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Full Length Research Paper

Detection of somaclonal variation by random amplified polymorphic DNA analysis during micropropagation of *Phalaenopsis bellina* (Rchb.f.) Christenson

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***Phalaenopsis bellina* (Rchb.f.) Christenson orchid species are known for their beautiful flower shape, graceful inflorescence and fragrance. Protocorm-like bodies (PLBs) of *P. bellina* were induced from leaf segments. The PLBs were then subjected to proliferation using ½ strength Murashige and Skoog (MS) media with two subcultures at three months intervals. Twelve decamer random amplified polymorphic DNA (RAPD) primers were used to study somaclonal variation among the mother plant, the initially induced PLBs and proliferated PLBs after 3 and 6 months in culture. Eight out of twelve primers produced 172 bands with 18 polymorphic bands in all the treatments. The amplified products varied between 125 to 8000 bp. Among the primers used, P 16 produced the highest number of bands (29), while primer OPU 10 produced the lowest number (15). The range of similarity coefficient was from 0.83 to 1.0 among the different sub-cultures and mother plant (MP). It was found that minimal or no changes occurred between the MP and the PLBs produced after 3 months of induction. The induced PLBs were then subcultured for six months for proliferation and this resulted in about 17% dissimilarity with MP. It is reported that micropropagation of *P. bellina* can be carried out successfully using ½ strength MS media for 6 months but further proliferation may result in somaclonal variation which might change the prolific characteristic of this orchids.**

Key word: Moth orchid, somaclonal variation, random amplified polymorphic DNA, protocorm-like bodies.

INTRODUCTION

Phalaenopsis 'Moth orchids' are among the most beautiful flowers in the world. This genus has economic value for pot plant and cut flower production and is distributed throughout Southeast Asia with a few species extending from Taiwan, Sikkhim to Australia and the Pacific (Teob, 1989). Taxonomically, the genus *Phalaenopsis* belongs to the family *Orchidaceae*, sub-family Epidendroideae, tribe *Vandeae* and subtribe *Aeridinae* which is divided into five subgenera, namely *Proboscidioides*, *Aphyllae*, *Parishianae*, *Polychilos* and *Phalaenopsis*. It comprises of approximately 66 species according to the latest classification of Christenson (Christenson, 2001). *Phalaenopsis bellina*, classified in subgenus *Polychilos*, is native to Malaysia and numerous commercial varieties have been bred because of its pleasant fragrance (Hsiao et al., 2006). Orchids can be propagated using different

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Abbreviations: MS, Murashige and Skoog medium; **TDZ,** 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea; **PLBs,** protocorm-like bodies; **RAPD,** random amplified polymorphic DNA; **MP,** mother plant; **RFLP,** restriction fragment length polymorphism; **PCR,** polymerase chain reaction; **CTAB,** cetyltrimethylammonium bromide; **EDTA,** ethylenediaminetetraacetic acid; **PCI,** phenol/chloroform/isoamyl alcohol; **TDZ,** thidiazuron.

methods such as division, back bulb, aerial cuttings, meristem, tissue culture of seeds and others. However, these methods can be time consuming and result in only a limited number of plants. The most popular method of propagation to date for orchids is through *in vitro* propagation as it is said to produce a large number of clones in relatively short duration. Despite its potential to produce numerous new plants from a single leaf segment, it is subject to unpredictable mutations or somaclonal variation which can occur during the process of multiplication. Somaclonal variation though hailed as a novel source of genetic variation which can result in improved material, is often undesirable when the objecttive is micropropagation of elite genotypes. Somaclonal variation can happen because of many reasons such as the type of media used, plant growth regulators and its concentration, the type of explant and number of subculture cycles (Reuveni et al., 1986). In the case of *P. bellina,* somaclonal variation may result in the loss of fragrance or change in the color of flowers which appears to be some of the important characteristics of this orchid.

