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An Efficient Micropropagation Protocol to Control Abnormality in Long-Term Shoot Cultures of *Spathiphyllum floribundum* (L.) 'Petite'

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ABSTRACT

An efficient *in vitro* propagation protocol for *Spathiphyllum floribundum* L. cv. 'Petite' was developed after refining different cultural parameters. Treatment with 0.5% sodium hypochlorite followed by 0.1% mercuric chloride, periodic washing in sterilized distilled water and culture in NB medium resulted in 53% contamination-free axillary buds, which served as the explants. White's (1963) medium, Murashige and Skoog (1962) medium, and its modification supplemented with or without different concentrations of plant growth regulators were tested in order to establish tissue cultures. Modified MS basal medium containing 2.5 μM N^6-benzyladenine (BA) and 1.0 μM α-naphthaleneacetic acid (NAA) was the most effective medium, with 72.50 ± 4.79% of cultures being established. When BA was increased to 10.0 μM, an average of 6.28 ± 0.52 axillary shoots/explant produced within 6 weeks of initial culture. Secondary subculture with this high concentration of BA in the multiplication medium significantly induced a large number of axillary shoots (8-10/explant) but these became morphologically abnormal after the second subculture. However, if 10.0 μM and 2.5 μM BA were alternated in each subculture, an average of 6.30 ± 0.89 shoots/explant with a mean shoot length of 39.40 ± 2.07 mm were generated. Based on this medium modification, from the third subculture onward an average of 10-15 plants/culture bottle could be directly harvested from the multiplication medium and transferred to the greenhouse for hardening. Small shoots (10-20 mm) present within the clusters were further elongated and rooted in the same basal medium fortified with 0.5 μM BA and 1.0 μM NAA before transfer to the field. The survival rate following acclimatization was 92.50 ± 1.44% and plants grew vigorously after transfer to earthen pots containing soil, coarse sand and cattle manure (1: 1: 1).

Keywords: axillary shoot proliferation, micropropagation, shoot abnormality
Abbreviations: 2iP, N^6-(2-isopentenyl)-adenine; **BA**, N^6-benzyladenine; **IBA**, indole-3-butyric acid; **KN**, kinetin; **MS**, Murashige and Skoog; **NAA**, α-naphthaleneacetic acid; **SE**, standard error; **TDZ**, thidiazuron

INTRODUCTION

Spathiphyllum floribundum var. 'Petite', belonging to the Araceae family, is native to tropical regions of the Americas and southeastern Asia. The plant is evergreen, herbaceous and perennial in nature and is very lucrative for its large leaves and attractive, long-lasting inflorescence consisting of a showy white spathe and spadix (Dewir *et al.* 2006). The commercial production of ornamental plants is growing worldwide. Its monetary value has increased significantly over the last two decades and there is a great potential for continued growth in both domestic and international markets. *Spathiphyllum* is one of the major import/export ornamental plants (Rout *et al.* 2006). Vegetative propagation of *Spathiphyllum* is slow and difficult, and seed production is irregular in temperate countries (Hennen and Hotchkiss 1995). Plant tissue culture industries mainly propagate this plant at a large scale using micropropagation because of its bushy and uniform growth habit and the tremendous growing demands in both domestic and export markets. The commercial production of ornamental plants for a competitive market requires cost reduction and this is possible either through reduced labor or by increasing the efficiency of the technology. Many studies have documented the successful micropropagation of *Spathiphyllum* using axillary bud culture (Fonnesbech and Fonnesbech 1979; Wand *et al*. 1995; Werbrouck *et al*. 1995; Watad *et al*. 1997; Malagon *et al*. 2001; Dewir *et al*. 2006). However, their observations were mainly restricted only to the first or second subculture and they did not study the long-term effect of the presence of a high concentration of cytokinin in the multiplication media. Wand *et al*. (1995) first reported abnormalities in *in vitro* generated *Spathiphyllum* plants after continuous culture in the presence of 1-2 mg/L of N^6-benzyladenine (BA). Cytokinins induce hyperhydricity in many plant species, usually in a concentration-dependent manner and when other conditions in the culture system are not optimized (Kataeva *et al*. 1991; Ochatt *et al*. 2002; Ivanova and van Staden 2008).

Parkinson *et al*. (1996) and Malagon *et al*. (2001) indicated that decontamination and culture establishment are major problems in *Spathiphyllum in vitro* propagation and they proposed different sterilization techniques. This report describes a simple efficient decontamination process of *Spathiphyllum* explants, *in vitro* shoot proliferation on six subsequent cultures by the controlled use of plant growth regulators (PGRs) to avoid morphological abnormalities, the use of a single medium for all three stages of micropropagation to minimize labor involvement and finally the optimization of a low-cost potting mixture for hardening *in vitro*-derived plantlets.

