to maximize the germination due to thecontent of inorganic salts, minerals, vitamin, and other supplements in different concentration and composition, that will give positive effects on the growth and development of orchid seeds into protocorm prior to germination.

Some media for orchid seeds germination are New Phalaenopsis (NP), Vacin and Went (VW), Murashige and Skoog (MS) (Murashige and Skoog, 1962;Vacin and Went,1949; Islam *et al*., 1998), however, each species needs specific culture medium for optimalgrowth and development oforchid embryos into protocorm (tuber-like developing orchid embryo) (Semiarti *et al*., 2014).

In this paper, large-scale propagation of *P.* "Sogo Vivien" from both normal and variegated leaves phenotype were carried out using seeds which were shown on various culture medium and the use of flower stalk's nodes to get an efficient method for production of hybrid plants as well as variegated plants.

2. Materials and methods

2.1. Plant material and culture conditions

Plant material used in this study was *P."*Sogo Vivien" orchid hybrid obtained from CV. Amabilis nursery, Kiaracondong, Bandung. The four years old plants were grown in pots filled with sphagnum moss as medium. The plants were reared in a *greenhouse* with natural lighting at temperature between 25 and 30°C. Seeds used as explants were obtained from four pods, two pods resulted from cross-pollinated between different variegated plants, and the other two pods from self-pollinated plants. For induction of variegated plants, the first to fifth nodes from the basal of selected flower stalks were used as explants.

2.2. Seed culture and data collection

A three-months-old orchid pods from self pollinated variegated *P.* "Sogo Vivien" were used as seed source. The pods were subsequently sterilized by dipping it in alcohol and then flame it on a spiritus lamp. Next, the seeds were sown on VW, NP and MS media. The observed parameters were the development phase of the seeds that grew to become protocorm, which was carefully examined using a stereomicroscope and digital camera (Nikon Cool Pix 5000), the percentage protocorm at each phase of development (Corel Draw version 11) and the leaf phenotypes appeared. The percentage of embryo of n-phase was calculated based on the number of embryos in n-phase divided by the number of observed embryos.

2.3. Flower stalk nodes culture and data collection

Surface sterilization of flower stalk was carried

outusing fungicide and bactericide (50 mg / 100 ml of each) for 10 minutes, followed by a treatment using NaClO 35% and 2 drops of tween for 5 min which was conducted twice. After being rinsed with sterile distilled water, the sterilized flower stalk nodes containing internode part were dried on filter paper and planted in medium VW + BA 10 mg L^{-1} + activated charcoal. Parameters observed included the speed of bud formation, the length of shoots and lamina, the color of formed leaf, as well as the speed of the leaf formation.

2.4. Histological Analysis

For histological study, bud of nodes were fixed in a mixture solution of formaldehyde: acetic acid: 70% ethanol (1:1:18, FAA) for 24 hours. Samples were washed once with gradual concentration of ethanol: 70% ethanol, 80% ethanol, 95% ethanol, twice with 100% ethanol, for 30 minutes each. Samples were dealcoholized in ethanol: xylol with a ratio of 3:1, 1:1, 1:3, respectively, for 30 minutes, then samples were put into xylol: parafin with ratio of 1:9 for 24 hours at 57°C. For embedding, the samples were infiltrated with 100% paraffin after replacing xylol/paraffin (1:9) and incubated at 57°C for 24 hours. The block of samples were longitudinally cut by microtome, following by staining with 1% safranin in 70% ethanol for 24 hours. The sections were observed under a light microscope for further analyses.

2.5. Data analysis

Experiments were performed in a randomized design and each treatment was carried out in five replications. Collected data were further analyzed using SPSS and tested for significancy using Duncan multiple range test (Duncan, 1955).

3. Result and discussion

3.1. The growth of embryo and seeds germination

The growth of *P*. "Sogo Vivien" embryo in NP medium showed 6 different developmental phases (Figure 1). Generally, orchid seeds show embryos that were protected by testa (phase 1), then the embryo would swell once planted in medium and later thebrown-colored stripes appeared indicating that testa were already broken (phase 2), over time most of testa were faded in color and embryo became round with white color (phase 3). The size of the protocorm became larger with bulbous shape and yellow color, and the absorbing hair began to appear (phase 4).The size of the protocorm became bigger with a round shape and the color changed to green with even more absorbing hair (phase 5). Further developments of orchids seeds showed the presence of Shoot Apical Meristem (SAM) followed by the formation of increasingly dense absorbing hair (phase 6) on 4 weeks after sowing (WAS).