Some orchid hybrids are more amenable than others to somaclonal variation. The percentage of the variations can range from 0 - 100% depending on varieties, with an average of 10% among *Phalaenopsis* (Tokuhara and Mii, 1993). The cause of somaclonal variation in higher plants has been reported during different biochemical and molecular events, including changes in DNA methylation pattern, activation of transposable elements and chromosome remodeling (Hirochika, 1993; Price et al., 2002). Therefore, molecular markers have been exploited for the detection of somaclonal variation, including random amplified polymorphic DNA (RAPD) (Chen et al., 1998; Rivalet al., 1998), methylation sensitive restriction fragment length polymorphism (RFLP) (Kubis et al., 2003; Jaligot et al., 2000, 2002) and microsatellite sequence variation (Alou et al., 2004).

The RAPD technique has several advantages such as the ease and rapidity of analysis, a relatively low cost, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (William et al., 1990). RAPD analysis using polymerase chain reaction (PCR) in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals. Somaclonal variation has also been reported in *Phalaenopsis* by RAPD analysis on regenerated plants, which showed morphological and physiological changes in the flowers (Chen et al., 1998).

In orchids, most of the work that utilized RAPD analysis has concentrated on population studies of one to a few species of orchids, e.g., *Goodyera proceracilis* and *Zeuxine strateumatica* (Sun and Wong, 2001), *Changnienia amoena*, *Paphiopedilum malipoense* and *Paphiopedilum micranthum* (Li et al., 2002). This study was conducted in order to verify the *in vitro* morphogenetic responses, as well as the genetic stability of the protocorm-like bodies (PLBs) produced during the process of subculture in *P. bellina* using RAPD markers.

MATERIALS AND METHODS

Plant materials

Induction of PLBs from the leaf of *in vitro* plantlets

Leaf of *P. bellina* (Rchb.f.) Christenson (Figure 1c) obtained from a single plant from nursery were originally sterilized and cultured with adaxial side on ½ MS (Murashige and Skoog, 1962) solidified medium. The ½ MS solidified medium was supplemented with 100 mg/l myo-inositol, 0.5 mg/l niacin, 0.5 mg/l pyroxidine HCL, 0.1 mg/l thiamine HCL, 2.0 mg/l glycine (Tokuhara and Mii, 2003; Chen and Chang, 2004), 3 mg/l TDZ, 2% sucrose and 3 g/l gelrite with 10% fresh ripen banana extract. The pH of the mixture was adjusted to 5.6 (Islam et al., 2003), before autoclaving for 15 min at 121°C. Leaves from this plant were also used to extract its DNA and termed mother plant (MP). These leaf cultures were maintained for 3 months in order to induce PLBs (Figure 1b). The PLBs obtained at the end of 3 months was termed subculture zero (S0).

The PLBs were than further maintained and allowed to proliferate for 6 months with 2 subcultures (S1 after 3 months and S2 after 6 months). Sample from the mother plant, S0, S1 and S2 were first washed with tap water and then surface sterilized with 10% (v/v) Clorox ® solution (Chlorox, Malaysia) for 5 min. Following this, they were rinsed three times with distilled water and blotted dry with paper towels. They were then wrapped with aluminum foil and stored at -80°C or used immediately for DNA extraction.

The PLBs induced on the adaxial side up of the leaf segments (Figure 1a) was isolated and placed on to fresh medium for proliferation of the PLBs. As *P. bellina* is slow growing, orchid subculture was only made after 3 and 6 months. A large number of PLBs (Figure 1b) can be obtained at the end of 6 months in order to detect the presence of somaclonal variation.

DNA extraction

Genomic DNA was extracted from leaf (MP) and PLBs (S0, S1 and S2) sample using a modification of the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990) with slight modifications. Quantification and purity analysis of DNA was made using spectrophotometer. Two hundred milligrams of fresh young material were lyophilized with liquid nitrogen and 700 µl of CTAB buffer [2% (w/v) CTAB; 1.4M NaCl; 0.5% (v/v) 2-mercaptoethanol; 20 mM ethylenediaminetetraacetic acid (EDTA); 100 mM Tris-Hcl, (pH = 8.0); 4% (w/v) PVP] was added to the tissue powder. The mixture was preheated at 65°C for 20 min and 5 ml of SEVAG [Chloroform-isoamylalchohol (24:1)] was added and centrifuged at 10000 rpm for 15 min at room temperature to obtain a clear supernatant containing the genomic DNA. The supernatant was transferred to a new tube, DNA was precipitated with 2/3 volume of isopropanol, and the mixture was centrifuged at 10000 rpm for 20 min at 4°C. The DNA pellet was washed with 5 ml wash buffer [76% (v/v) ethanol, ammonium acetate 10mM] and retained in the wash buffer for 20 min at room temperature. Subsequently, the mixture was centrifuged at 10000 rpm for 5 min, and the pellet was dissolved with 500 µl of TE (Tris-Hcl 10 mM, EDTA 1 mM) buffer. Then, 1 µl of RNAase (10 mg/ml) was added to the DNA solution, centrifuged at 12000 rpm for 5 min at room temperature, and phenol/chloroform/isoamyl alcohol (PCI) extraction was repeated for the top aqueous layer. Subsequently, 3 M sodium acetate (pH 5.2) and equal volume of cold isopropanol were added to the PCI extraction, centrifuged at 12000 rpm for 10 min at room