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MATERIALS AND METHODS

Source material collection and sterilization

Nodal explants were collected from mature plants grown in a greenhouse at the National Agricultural and Scientific Research Foundation, Kolkata, West Bengal, India. The explants were collected from the field and immediately placed in a solution containing 1% (v/v) aqueous teepol (Qualigen, India), 0.2% (w/v) Bavistin (M/S BASF India Pvt. Ltd., India) and 0.05% (w/v) Streptomycin sulphate (Jayco Chemicals, India). The excised nodes (0.5-1 cm long) were washed thoroughly under running tap water and then rinsed with double distilled water (DW) and placed under a laminar airflow hood for further surface sterilization treatments. The explants were then immersed in 90% ethanol for 1 min and washed with sterilized double DW. To decontaminate the explants, two surface sterilants – sodium hypochlorite (0.5 and 1.0%, v/v) and mercuric chloride (Himedia, India) (0.1 and 0.2%, w/v) – were used either alone or in the following combinations: NaOCl (0.5%, v/v) + $HgCl_2$ (0.1%, w/v), NaOCl (0.5%, v/v) + $HgCl_2$ (0.2%, w/v), NaOCl (1.0% v/v) + $HgCl_2$ (0.2%, w/v). Three drops of Tween-20 (Sigma-Aldrich, St. Louis, MO, USA), a wetting agent, were added to every 100 ml of sterilant solution and explants were treated in this solution for 5 min. Surface sterilization was carried out within a sterilized desiccator attached to a vacuum pump to withdraw air trapped within the explant. After each treatment the explants were washed with sterile DW for 5 min and, as a final step, the explants were thoroughly and repeatedly washed with fresh changes of sterilized water after 5-, 10-, 15-, and 30-min intervals prior to being dried on pre-sterilized Whatman No. 1 filter paper.

The explants were initially inoculated into solidified NB medium (Fisse *et al.* 1987) containing 1200 mg l^{-1} NH_4NO_3, 1200 mg l^{-1} K_2HPO_4, 480 mg l^{-1} $MgSO_4{\cdot}7H_2O$, 1200 mg l^{-1} NaCl, 2400 mg l^{-1} yeast extract, 24 g l^{-1} glucose and 7 g l^{-1} agar-agar (Sigma-Aldrich). Medium pH was adjusted to 7.2 before autoclaving at 121.8°C, 1.1 kg cm^{-2} for 15 min. All culture establishment experiments were carried out in 150 mm × 18 mm glass culture tubes sealed with non-absorbent cotton wool wrapped with gauze. Data on infection was recorded after 2 weeks.

Culture media

The basal media used in the first set of experiments consisted of White's (1963), Murashige and Skoog (1962) (MS) and modified MS mineral salts along with vitamins, 3% (w/v) sucrose and 0.8% (w/v) bacteriological grade agar (Himedia). In the modified MS basal medium, the concentration of ammonium nitrate and potassium nitrate was reduced as much as 50% while keeping the other mineral constituents unaltered from the original basal full-strength MS. A range of concentrations of BA (0, 2.5 and 5.0 µM), either alone or in combination with 0.5 µM α-naphthaleneacetic acid (NAA) were supplemented in the basal culture media to induce sprouting of shoots from explants.

In the second set of experiments, modified MS basal medium was supplemented with vitamins, 3% sucrose, 0.8% agar and one of three cytokinins [Kinetin (Kn), BA, or N^6-(2-isopentyl) adenosine (2iP)] at different concentrations (0, 2.5, 5.0, 7.5, 10.0 µM) to multiply shoots.

In a third set of experiments, small shoot clusters having shoots 5-10 mm in length were cultured in modified MS basal medium containing the same concentration of vitamins, sucrose and agar, but supplemented with BA (0, 0.5, 1.0, 2.5 µM) either alone or in combination with 1.0 µM NAA for shoot elongation and rooting.

In all experiments, medium pH was adjusted to 5.8 with 1.0M KOH before autoclaving at 1.1 kg/cm^2 pressure (121°C) for 15 min. In each experiment, 50 ml of medium was poured into 400-ml glass bottles, each covered with a polypropylene cap.

Plantlet acclimatization

Young plantlets were carefully removed from culture medium and their roots were washed with sterilized DW and initially acclimatized to a controlled *ex vitro* environment (primary hardening). The plantlets were first placed in perforated plastic cups (6 cm in diameter) containing four different sterilized potting mixtures: coco peat: perlite (1: 1), coco peat: perlite (2: 1), peat moss: perlite (1: 1) and peat moss: perlite (2: 1). Plantlets were then incubated in a closed growth chamber at 20-28°C and 70-90% relative humidity (RH). Light was provided by cool fluorescent tubes (16-h photoperiod; photosynthetic photon flux density (PPFD) = 55 µmol m^{-2} s^{-1}, Philips, India). Plants were watered with a hand sprayer when required. After 2 weeks, plantlets were transferred to a separate polythene growth chamber with a PPFD of 200 µmol m^{-2} s^{-1} and 70-100% RH, and maintained at 20-28°C.The hardened plants (primary) from each treatment were transferred to a greenhouse into 15-cm earthen pots, the process of secondary hardening, in which 40 plants were randomly selected and potted in a mixture of soil, coarse sand and cattle manure (1: 1: 1, v/v) without disturbing the root system.

