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Full Length Research Paper

Optimization of growth regulators in organogenesis of *Bletia purpurea* (Lam.) using response surface design and genetic evaluation

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This paper reports the optimal concentrations of indol acetic acid (IAA) and benzylaminopurine to stimulate morphogenetic induction *Bletia purpurea* (Lam.) and the application of random amplification of polymorphic deoxyribonucleic acid (DNA) (RAPD) for genetic evaluation of micropropagated plantlets. Organogenesis was induced from *B. purpurea* (Lam.) explant pseudobulbs in Murashige and Skoog (MS) medium at full ionic strength and supplemented with 20 g/l sucrose, 2 g/l activated carbon and 2.2 g/l Gel Rite. Optimum growth regulator concentrations were determined by response surface design, according to Box-Benhken. At 15 days after morphogenic induction was observed, direct shoots formation on the explant was attained, and optimum concentrations for the greatest number of shoots was 1.8 mg/l Indol acetic acid and no benzylaminopurine. Comparison of RAPD genetic profiles for mother plantlets and regenerated daughter plantlets showed no changes in the genome based on the oligonucleotides used. The conditions applied in this protocol produces plantlets which maintain the genetic traits of the first generation.

Key words: Genetic evaluation, polymerase chain reaction (PCR), random amplification of polymorphic DNA (RAPD) analysis, *Bletia purpurea*, pseudobulbs, direct organogenesis, response surface design, *in vitro*.

INTRODUCTION

Urban growth and habitat alteration threaten the wild orchid *Bletia purpurea* (Lam.), making development of alternative propagation techniques vital to reversing decreases in wild populations (Dutra et al., 2008). In a natural setting, *B. purpurea* propagates sexually and asexually, but at rates far too slow to overcome the threat of extinction. Plant tissue culture is an effective tool for large-scale propagation since it produces a complete plant from a cell or group of cells. However, successful propagation requires that optimum conditions be identified for nutrients and plant growth regulators such as auxins and/or cytokinins.

Response surface methodology is a collection of statistical techniques for designing experiments, developing and adjusting mathematical models, evaluating the effect of factors and identifying optimum factor condition for the desired response (Montgomery, 2007). It has been applied in biotechnology areas such as optimization of biosurfactant production by probiotic bacteria (Rodríguez et al., 2006); kinetic analysis for rehydration of lyophilized carrots (*Daucus carota*) (Zambrano et al.,

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Abbreviations: PGR, Plant growth regulators; **PCR,** polymerase chain reaction; **RAPD,** random amplification of polymorphic DNA; **RSM,** response surface methodology; **$AgNO_3$,** Silver nitrate; **IAA,** Indol-3-acetic acid; **BAP,** 6-Benzylaminopurine; **EDTA,** Ethylenediaminetetra-acetic acid; **TRIS,** Hydroxymethylaminomethane (2-Amino-2-hydroxymethyl-propane-1,3-diol); **NaCl,** Sodium chloride; **β-ME,** Mercaptoethanol; **rpm,** revolutions per minute; **NaOAc,** Sodium acetate; **TDZ,** Thidiazuron; **NAA,** Naphthalenacetic acid.

Table 1. Morphogenic induction optimization was done using ten treatments with different quantities of IAA and BAP.

Design	Treatment	Coded Variable		Real Variable	
		X_1	X_2	IAA	BAP
First design	1	-	-	0	0
	2	+	-	2	0
	3	-	+	0	2
	4	+	+	2	2
	5	0	0	1	1
	6	0	0	1	1
	7	0	0	1	1
Second design	8	+ √2	0	2.41	1
	9	- √2	0	0	1
	10	0	+ √2	1	2.41
	11	0	- √2	1	0

2007); and optimization of plant growth hormones (PGR) and silver nitrate ($AgNO_3$) in micropropagation of *Dianthus caryophyllus* L. (Gutiérrez et al., 2010).

Conditions in plant tissue culture can be stressful to cells, leading to possible genetic variation in the regenerated plants. Somaclonal variation is genetic or epigenetic variation generated during *in vitro* plant culture from somatic cells (Sánchez-Teyer et al., 2003; Noro et al., 2007). When the objective is to maintain plant uniformity and traits, somaclonal variation is undesirable (Ahloowalia, 1998; Brar and Jain, 1998; Sahijram et al., 2003). Variations can be detected by molecular markers, which identify genetic differences between individuals and produce a molecular profile or genetic fingerprint for each variety independent of plant growth conditions (Narváez et al., 2000). The many polymerase chain reaction (PCR) based methods identify the unique profiles of deoxyribonucleic acid (DNA) fragments by varying the nature of the oligonucleotides used and the alignment conditions.