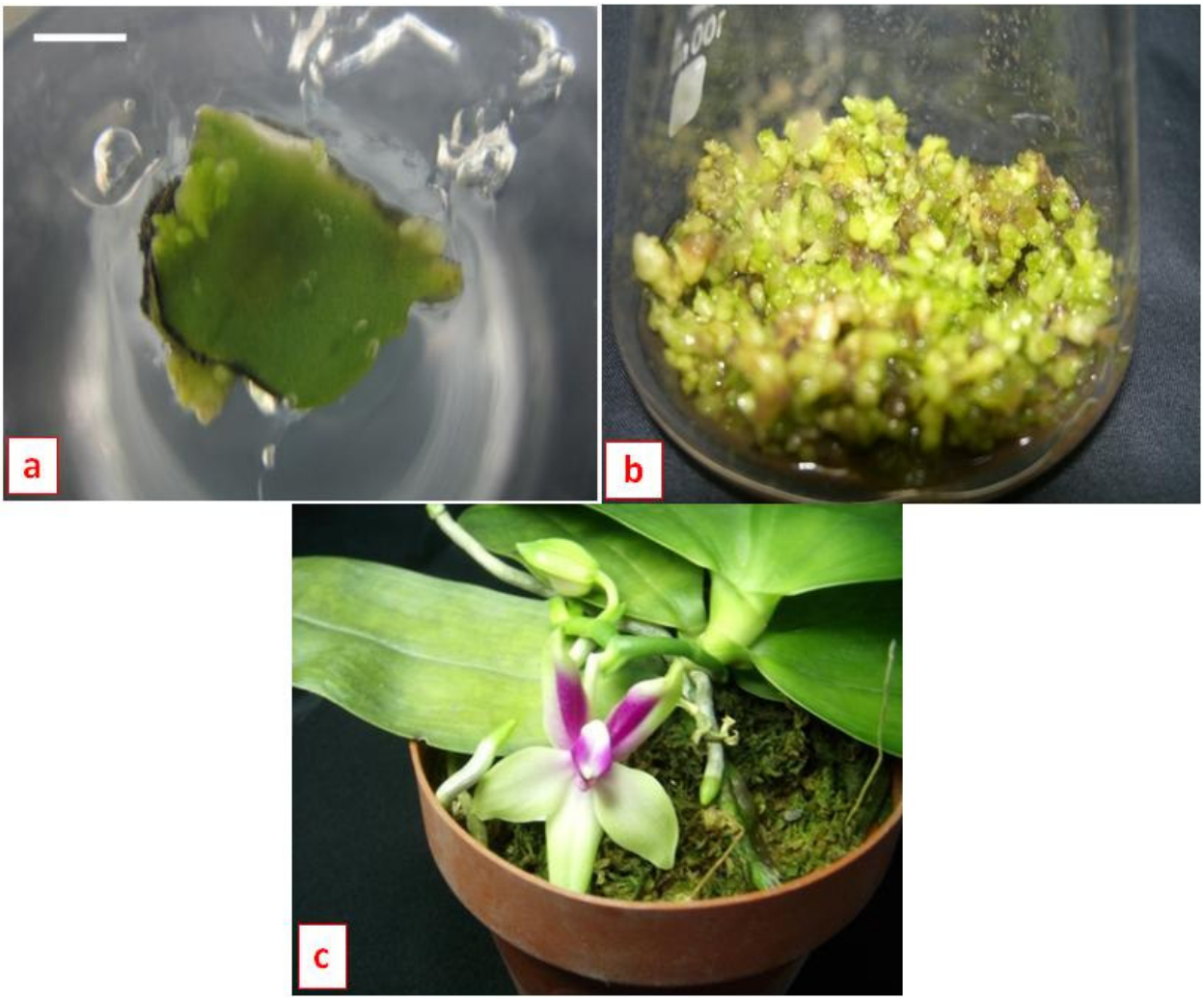

Figure 1. Inducuction of protocorm-like bodies (PLBs) from leaf explant of *P. bellina* (bar in upper left refers to all panels). (a) ½ strength MS medium supplemented with 3 mg/l TDZ (bar = 0.5 cm), (b) PLBs induced from PLBs (bar = 1cm), (c) six month old mother plant (bar = 2.5 cm).

temperature. The DNA pellet obtained was washed with 500 µl of 70% ethanol, centrifuged at 12000 rpm for 5 min at room temperature, and the pellet was dried. The pellet was dissolved in 500 µl TE buffer.

Screening of primers

Twelve decamer oligonucleotide RAPD primers were chosen (Table 1): (OPU 08, OPU 09, OPU 10, OPU 11, OPU 12, OPU 13, OPU 14, OPU 15, OPU 16, P 12, P 14 and P 16) (1st base company, England and Wales) and used for analysis on the genetic stability between the mother plant and plants produced after induction and proliferation namely: S0, S1 and S2. Primers that produced reproducible polymorphic bands were scored, followed by determination of the fragment size in base pairs.

PCR amplification

The random amplified polymorphic DNA reaction mixture contained 1 µl DNA sample, 500 mM PCR buffer, 20 µl deoxynucleotide triphosphates (dNTPs), 2.5 units of Taq DNA polymerase (Fermentas) and 2 µml of $MgCl_2$ (Fermentas), 1.4 µml of each primer (1st base company, England and Wales) made up to a final volume of 25 µl. Amplification was conducted in a thermal cycler (Biometra Thermal Cycler, Whatman, USA) programmed for 1 cycle of 94°C for 5 min, 94°C for 30 s and 42°C for 1 min, followed by a program run through 35 cycles of 72°C for 2 min. The final extension was at 