Figure1. Development of Phalaenopsis "Sogo Vivien" embryos from fruit aged 3 months and 1weeks after pollination, which were grown in NP medium. (a) Phase 1 = Embryo of orchid before beingplanted (0 day after sowing/ DAS); (b) Phase 2 = Swelled embryo with brown-colored stripes indicating broken testa (5DAS); (c) Phase 3 = Round/ oval embryo with white color, protected by a few testa (11 DAS); (d) Phase 4 = Larger embryo with bulbous shape and yellow color, absorbing hair started to appear (16 DAS); (e) Phase 5 = Larger embryo with bulbous shape, green color and more absorbing hair (22 DAS); (f) Phase 6 = Shoot Apical Meristem (SAM) detected with green color and bushy absorbing hair (29 DAS).Bars = 0,1 mm.

Embryo development has also been analyzed on *Phalaenopsis amabilis* (Semiarti *et al.,* 2007). The formation of large nuclei at one pole of the embryo occurs after the seed has been planted in the medium for 2 weeks, followed by formation of shoot apical meristem, absorbing hair and single leaf primordium after 3 WAS. After 4-6 WAS, leaves and rod monopodial system are formed. The growth of *P.*"Sogo Vivien" embryos was slower than that of *P. amabilis*. It means that the hybrid have lower potential of growth rate compared to the parent. Development of protocorms were indicated by color changes of the protocorm during its development, initially the protocorms were white in color, then they changed into yellow, and finally they become green (Semiarti *et al.,* 2007). The result of this research was similar to the work of Mohanty *et al.* (2012) on the development of *Cymbidium mastersii* protocorms, that embryos consist of undifferentiated cells in the form of irregular cell mass from testa (spherules) in 5-9 WAS. Spherules will develop into green globular shape during 3-4 weeks. After 12 weeks, vegetative apex and rhizoid were seen in protocorm. After 14 weeks, 2-3 leaf primordia were emerge followed by generation of 1-2 roots.

One of the important factors that support the growth of embryo is macronutrients and micronutrients content of medium (Mohanty *et al*., 2012). In this study three types of media VW, NP and MS consist of different composition in either macronutrients or micronutrients. Among those culture medium, NP was the best medium to induce the growth and development of protocorms (Figure 2, Table 1). Protocorm grown in NP medium showed larger size (Figure 2a) compared to that of protocorm growth on VW medium (Figure 2b) or MS medium (Figure 2c). However, the number of germinating protocorms decreased significantly when seeds were planted on MS medium (Figure2 and Table 1).

Figure2. The growth of Phalaenopsis "Sogo Vivien" protocorms from seeds on various medium. (a) VW , (b) NP, (c) MS, at 3 months old after seeds sowing. Bars 0.5 cm

Table1. The growth of Phalaenopsis "Sogo Vivien"embryo during seed germination on various medium (3 months after sowing).

Type of medium	No. of seeds for sowing	Percentage of survival protocorms (%)				No of variegated plants
		Phase 3	Phase 4	Phase 5	Phase 6	
NP	1344	8.25 ± 0.13 c	17.05 ± 0.13 b	50.36 ± 0.14 a	24.34 ± 0.14 a	1 (0.001 %)
VW	1291	16.83 ±0.25 b	21.02 ± 0.25 a	44.04 ±1.70 b	18.11 ± 1.70 b	0
MS	1376	99.59 ± 0.01 a	0.01 ± 0.89 c	0.01 ± 0.01 c	0.41 ± 0.01 c	0

*Notes:Values in columns preceding the same letter are not significantly different according to SPSS and Duncan test at 5% significance level.

These results were in contrast to the results of the growth of *Cymbidium mastersii* protocorms, where the best medium was MS (Mohanty *et al*., 2012). Seeds of *Phalaenopsis* sp. were highly germinated on 1/10, 1/4 and 1/2 strength of MS media, but the survival percentage of protocorms were varied due to the species and concentration of MS medium (Lee *et al*., 2010). The optimum strength of medium for protocorm survival of *P. aphrodite* subsp. *formosana* and *P. philippinensis* was 1/10 MS salts (Lee *et al*., 2010).