A common method is random amplification of polymorphic DNA (RAPD), which has been used to evaluate genetic variability in important micropropagated crop plants such as *Prunus dulcis* (Martins, 2004); to study genetic diversity in grape and orchid cultivars (Narváez et al., 2000; Taniguchi et al., 2008); to determine genetic stability in micropropagated plantlets such as *Zingiber oficinales* Rose (ginger) (Rout et al. 1998), and *Dendrobium* Hybrid (*Orchidaceae*) (De Melo et al., 2006); and to analyze possible genetic variations in somaclons of *Phalaenopsis* True Lady "B79-19" (Chen et al., 1998). The present study objective was to optimize indol-3-acetic acid (IAA) and 6-benzylaminopurine (BAP) concentrations by applying the response surface methodology (RSM), and use RAPD to generate genetic profiles of the mother plantlets and the structures produced with the validated optimum treatment for morphological induction of *B. purpurea*.

MATERIALS AND METHODS

Explant pseudobulbs were taken from seven-month old, *in vitro* micropropagated *B. purpurea* plants of uniform size.

Culture media and conditions

In vitro morphogenic induction of *B. purpurea* was done in MS base medium (Murashige and Skoog, 1962), supplemented with 20 g/l sucrose, 2 g/l activated carbon and 2.2 g/l Gel-Rite. Morphogenic induction optimization was done using ten treatments with different quantities of IAA and BAP (Table 1). Adjustment of pH to 5.7 was done by adding 1 N NaOH or HCl. Culture medium (20 ml) was placed in 25 x 150 mm PYREX® test tubes and sterilized by heating to 121°C at 1.5 atmospheres for 15 min. For each treatment, the tubes with explants were cultured at 23 ± 2°C with a 16/8 (light:dark) photoperiod produced by a 40 W white fluorescent lamp. Response variables were number of shoots, leaves and roots, with evaluations done at 15, 30 and 45 days of culture.

Statistical analysis

A Box-Behnken response surface design (2^2 compound factor designs) was used with two factors (IAA and BAP), three central points and two designs. Factor levels were 0 and 2 mg/l, four replicates were done for all treatments, experimental unit was one explant per tube and sampling was random. Evaluated response variables were number of shoots, number of leaves and number of roots on the main shoots. Linear and quadratic values for all factors and their interactions were tested using a 95% confidence level. All statistical analyses were run with the Stat Graphics Centurion XV.1 package. The optimum treatment was used to validate the experimental design and the resulting plantlets were genetically evaluated.

Genetic analysis with RAPD

DNA extraction

Extraction of DNA was done following Allen et al. (2006), which is based on the hexadecyltrimethylammonium bromide (CTAB)

method. The DNA was extracted from 200 to 400 mg tissue with extraction buffer (2% CTAB; 1 M Tris base, pH 8.0; 0.5 M EDTA; 5 M NaCl; and 1% β-ME). Samples were macerated with the buffer, placed in 1.5 ml Eppendorf tubes, and incubated at 65°C for 30 min, mixing every 10 min. The tubes were centrifuged at 13,500 rpm for 10 min, one volume of phenol/chloroform/isoamylic alcohol (25:24:1) added, and then mixed by inversion before being left to stand for 20 min at room temperature. They were centrifuged as aforementioned, the aqueous phase recovered, the tubes washed and the aqueous phase recovered again.One volume of cold isopropanol was added to each tube, which was then mixed for 10 min at room temperature and centrifuged.

The pellet was recovered, 250 µl TE (2 M Tris and 0.5 M EDTA) and 2 µL RNAse added, and the tube incubated at 37°C for 30 min. An additional 25 µl 3 M NaOAc and cold 100% EtOH were added and the mixture incubated at -20°C for 20 min to precipitate the DNA. Two washes with 70% EtOH were done; the DNA was resuspended in sterile distilled water and stored at -20°C until use. Genomic DNA concentration was estimated using spectrophotometry (Eppendorf Biophotometer) at 260 and 280 nm. Agarose (1%) gels stained with 0.5 µg m/l ethidium bromide were produced and images taken with a gel photodocumenting system (UVP DIGIDOC-IT).