72°C for 5 min. Amplification products were separated in 2% (w/v) agarose gels in 1×TAE buffer at 80 V for 1 h. The size of the amplification products were estimated using 1 kb DNA ladder (Fermentas). The gel was stained with 2 mg/L ethidium bromide for 5 min, destained in sterilized distilled water for 1 min. The RAPD analyses were repeated three times in order to check reproducibility.

Gel scoring

Bands were viewed under UV light and photographed. Bands on photos were scored using GENE TOOLS of Syn Gene package

Table 1. List of 12 RAPD primers used for amplification of different DNA obtained from the MP, S0, S1 and S2 in *P. bellina.*

Primer	Sequence (5'-3')	Melting temperature (Tm °C)	GC content (%)
OPU 08[a]	GGCGAAGGTT	32	60
OPU 09	CCACATCGGT	32	60
OPU 10 [a]	ACCTCGGCAC	34	70
OPU 11	AGACCCAGAG	32	60
OPU 12 [a]	TCACCAGCCA	32	60
OPU 13 [a]	GGCTGGTTCC	34	70
OPU 14	TGGGTCCCTC	34	70
OPU 15	ACGGGCCAGT	34	70
OPU 16 [a]	CTGCGCTGGA	34	70
P 12 [a]	CCAAGCTTGC	32	60
P14 [a]	AGGATACGTG	30	50
P 16 [a]	GGATCTGAAC	30	50

(Gene Genius, Bio Imagine, USA).

RAPD data and cluster analysis

Clear RAPD bands were scored '1' if they were present and '0' for absence of the band for each DNA samples. The binary data obtained was analyzed with the program NTSYS-PC version 2.0 (Rohlf, 1993). The SIMQUAL module was used to generate a similarity matrix using the Jaccard coefficient with the formula:

$$Sij = a / a + b + c$$

Where, Sij is the measure of genetic similarity between two individuals, i and j. 'a' is defined as the number of bands present in both i and j; 'b' is defined as the number of bands present in i.