Potted plants were maintained in a shade house under 50% shade provided by an Agro shade net (B and V Agro Irrigation Co., Mumbai, India), a 10-h photoperiod, 200 µmol m^{-2} s^{-1} PPFD, 70% RH and at 28 ± 2°C. Water and fertilizer (foliar application of 19: 19: 19, N: P: K) were applied to individual plants by drip irrigation (Netafilm, Israel) at 200-300 ml of water/pot/day. The hardening experiments were conducted during the rainy season (July–October).

Experimental design

The decontaminated explants from NB medium were inoculated vertically in different culture media to evaluate the initial response regarding the sprouting of shoots. The six-week-old shoots that sprouted from any media were transferred onto fresh multiplication media containing different concentrations of three cytokinins (Kinetin, BA and 2iP). The average number of shoots/explant was counted after 6 weeks in culture. The excised nodal segments from the *in vitro* raised shoots were subcultured consecutively 4 times in multiplication medium containing a high concentration of BA (10.0 µM) to evaluate the effect on shoot multiplication (average number of shoots/explant), shoot length (mm) and shoot morphology (normal or abnormal). In a preliminary trial, the nodal portion of *in vitro* generated shoots were cultured in modified MS basal media with different concentrations of BA (2.5, 5.0 and 10.0 µM) in all possible permutations in successive subcultures on multiplication medium with the purpose of avoiding abnormal shoot cluster formation during multiplication. Initial findings showed that the experiment was designed in such a manner that nodal explants from the 1st multiplication medium containing 10.0 µM BA could be transferred to the 2nd multiplication medium containing 2.5 µM BA after 6 weeks without the formation of abnormal shoots, which would take place if subcultures were indefinitely maintained on medium with a high level (10.0 µM) of BA. Using this logic, two multiplication media were used in an alternate way over six successive cultures for 6-weeks each. At the end of each subculture, data on morphological abnormalities, number of shoots/node and shoot length were scored. To investigate shoot elongation and rooting, clusters of small shoots 8-10 mm long obtained after the 6th subculture were transferred to modified MS medium containing different concentrations of BA (0.5, 1.0 and 2.5 µM) either alone or in combination with NAA (1.0 µM). The average shoot length and the number of roots/plant were scored after 6 weeks of culture.

All cultures were maintained at 22 ± 2°C under a 16-h photoperiod (50 µmol m^{-2} s^{-1} PPFD) provided by white fluorescent tubes (Phillips, Gurgaon, India). After initial screening, modified MS medium was selected for further studies unless otherwise noted. All culture establishment experiments were carried out in 150 × 18 mm glass culture tubes capped with non-absorbent cotton wool wrapped in gauze.

Statistical analyses

A complete randomized block (CRD) design was used for all experiments. Ten explants were inoculated per treatment and each treatment contained three replications. Treatment effects were evaluated after 2–6 weeks.

For statistical analysis of the acclimatization process of

regenerated plants, three comparable experiments were performed for primary hardening, each with 50 plantlets; the percentage survival was scored after 30 and 60 days. During field establishment (i.e., secondary hardening) 40 plants were randomly selected and transferred to earthen pots. The experiment was repeated in triplicate and survival percentage of the plants was recorded after 60 and 120 days following transfer to the field.

Pooled data from repeated experiments was subjected to statistical analysis. Percentage data was arcsin transformed before analysis and converted back into percentage form for presentation in tables. Data was analyzed using SPSS for Windows version 16. Significant differences were assessed using Duncan's multiple range test at $P < 0.05$.

RESULTS

Surface sterilization

The maximum number of contamination-free explants survived (53%) after 2 weeks of culture on NB medium when they were sequentially exposed to 70% ethanol (1 min), 0.5% NaOCl (5 min), 0.1% $HgCl_2$ + 3 drops of Tween-20/100 ml (5 min) with repeated washes with sterile DW (**Fig. 1**).

Effect of medium formulations on establishment of cultures and shoot bud development

The trials conducted with the three basal media formulations revealed the superiority of modified MS medium over MS and White's media with respect to culture establishment and shoot bud induction (**Table 1**). White's (1963) medium was clearly unsuitable for culture establishment, although initially a few explants responded and remained green but, later on, shoot initiation did not progress.

Besides the basal culture medium, the concentration and type of PGRs used in media influenced explant establishment and axillary shoot bud induction. Callus formation from the base of the explant was a prerequisite for the establishment of a culture and this callus developed within 2 weeks on modified MS medium supplemented with BA (2.5 and 5.0 μM) and NAA (0.5 and 2.5 μM). A similar culture medium devoid of PGRs induced callus formation from significantly fewer explants after 3-4 weeks of culture. Shoot buds could be induced to sprout from explants with callus at the base within 3-6 weeks of culture depending on the PGR used. Use of either BA or NAA in the culture medium did not favor shoot bud induction from most of the explants tested, although significantly more explants responded in culture media supplemented with both NAA and BA (**Table 1**). Modified MS medium supplemented with BA (2.5 μM) and NAA (1.0 μM) produced the best response with respect to culture establishment and shoot bud induction and stimulated most of the explants (72.50 ± 4.79%) to form axillary shoots.