Most of plantlets derived from seeds exhibited non-variegated leaves (Figure 2, 3a). Among them, only one plantlet (out of 1344 seeds) that produced variegated leaves (Figure 3c), although it has variegated pattern but it showed not so severe phenotype as the parent (Figure 3b).

Figure 3. Phenotype of mini hybrid P. "Sogo Vivien"orchids. (a) P. "Sogo Vivien" with normal green leaves; (b) P. "Sogo Vivien" with variegated leaves; (c) Plantlet of variegated P. "Sogo Vivien" generated from seed. Bars: 1 cm

Variegated traits may becaused by mutation in nuclear genes that could be distinguished from mutation in the chloroplast genome by examining the inheritance pattern of the variegated traits. Inheritance to mutation in the chloroplast is due generally non-Mendelian and it will show maternal inheritance after reciprocal crosses was carried out (Kirk and Tilney-Basset, 1978; Hatfield *et al.*, 1985; Shoemaker *et al.*,1985; Cianzio and Palmer, 1992). In tobacco, after reciprocal crosses of variegated plants, the result showed non maternal inheritance,that resulted *periclinal chimera* mutation (Bae *et al.*, 2000).

3.2. Regeneration of plantlets from flower stalk nodes

The result of growing plantlets from nodes as explants showed that the buds on the second node (Figure 4b) exhibits the best growth response compared to the other nodes, although the speed of bud formation on the second node (13 days) was longer than that of the third node (11 days) and fourth node (12 days) (Table 2).The slowest growth of plantlets was bud that developed from the first node (Figure 4a, Table 2). Buds on the fifth node (Figure 4e, Table 2) showed slow growth due to the browning reaction as results of the most production of phenolic compounds that easily oxidized (Chugh *et al.,* 2009). Leaf lamina emerged only from buds on second node (day 42) and third node (day 115) (Table 2). In this case, the second node from the basal part represented a portion of the enlarged dormant bud, consisting of young, rapidly dividing cells or tissue, which can regenerate much easier and quicker than the buds of the other nodes. Histological analysis showed that at 11-13 days after shoot segment plantation on NP medium, the shape of apical cells in the nodes was changed, then followed by the change of cell shape in the basal part of the nodes, produced bipolar pattern, then gradually developed into shoot (Figure 4f-j).

Murthy and Pyati (2001) also reported that the physiological age of an explant is an important factor in plant regeneration. In previous study, Balilashaki *et al*. (2014) reported that buds on the swollen nodes started to grow 2 weeks after emergence, but some other buds remained dormant.

The different result was showed by Kosir *et al*. (2004), that direct shoot regeneration from nodes of *Phalaenopsis* orchids was not significantly affected by the position of the dormant buds from top down to base of a flower stalk.

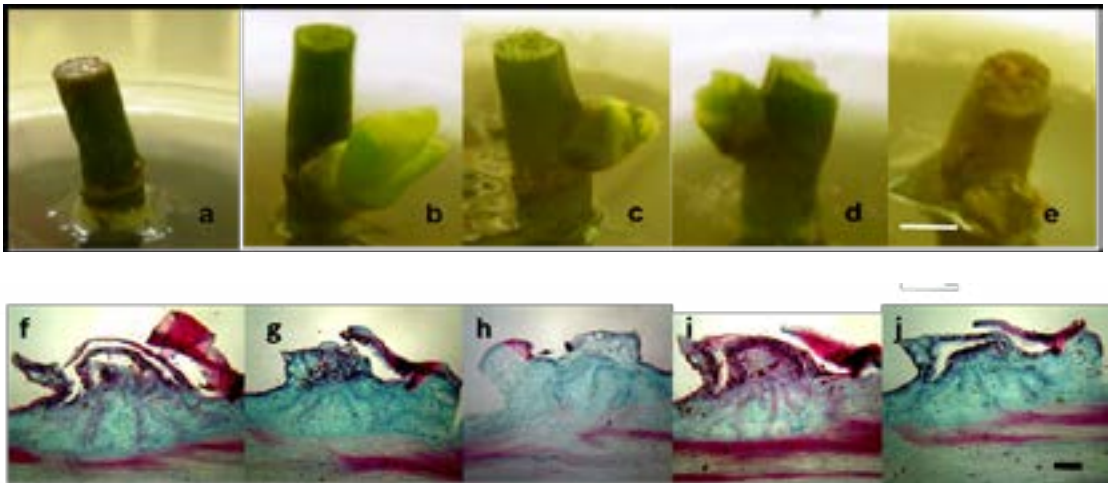

Figure 4. The growth of adventitious buds from flower stalk nodes of P. "Sogo Vivien". (a-e) Bud development at flower stalk nodes; (f-j) Longitudinal section of buds on node 1 (a, f), node 2 (b,g), node 3 (c,h), node4 (d,j) and node 5 (e, j) of the base of the flower stalks, which were planted in medium VW + BA 10 mg.L^{-1} + activate carbon, at 37 days after plantation (DAP). Bars 0.5 cm for a-e and 100 µm for f-j. Arrows point to shoot axiler meristem.