The distance matrix was then used for cluster analysis, and the sequential Agglomerative, Hierachila and Nested clustering (SAHN) (Sneath and Sokal, 1973) module was used to produce a dendrogram with the unweighted pair-group method with arithmetic mean (UPGMA) clustering strategy. The experiments were carried out in the laboratory of Agribiotechnology, Faculty of Agriculture, Universiti Putra Malaysia.

RESULTS

Assessment of variation using RAPD analysis

Optimization of RAPD protocols and selection of primers ensured that the RAPD profiles were reproducible. Among the 12 random primers (Table 1) used for the initial screening, four failed to amplify any bands, while the other eight primers provided clear and scoreable amplification products in all the treatments (Figure 2). A total of 172 bands were produced using eight primers with 154 monomorphic and 18 polymorphic bands (Table 2). The decamer DNA fragment identified an average of 21.5 bands per primer. The highest number of bands produced was for P 16 with 29 bands, while the lowest number of bands was produced for OPU 10 (15 bands). The primers produced a unique set of amplification products ranging in size from 125 bp in OPU 10 to 8000 bp in OPU 08 (Table 2). The primer OPU 08 produced 28 bands with 0% polymorphism (Plate 1). Similarly, primer P 12 with 23 bands also resulted in 0% polymorphism, indicating that proliferation up to 6 months in *P. bellina* did not result in somaclonal variations. Other primers namely OPU 10, OPU 12, OPU 16 and P 14 had low polymorphism ranging from 6 - 12%. Primer OPU 12 identified 18 bands with two polymorphic bands at 3000 and 4000 bp in samples representing the initial subculture. This could be due to the material at this stage not haven differentiated completely as the initial subculture consist of newly induced PLBs from the leaf explants or indication that the detected variations do not necessarily correspond to alterations in the DNA sequence. Ten additional bands were present in the initial and second subculture (1 and 3) and one band (560 bp) was missing from the MP.

The occurrence of specific bands/loci in the PLBs of different sub-cultures showed high similarity among different PLBs and MP and usage of tissue culture caused minimal dissimilarity in *P. bellina*. It also seems that the genetic variations induced in the PLBs increase with the time-period of the sub-culture. For example, the PLBs of the first sub-culture showed comparatively lower degree of genetic dissimilarity from the parental plants as they are placed in the clusters much closer to each other when compared to the PLBs of the last sub-cultures.

Genetic similarity and multivariate analysis

Among treatments which were produced from different subcultures and mother plant, the range of similarity coefficients was from 0.83 to 0.97 (Figure 2). Based on the dendrogram, 2 main clusters from 4 treatments were obtained. Cluster 1 contained MP and PLBs corresponding to the initial subculture (S0) which had similarity at the coefficient level of 0.97 and a sub cluster with PLBs