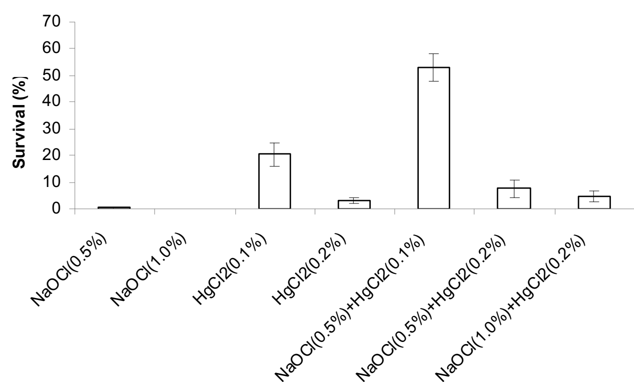

Fig. 1 Effect of two surface sterilants, their concentrations and combinations on the survival percentage of nodal explants of *Spathiphyllum floribundum* cv. 'Petite' after culturing in 'NB' (Fisse *et al.* 1987) medium for 2 weeks. Values are mean ± standard error of the mean (SE) with three comparable experiments. Data were taken after 2 weeks.

Shoot multiplication

This experiment, conducted to assess the optimal cytokinin concentration in shoot multiplication medium, revealed that by supplementing modified MS medium with 2iP (5.0-10.0 μM) or BA (10.0 μM) produced significantly more shoots than the other concentrations tested (**Table 2A**). KN in the culture medium induced comparatively fewer axillary shoots

Table 2A Effect of different concentrations of three cytokinins [N⁶-benzyladenine (BA), kinetin (KN) and N⁶-(2-isopentyl) adenosine (2iP)] on axillary shoot proliferation (number of shoots/explant) of *Spathiphyllum floribundum* cv. 'Petite' after 6 weeks of culture.

Concentration (μM)	BA	KN	2iP
0	-	-	-
2.5	1.84 ± 0.16 g*	1.52 ± 0.25 g*	1.52 ± 0.25 g*
5.0	3.18 ± 0.30 def	2.72 ± 0.13 efg	2.72 ± 0.13 efg
7.5	3.18 ± 0.30 def	3.60 ± 0.44 cde	3.60 ± 0.44 cde
10.0	6.28 ± 0.52 ab	4.28 ± 0.65 cd	5.36 ± 0.77 ab

Data is expressed as mean ± S.E. (n = 10). 10 replications were used for each treatment and the experimental trial repeated in triplicate.
* Values followed by different letters within column are significantly different at $P < 0.05$ by Duncan's multiple range test.

Table 2B Effect of different concentrations of three cytokinins [N⁶-benzyladenine (BA), kinetin (KN) and N⁶-(2-isopentyl) adenosine (2iP)] on average shoot length (cm) of *Spathiphyllum floribundum* cv. 'Petite' after 6 weeks of culture.

Concentration (μM)	BA	KN	2iP
0	-	-	-
2.5	2.73 ± 0.21 de*	3.53 ± 0.20 bc*	2.70 ± 0.23 de*
5.0	2.43 ± 0.21 e	3.40 ± 0.32 bcd	2.66 ± 0.33 de
7.5	2.66 ± 0.26 de	3.80 ± 0.11 ab	2.93 ± 0.20 cde
10.0	2.73 ± 0.06 de	4.33 ± 0.24 a	3.06 ± 0.18 cde

Data is expressed as mean ± S.E. (n = 10). 10 replications were used for each treatment and the experimental trial repeated in triplicate.
* Values followed by different letters within column are significantly different at $P < 0.05$ by Duncan's multiple range test.

Table 1 Effect of different basal media supplemented with N⁶-benzyladenine (BA) and 1-naphthalene acetic acid (NAA) on the culture establishment percentage of nodal explants based on shoot formation of *Spathiphyllum floribundum* cv. 'Petite'.