Table 2. Growth of buds and leaf lamina from the nodes of P. "Sogo Vivien" flower stalks on VW medium

Parameters	Number of buds on node-n (n=1,2,3...)				
	1	2	3	4	5
Buds are formed (days)	15±1.00a	13±1.00b	11±1.14c	12±1.00b	13±0.70b
Length of bud aged 15 days (mm)	1±0.25d	2±0.25c	4±0.36b	5±0.5a	2±0.35c
Length of bud + lamina aged 42 days (mm)	2±0.66c	10±0.82a	6±0.51b	6±0.76b	2±0.35c
Length of bud + lamina aged 54 days (mm)	2±0.35c	21±0.35a	9±0.35b	8±0.57b	4±0.43c
Chapped buds with yellow outskirts (days)	nd	30±2.00b	38±3.6a	40±1.00a	nd
Appearance of leaf lamina with yellow outskirts (days)	nd	42±2.54b	115±4.12a	nd	nd

*Notes: Values in rows preceding the same letter are not significantly different according to SPSS and Duncan test at 5% significance level, nd= not detected.

In this study,the growth of bud at the second node from flower stalk on the medium VW + BA 10 mg.L^{-1} and active carbon was initiated by increasing the size of bud (Figure 5a), and followed by chapping bud with yellow outskirt (Figure 5b). Then, the bud forms several leaf laminas on 42 days after plantation (DAP) (Figure 5c). The growth speed of leaf lamina in apical much higher than the growth of leaf lamina in the basal (Figure 5d). In this stage, pattern of variegated leaf can be easily shown as yellow sector that wider than the green sector. After 51 DAP, leaf lamina increased in size and the leaf was dominated by green color in the center with yellow stripe in the margin of the leaf (Figure 5e). The formation of variegated pattern was completed on fully opened leaves (Figure 5f).