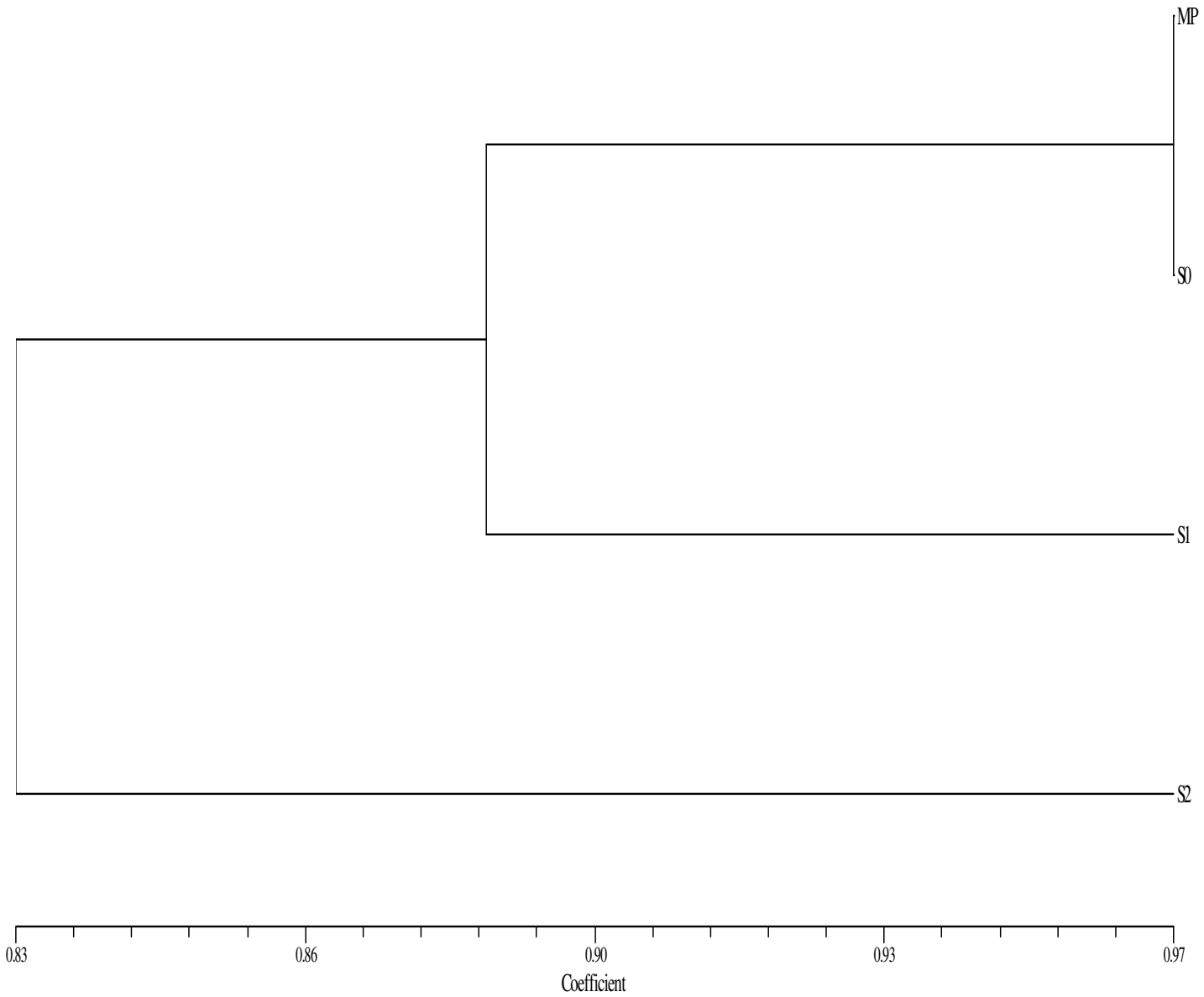

Figure 2. Dendrogram of genetic similarity constructed using the jaccard coefficient and the UPGMA method for mother plant (MP), corresponding to the initial subculture (S0), second subculture (S1) and third subculture (S2) of *P. bellina*

Table 2. Distribution of RAPD in eight selected primers among MP and PLBs achieved from various subcultures in *P. bellina*.

Primer	Sequence (5'-3')	No. of bands	Ranges		Polymorphic band	Polymorphism (%)
			High	Low		
OPU 08	GGCGAAGGTT	28	8000	3500	0	0.0
OPU 10	ACCTCGGCAC	15	740	125	1	6.66
OPU 12	TCACCAGCCA	18	7750	2030	2	11.11
OPU 13	GGCTGGTTCC	17	975	560	6	35.29
OPU 16	CTGCGCTGGA	24	4160	710	2	8.33
P 12	CCAAGCTTGC	23	3035	910	0	0.0
P14	AGGATACGTG	18	975	560	2	11.11
P 16	GGATCTGAAC	29	3680	400	5	17.24
Total		172			18	