Medium	BA (μM)	NAA (μM)		
White		**0.0**	**0.5**	**2.5**
	0	1.25 ± 1.25 k*	1.25 ± 1.25 k*	13.75 ± 2.39 ghijk*
	2.5	7.50 ± 1.44 ijk	17.50 ± 3.23 fghij	21.25 ± 4.37 fghi
	5.0	10.00 ± 2.04 hijk	18.75 ± 4.27 fghij	12.50 ± 3.23 ghijk
MS	0	25.00 ± 2.04 fg	5.00 ± 2.04 jk	8.75 ± 3.15 ijk
	2.5	31.25 ± 4.27 ef	50.00 ± 4.08 cd	38.75± 5.15 de
	5.0	23.75 ± 2.39 fgh	48.75 ± 4.27 cd	55.00 ± 6.45 bc
Modified MS	0	7.50 ± 1.44 ijk	11.25 ± 3.15 ghijk	6.25 ± 2.39 jk
	2.5	55.00 ± 6.45 bc	72.50 ± 4.79 a	62.50 ± 8.54 abc
	5.0	57.50 ± 4.75 bc	65.00 ±6.45 ab	60.00 ± 9.13 abc

Data were taken after 6 weeks. Data is expressed as mean ± S.E. (*n* = 10). 10 replications were used for each treatment and the experimental trial repeated in triplicate.
* Values followed by different letters within column are significantly different at $P < 0.05$ by Duncan's multiple range test.

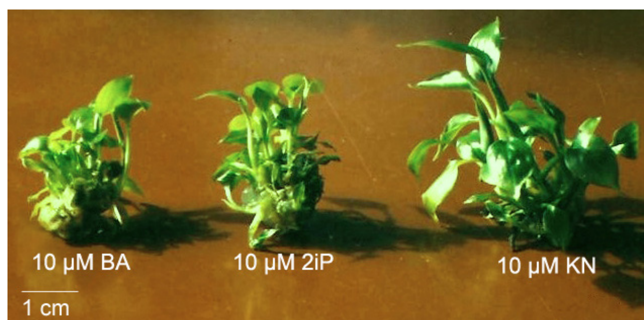

Fig. 2 Comparison on shoot multiplication and shoot length of *Spathiphyllum floribundum* cv. 'Petite' after 6-weeks of culture on three different media consisting of modified MS constituents with 10 µM BA, 2iP and KN, respectively.

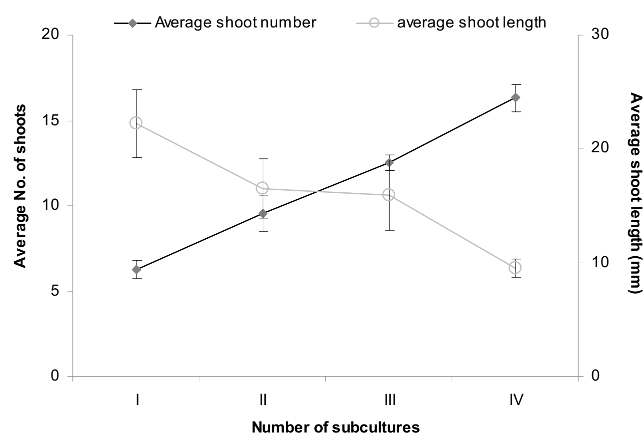

Fig. 3 Relationship between average shoot number and shoot length in four consecutive subcultures of *Spathiphyllum floribundum* cv. 'Petite' in modified MS medium supplemented with 10 µM BA. Values are mean ± standard error of the mean (SE) with three comparable experiments. Data were taken after 6 weeks.

Fig. 4 Abnormal shoot clusters of *Spathiphyllum floribundum* cv. 'Petite' after three repeated sub-culturing of 6 weeks each in the modified MS medium containing 10 µM BA.

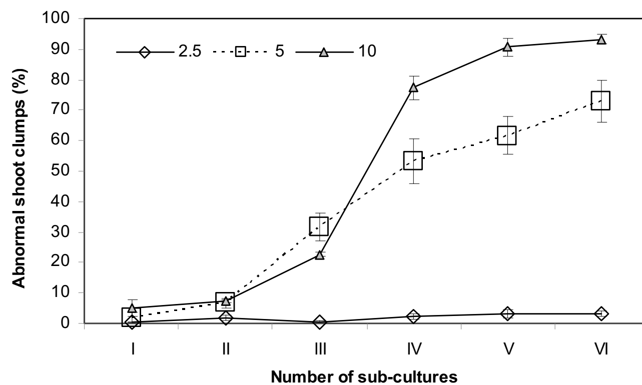

Fig. 5 The co-relationships among the formation of abnormal shoot clusters, number of sub-cultures and concentrations of BA (2.5, 5 and 10 µM) in modified MS medium. Values are mean ± standard error of the mean (SE) with three comparable experiments. Data were taken after 6 weeks.