Oligonucleotide selection

The 20 oligonucleotides included in the OPJ kit (Operon Technologies, Inc.) were analyzed following Williams et al. (1990) with modifications proposed by Scovel et al. (1998). Reproducible bands were selected from the amplified oligonucleotides by determining fragment size in base pairs. Six oligonucleotides were selected: **OPJ-4** (5′ CCG AAC ACG G 3′); **OPJ-7** (5′ CCT CTC GAC A 3′); **OPJ-13** (5′ CCA CAC TAC C 3′); **OPJ-15** (5′ TGT AGC AGG G 3′); **OPJ-16** (5′ CTG CTT AGG G 3′); and **OPJ 18** (5′ TGG TCG CAG A 3′).

Genomic DNA amplification conditions

The reaction mixture (25 µl final volume) included the following concentrations: 0.625 U Taq Polimerase (BioLabs, Inc., New England); 10 mM dNTPs; 10X buffer (20 mM Tris-HCl, 10 mM $[NH_4]_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100; pH 8.8 at 25 ºC); 100 ng oligonucleotides; and 100 ng plant DNA. Reproducibility was confirmed by running each amplification two times. Amplification was done with a PXE 0.2 Thermal Cycler (ThermoElectron Corporation, USA) under the following conditions: initial denaturation at 94 ºC/5 min.; 40 cycles (94°C/1 min, 40°C/1 min, 72°C/2 min); final extension at 72°C/5 min. The PCR products were separated by electrophoresis on 2% agarose gels in 1X TAE buffer at 80 V for 120 min. A 100 bp molecular weight marker (Invitrogen, USA) was used to identify PCR product size. Finally, they were stained with 0.5 µg ml ethidium bromide and viewed in a gel photodocumentation system. Analysis was done with RAPD gel images in which each individual exhibits a specific banding pattern. Bands with the same molecular weight in different individuals (mother and daughter) can be assumed to be identical.

RESULTS AND DISCUSSION

At day 15 of the morphogenesis trial, the pseudobulbs had formed direct shoots without callus formation (Figure 1a): direct organogenesis was attained. This coincides species such as *Dendrobium microbulbon* A. Rich (Sharma et al*.,* 2007), *Dendrobium transparens* (Sunitibala and Kishor 2009) and *Lycaste aromatic* (Mata et al*.,* 2010). At 30 days, plantlet development was clearly visible (Figure 1b), and by 60 days a complete plantlet with leaves and roots could be separated from the explant (Figure 1c). At 45 days, plantlets in the treatments of the first design had produced an average of 1 to 3 shoots per plant, 3 to 4 leaves and 1 to 2 roots per shoot, with a shoot height of 50.32 to 69.84 mm. In the treatments of the second design, the plantlets produced an average of 1-2 shoots, measuring 27.94 to 37.91 mm, with 2 to 3 leaves and 1 root per shoot (Table 2). These are comparatively lower numbers than reported in morphogenesis trials with other species. Sharma et al. (2007) reported 8 to 9 shoots per explant and a 0.5 cm shoot length in *Dendrobium pseudobulbs* when using 7.5 mg/l IAA and 20 mg/l BAP. Sunitibala and Kishor (2009) observed 7 shoots per explant in *D. transparens* L. pseudobulbs with a combination of 2 mg/l BAP and 1 mg/l naphthalenacetic acid (NAA), while Mata et al. (2010) reported 9.9 shoots per explant with 4.4 µM thidiazuron (TDZ), 8.6 shoots with 8.87 µM and 7.3 spouts with 2.2 µM.

The regression analysis and ANOVA run with data from the first design showed the evaluated variables (that is, number of shoots, and number of leaves and roots on the main shoot) to have p values greater than 0.05, meaning the hypothesis of no fit to the linear model was accepted, indicating it to be near the optimum zone. The results were then fit to the quadratic model by adding the data from the second design. In the quadratic equations for each variable (Table 3), the p value exhibited a fit to a second-order model. These models aid in predicting how response will vary as a function of the values assigned each variable (X_1, X_2) corresponding to growth regulator concentration.

Shoot number was positively affected by IAA concentration (Figure 2a). Surface shape was that of a minimax or “saddle” point (Figure 2b). The estimated optimum zone for IAA was 1.8 mg/l and that for BAP was 8.1×10^{-10} mg/l; indeed, the model showed BAP to have nearly no effect because its level was almost zero (Figure 2c). A movement from the right or left towards the center of the array decreased response, while moving from the center upwards or downwards increased response. Surface shape for the number of leaves and number of roots variables was a minimum or plateau point (results not shown). The optimum zone for number of leaves was estimated as 2.4 mg/l IAA and 2.5×10^{-8} mg/l BAP; again, the model showed BAP to have no effect because it was near zero. If BAP concentration was raised, number of leaves decreased. For number of roots, the optimum zone was estimated at 2.4 mg/l IAA and 2.4 mg/l BAP. Movement towards the center from the edge decreased response, although if concentrations were raised in turn the response increased. This would not be viable in practice, however, given that it would increase