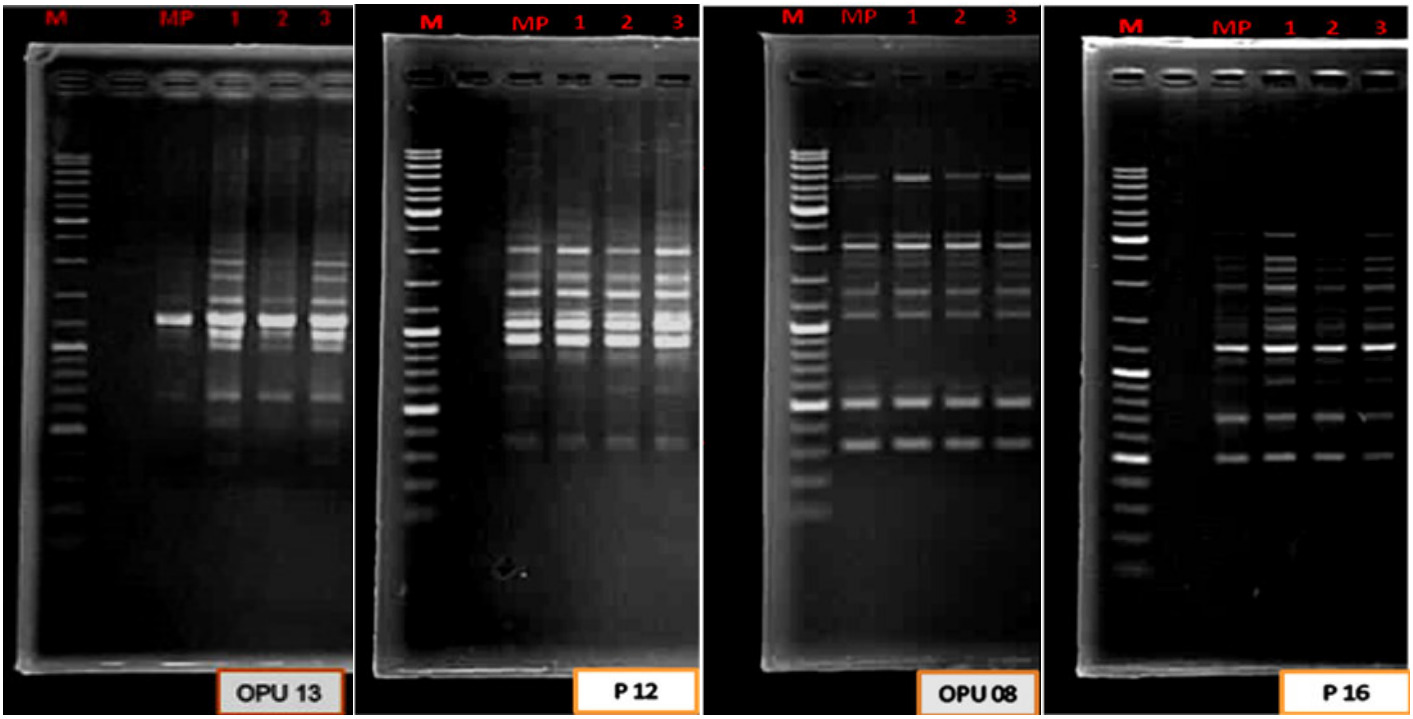

Plate 1. RAPD analysis in mother plant (MP), corresponding to the initial subculture (1), corresponding to the first subculture (2), corresponding to the second subculture (3) of *Phalaenopsis bellina* for detection of somaclonal variation; a) OPU 08, b) OPU 12, c) P 12, d) P 16.

Table 3. Summary of RAPD amplified products examined in the study.

Description	RAPD
Total bands scored	172
Number of monomorphic bands	154
Number of polymorphic bands	18
Percentage of polymorphism	10.46
Number of primers used	8
Average polymorphism per primer	2.25
Average number of fragments per primer	21.5
Size range of amplified fragments	125 - 8000

RAPD, Random amplified polymorphic DNA.

corresponding to the first subculture (S1) which showed 0.88 coefficient level with MP and S0. Cluster 2 was occupied by PLBs corresponding to the second subculture (S2) which showed similarity at the coefficient level of 0.83 with cluster 1. The closest similarity with the mother plant was observed in the case of S0, which gave 97% similar RAPD profile to its mother plant. On the contrary, S2 showed highest divergence with similarity value of 0.80 (Table 4) from MP. Among the PLBs produced after different subculture cycles, S0 and then S1 had closer RAPD profile relationship to MP (similarity value 0.96 and 0.87), while S2 was 83% similar to MP (Table 3 and Figure 2).