Fig. 6 *Spathiphyllum floribundum* cv. 'Petite' micropropagation and acclimatization. (**A**) The consecutive application of 10 and 2.5 µM BA in modified MS media up to the 6th round of multiplication of 6-week each produced normal shoot clusters, elongated shoots and (**B**) rooted plants from the same culture bottle. (**C**) The small shoot clusters elongated and rooted after 6-weeks of culture in modified MS medium supplementation with BA (0.5 µM) and NAA (1.0 µM) developed rooted plants from the shoot cluster. (**D**) 60-days-old primary hardened normal plants in perforated plastic cup with coco peat: perlite (1:1). (**E**) 120-days-old well developed plants in earthen pot containing a mixture of soil, coarse sand and cattle manure (1: 1: 1).

than all other cytokinins tested. Medium supplemented with BA and 2iP at 10.0 µM produced 6.28 ± 0.52 and 5.36 ± 0.77 shoots/explant, respectively after 6 weeks in culture although these values were statistically insignificantly (**Table 2A**). However, the average shoot length in KN-containing medium was significantly higher than medium containing 2iP or BA (**Fig. 2; Table 2B**). Therefore, for further studies on the consequence of subsequent cultures in shoot multiplication medium, modified MS medium supplemented with 10 µM BA was more appropriate than 2iP because 2iP is costlier than BA (cost of 2iP = 110.60 US$/g and of BA = 25.20 US$/g; Sigma 2011 price list).

Comparative data for four subsequent cultures in shoot multiplication medium showed that the shoot multiplication ratio (i.e., number of axillary shoots/explant) was directly proportional to the number of subcultures while shoot length was inversely proportional to it (**Fig. 3**). An "accep-

Table 3 Consequences of the use of two dissimilar concentrations (10.0 and 2.5 µM) of N^6-benzyladenine (BA) on shoot proliferation and shoot elongation of *Spathiphyllum floribundum* cv. 'Petite' for successive six subcultures of 6 weeks each.

No. of subcultures	BA concentration (µM)	Mean No. of shoots	Mean shoot length (mm)
1	10.0	6.28 ± 0.52 a*	22.20 ± 2.95 c*
2	2.5	5.40 ± 0.70 a	33.20 ± 2.04 ab
3	10.0	7.40 ± 0.74 a	30.70 ± 2.38 b
4	2.5	6.18 ± 0.78 a	33.30 ± 3.12 ab
5	10.0	7.60 ± 0.91 a	36.70 ± 2.58 ab
6	2.5	6.30 ± 0.89 a	39.40 ± 2.07a

Data is expressed as mean ± S.E. (n = 10). 10 replications were used for each treatment and the experimental trial repeated in triplicate.
*Values followed by different letters within column are significantly different at $P < 0.05$ by Duncan's multiple range test. Data were taken after 6 weeks at each subculture.

Table 4 *In vitro* shoot elongation and rooting of *Spathiphyllum floribundum* cv. 'Petite' shoot clumps (5-10 mm) as influenced by modified MS medium supplemented with various concentration and combinations of N^6-benzyladenine (BA) and 1-naphthalene acetic acid (NAA).

BA (µM)	NAA (µM)	Mean shoot length (mm)	Mean number of roots/plant
0	0	19.10 ± 1.00 d*	1.70 ± 0.30 b*
0.5	0	26.20 ± 1.54 c	1.75 ± 0.36 b
1.0	0	20.40 ± 1.37 d	1.45 ± 0.26 b
2.5	0	21.22 ± 1.52 d	1.55 ± 0.30 b
0.5	1.0	48.20 ± 1.99 a	2.90 ± 0.23 a
1.0	1.0	40.00 ± 1.50 b	2.02 ± 0.21 b
2.5	1.0	21.30 ± 1.35 d	1.45 ± 0.26 b

Data recorded after 6 weeks of culture. Data is expressed as mean ± S.E. (n = 10). 10 replications were used for each treatment and the experimental trial repeated in triplicate.
*Values followed by different letters within column are significantly different at $P < 0.05$ by Duncan's multiple range test.

table" number of shoots (12.52 ± 0.47) with an average shoot length of 15.9 ± 3.08 mm could be obtained by the third subculture. However, repeated subculture in multiplication medium containing 10 µM BA generated abnormal shoot clusters (**Fig. 4**). This abnormality was characterized by longer petioles than normal shoots and, as a result, the leaf became twisted by 180°. Naturally, these plants are not suitable for further multiplication since they are not acceptable to customers. Efforts were made to minimize morphological abnormalities; normal *in vitro* grown shoots were studied for 6 successive cultures in modified MS medium supplemented with 2.5, 5.0 and 10.0 µM BA. Comparative studies revealed that shoot abnormalities were not observed up to the second subculture for all media tested and medium containing 2.5 µM BA induced a negligible amount of morphological abnormalities up to the 6th subculture (**Fig. 5**). However, the application of 2.5 µM BA in multiplication medium at every subculture only produced an average of 2-3 shoots/explant (data not shown), which is insufficient for large-scale commercial production. Therefore, initiatives were taken to produce healthy, morphologically normal as well as many shoots/explant. Among the several preliminary trials (data not shown), alternate applications of 10 µM and 2.5 µM BA successively in shoot multiplication medium did not produce morphological abnormalities up to the 6th subculture. In addition, the concentration of BA in culture medium and the number of subcultures also influenced shoot length. The application of 2.5 µM BA in alternate multiplication media did not hamper the shoot proliferation ratio as well as the growth of shoot clusters (**Fig. 6A; Table 3**). No significant difference in the average number of shoots/explant was observed up to the 6th subculture. The average shoot length was significantly higher by the 2nd subculture relative to the 1st one but no significant changes were observed among the subcultures from the 2nd to the 6th subculture. The alternate use of two concentrations of BA (2.5 and 10.0 µM) in multiplication medium yielded 5 to 7 shoots/explant with a shoot length of 30-40 mm. Shoot

Table 5 Effect of different potting mixtures on primary hardening of *in vitro* generated plants of *Spathiphyllum floribundum* cv. 'Petite' in a greenhouse.