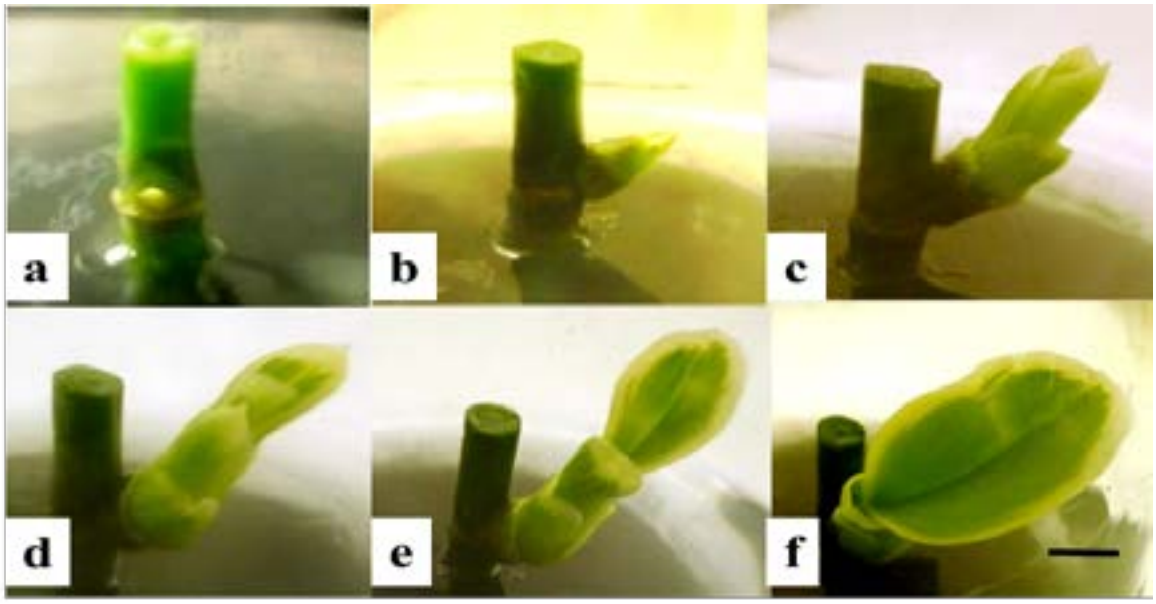

Figure 5. The growth and development of bud on the second node from flower stalk in medium VW + BA 10 mg l^{-1} + active carbon. (a) The bud on second node, 0 day after plantation (DAP). (b) Chapped buds with yellow outskirt, 30 DAP. (c) Appearance of leaves lamina with yellow outskirts, 42 DAP. (d). The growth of apical leaf was prominent, 49 DAP, (e) The apical leaf was bigger in size, 51 DAP. (f) Optimum growth of apical leaf, 62 DAP. Bars 0.5cm.

All regenerated plantlets from buds of the nodes of flower stalks displayed variegated phenotype, although the variegated pattern varied greatly depending on the age of the leaves. The young leaves later showed yellow sector as more dominant part than the green sector. Along with the growth of leaves, yellow sector will be shifted to the outskirts of the leaves and the green sector will eventually dominate the leaves (Figure 6a). The variegated phenotype was inherited permanently that indicated by no change on the pattern of leaves after acclimatization of the plantlets *ex vitro* (Figure 6b). Propagation of orchids with flower stalk explants of *Dorietanopsis* Purple Gem 'Ching Hua' using a combination of plant growth regulator NAA and TDZ produce plantlets, which are morphologically similar to their parent (Vendrame and Maguire, 2007).

Micropropagation using node explants from flower stalks have been carried out many times on *P. amabilis*, *Phalaenopsis* hybrid and adventitious shoots at the pseudobulb that called keiki in *Dendrobium* hybrid to produce plantlets, directly or indirectly, which resulted in identical phenotype as their parent in a shorter period of time (Park *et al*., 2003; Kosir*et al*., 2004; Martin *et al*., 2004; Balilashaki*et al*., 2014). This method could minimized the cost for micropropagation (Markovi*ćet al*., 2012). This method could also be used for propagation of orchids, which are recalcitrant, such as *P. violacea*, and it could minimize somaclonal variation (Subramaniam*et al*., 2009).

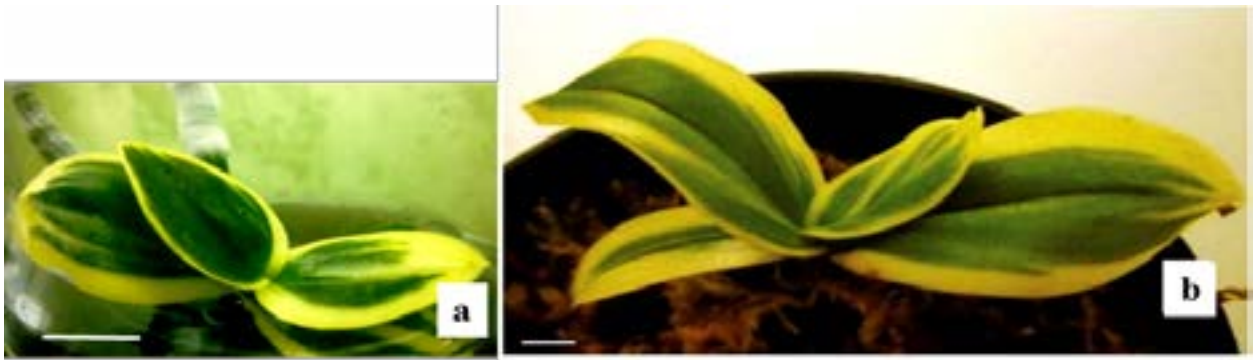

Figure 6. Plantlets of Phalaenopsis"Sogo Vivien" produced from buds on nodes of flower stalks, aged 5.5 months after plantation (a), and from acclimatized plants, aged 9 months (b). Bars: 1cm.

4. Conclusions

Large number of *P.* "Sogo Vivien" can be produced by embryo culture using seeds sown on NP medium. Variegated *P.* "Sogo Vivien" plantlets can be regenerated from *in vitro* culture of the second and third nodes (from the basal part) of variegated plant's flower stalk.

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