DISCUSSION

Plant cells grown *in vitro* have been shown to be susceptible to genomic variations, a phenomenon often referred to as somaclonal variation. The frequency of variation depends on the genotype, culture medium, growth hormones and the way of multiplication. Inherited modifications in plants regenerated from tissue culture (somaclonal variation) have been reported frequently for example in microproagated plants of *Populus deltoides* (Rani et al., 1995), *Coffea arabica* as well as diploid and triploid elite tea clones representing *Camellia sinensis*, *Camellia assamica* (Devarumath et al., 2002). Quantification of the genetic variations derived from tissue culture regenerants has also been reported in many plant species (Bajaj, 1990; Larkin and Scowcroft, 1981) such as *Musa acuminate* (Damasco et al., 1996; Gimenez et al., 2001), *Picea glauca* (Isabel et al., 1996), *Beta vulgaris* (Munthali et al., 1996), *Elaeis guineensis* (Rival et al., 1998), *Dieffenbachia* (Shen et al., 2007) and sugarcane (Rajeswari et al., 2009). Although reports on the presence of somaclonal variation in tissue culture derived systems are numerous, reports for orchids in general are scarce. To the best of our knowledge, to date there is no report on somaclonal variation in *Phalaenopsis*

Table 4. Similarity matrix of the somaclonal variation between mother plant and different subcultures in *P. bellina.*

Samples	MP	S0	S1	S2
MP	1.00			
S0	0.96	1.00		
S1	0.87	0.88	1.00	
S2	0.80	0.83	0.84	1.00

MP = Mother plant, S0 = initial subculture, S1 = first subculture, S2 = second subculture of *P. bellina*.

in response to subculture cycles induced by the culture conditions. Tokuhara (1992) however, wro-te on the phenotypic and genetic variations among the different clones of *Phaleanopsis*, concluding that about 10% variation was present among the various clones.

The use of RAPD in this study was able to differentiate between the mother plant and product of the various subcultures in *Phalaenopsis* orchid. Out of the 12 randomly selected primers, eight primers were able to generate highly specific amplification profiles. The identification of the specific primers can be useful for future studies in this orchid. Variations observed in this study on the total number of RAPD bands as well as the number of specific bands among the parental plants and protocom-like bodies of different sub-cultures indicate minimal genetic differences of the PLBs when compared to the MP. Even after 6 months in the culture condition containing thidiazuron (TDZ), the percentage of dissimilarity obtained was only 17. The presence of specific band/loci in the parental plants and loss of it in the PLBs of different sub-cultures indicate the loss of certain loci during tissue culture due to somaclonal variation, while the occurrence of specific bands/loci in the PLBs of different sub-cultures and their absence in mother plants may indicate the occurrence of genetic changes leading to formation of new binding sites in these plants. Modgil et al. (2004) reported that none of the micropropagated apple clones was exactly similar to the parental line with up to 17% dissimilarity in terms of RAPD profile. In banana cultivar Valery, Sheidai et al. (2008) found that the genetic variation was induced in the regenerated plants that increased with the time-period of the subculture after nine subcultures at 45 days each. Similar findings on the effect of subculture cycles on genetic variations has also been reported in *Apium* and *Prunus* species (Soniya et al., 2001) as well as in cotton (Sheidai et al,. 2008). It appears that, the presence of variations *in vitro* is common and what have to be determined are the frequencies in relation to the type of growth regulator and the subculture period. Some species are more amenable to variations than others and understanding this will enable the producer to limit the subculture cycles in order to maintain the clonal characteristics.

Conclusions

In conclusion, this study looked at the level of polymorphism which can show somaclonal variations during the process of tissue culture multiplication. A number of random primers, commonly used for higher plants were used to obtain a reliable estimate of the variation. It was found that minimal or no changes occurred between the MP and the PLBs produced after 3 months of induction. The induced PLBs were then subcultured for six months for proliferation and this resulted in about 17% dissimilarity with MP. It is reported that micropropagation of *P. bellina* (Rchb.f.) Christenson can be carried out successfully using ½ strength MS media for 6 months but further proliferation may result in somaclonal variations resulting in changes to the prolific characteristic of this orchid.

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