Potting mixture	Survival (%)	
	after 30 days	after 60 days
Coco peat : perlite (1:1)	84.67 ± 2.90 a*	80.33 ± 4.16 a*
Coco peat : perlite (2:1)	87.33 ± 2.90 a	80.67 ± 1.76 a
Peat moss : perlite (1:1)	87.33 ± 2.90 a	83.33 ± 1.76 a
Peat moss : perlite (2:1)	90.00 ± 5.03 a	84.66 ± 4.37 a

Each experiment (different potting mixtures) repeated in triplicate with 50 replicates each (n = 150 total).
* Values followed by different letters within column are significantly different at $P < 0.05$ by Duncan's multiple range test.

Table 6 Establishment of primary hardened plants (i.e., transferred 60 days after primary hardening) in earthen pot containing a mixture of soil, coarse sand and cattle manure (1:1:1).

Survival (%)	
after 60 days	after 120 days
95.83 ± 1.44 a*	92.50 ± 1.44 a*
91.66 ± 1.44 ab	86.66 ± 2.20 ab
83.33 ± 3.81 b	81.66 ± 2.20 b
95.00 ± 2.50 a	89.16 ± 2.20 a

Each field trial repeated in triplicate having 40 replicates each (n = 120 total). The samples were maintained under natural field conditions under 50% agro shade net.
* Values followed by different letters within column are significantly different at $P < 0.05$ by Duncan's multiple range test.

clusters cultured in medium containing 2.5 µM BA induced 2-3 strong roots at the base of 80% of plants (**Fig. 6B**). However, it was noticed that roughly 10-15 plants/culture bottle could be directly harvested from the multiplication medium from the 3rd subculture and transferred directly to the greenhouse for hardening. The clusters of small shoots 8-10 mm long were directly cultured in PGR-free modified MS medium or medium supplemented with different concentrations of BA and NAA. Supplementation of medium with a lower concentration of BA (0.5 µM) enhanced shoot growth but higher concentrations (1.0 and 2.5 µM) influenced axillary shoot proliferation (**Table 4**). The combination of BA (0.5 µM) and NAA (1.0 µM) yielded an average of 48.20 ± 1.99 mm long shoots with an average of 2.90 ± 0.23 roots/plant, proving to be the best treatment among all the conducted trials (**Fig. 6C**).

Hardening

Plantlets with shoots 30-50 mm in length with 2-4 strong roots were subjected to primary hardening in four different types of sterilized potting mixture containing coco peat: perlite (1: 1), coco peat: perlite (2: 1), peat moss: perlite (1: 1) and peat moss: perlite (2: 1). No significant differences were identified among the four potting mixtures and about 85% of plants survived after 60 days of primary hardening (**Table 5**). However, primary hardened plants (**Fig. 6D**) from the respective potting mixtures, after transfer to earthen pots containing a mixture of soil, coarse sand and cattle manure (1: 1: 1), showed significant differences in survival percentage after 60 and 120 days (**Table 5**). The highest number of plants that survived was in coco peat: perlite (1: 1) and peat moss: perlite (2: 1) during primary hardening and subsequent transfer after acclimatization was 92.50 ± 1.44 and 89.16 ± 2.20%, respectively, in which plants grew vigorously (**Table 6; Fig. 6E**).

DISCUSSION

Simple surface sterilization procedures carried out (Kunisaki 1980; Zimmerman and Read 1986) for decontamination of ornamental plants was not effective for *Spathiphyllum* axillary bud culture. Parkinson *et al.* (1996) recommended the use of sodium dichloroisocyanurate (500 mg/l) for the elimination of some bacteria from the explants of *Spathiphyllum* sp. Rhizome segments of *S. wallisi* were dis-

infected using calcium hypochlorite and sodium hypochlorite at 2% with or without immersion in 70% alcohol (Diniz *et al*. 2008). In our study, 70% ethanol (1 min), 0.5% NaOCl (5 min), 0.1% $HgCl_2$ + 3 drops of Tween-20/100 ml (5 min) with repeated washes in sterile DW was the most suitable protocol for sterilization. The surface-sterilized buds were initially cultured in NB medium to ascertain the presence of bacterial and fungal contamination. Fisse *et al*. (1987) used the same medium to screen bacterial contamination of *Syngonium*, *Philodendron* and *Ficus elastica* shoot tips. The constituents of NB medium are mainly yeast extract and glucose, which are best for indicating the presence of hidden contaminants of potato (Long *et al*. 1988).

Medium formulation displayed a strong effect on the establishment of cultures (**Table 1**). One possible explanation for the result are the differences in nitrogen and potassium content between MS, modified MS and White's media. In White's medium, nitrogen was solely supplied in the form of nitrate and the total amount of N (3.3 mM) was much less than MS and modified MS media. Morphogenesis is influenced by the total amount of N (60.01 mM) provided in the medium and for most purposes, a supply of both a reduced form of N and nitrates seems to be necessary (Qaoud *et al*. 1991). Teixeira da Silva *et al*. (2006), Malagon *et al*. (2001) and Dewir *et al*. (2006) recommended the use of MS basal medium for the micropropagation of *Spathiphyllum*. However, in this experiment, the best response was observed in modified MS basal medium in which the concentration of total N was decreased (30 mM) but the nitrate/ammonium ion balance remained the same. Watanabe *et al*. (1990) established the effect of the ratio of ammonium to nitrate in the culture medium on the growth of *Spathiphyllum*, but in our experiment, a reduced amount of total N improved the sprouting of shoot buds, shoot multiplication and simultaneous rooting from the base of shoots. Improved shoot growth or elongation by reducing the N and potassium concentration in the culture medium was also observed in *Citrus sinensis* (Bespalhok *et al*. 1992; Kobayashi *et al*. 2003).

Shoots did not proliferate in the absence of cytokinin. The exogenous requirement of PGRs depends on the endogenous levels of phytohormones in plant tissues. Despite the occurrence of endogenous cytokinins in whole plants, many tissues and small organs isolated *in vitro* are unable to synthesize these substances to sustain growth. Malagon *et al*. (2001) reported that the incorporation of IAA, NAA and 2,4-D along with BA (1-5 mg/l) formed most shoots of *Spathiphyllum floribundum* in *in vitro* culture. However, medium supplemented with NAA + BA or 2iP induced the proliferation of 10-15 axillary shoots with considerable callus at the base. Shoots regenerated from callus may be abnormal due to somaclonal variation.

In general, cytokinins influence numerous developmental and physiological processes in plants. Exogenously applied cytokinins are essential for *in vitro* shoot development (Krikorian 1995). Most of the protocols for micropropagation of *Spathiphyllum* proposed a higher concentration of cytokinin (1-10 mg/l) either alone or in combination with an auxin for the shoot multiplication stage (Fonnesbech and Fonnesbech 1979; Werbrouck *et al*. 1995; Malagon *et al*. 2001; Dewir *et al*. 2006). In the present study, shoot abnormalities were prominent after the 3rd subculture when axillary shoots were placed on MS medium with higher concentrations of BA (5 and 10 μM) for shoot multiplication. Although the morphological abnormalities of *in vitro* generated shoot clusters were not mentioned by most scientists who worked on *Spathiphyllum*, Wand *et al.* (1993) reported the same type of abnormalities among *in vitro* generated plants after continuous culture in the presence of 1-2 mg/L of BA.

Cultures maintained at high concentrations and over long incubation periods with cytokinin can result in damage to shoot growth and form abnormal leaves, swollen bases, frequent callus formation and induction of buds that do not elongate into shoots, as observed for *Aloe polyphylla* (Ivanova and van Staden 2008), and can cause a higher percentage of hyperhydric shoots (Ziv 1991). In the present study, the alternate use of 10 μM and 2.5 μM BA in multiplication media during each subculturing not only induced a good number of normal shoot clusters but also stimulated shoot elongation and rooting of some plants. The small shoot clusters derived from multiplication media could be elongated and simultaneously rooted in modified MS medium augmented with BA (0.5 μM) and NAA (1.0 μM) within 6-weeks of culture. A low concentration of BA in the presence of IBA during shoot elongation and root formation is also necessary for *Limonium sinensis* (Dam *et al*. 2010).

The rooted *Spathiphyllum* plants were easily transferred to earthen pots through primary and secondary hardening in a greenhouse with a 90% success rate and the plants were morphologically normal, uniform and with a bushy *habitus*. Van Huylenbroek and De Riek (1995) studied how sugar reserves of *Spathiphyllum* 'Petite' accumulated *in vitro* could allow the plantlets to survive the acclimatization process.

CONCLUSION

This study is one of the few in which the stage of shoot multiplication has been carefully manipulated to prevent morphological abnormalities. Optimizing this stage in *Spathiphyllum floribundum* has improved the protocols previously described for this species (Werbrouck *et al*. 1995; Malagon *et al*. 2001). The controlled use of cytokinin concentration in the multiplication stage ensured the long-term culture of this species in which the establishment of a contamination-free culture is difficult. The improvement reported here has the potential to lead to the formation of one million plantlets from decontaminated cultures established from 10 explants within one year, thereby increasing the possibility of the method being used for *in vitro* mass clonal propagation of this popular ornamental, commonly termed the peace lily.

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