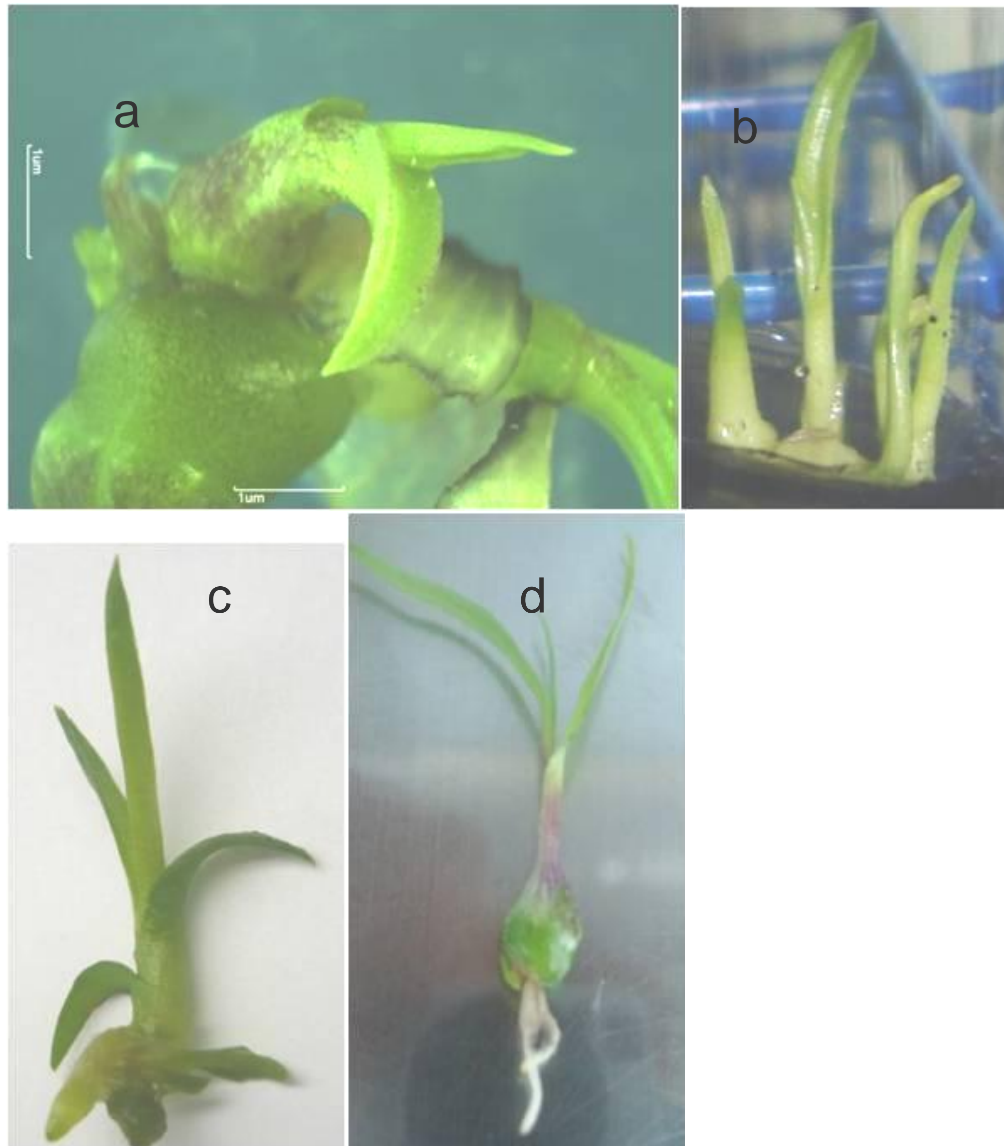

Figure 1. Organogenesis in *Bletia purpurea* (Lam.). a) at 15 days from morphogenic induction, direct formation of shoots on pseudobulb explants (stereoscope image); b) regenerated plantlets at 30 days; c) regenerated plantlet at 45 days d) regenerated plantlet at 60 days, with well-developed root and leaves.

costs due to the amount of plant growth regulators (PGR) required.

The optimum canonical analysis value for each response variable was tested experimentally (Figure 2c). For number of shoots the error was 0%, indicating that the optimum model for organogenic induction in *B. purpurea* was the treatment with 1.8 mg/l IAA and no BAP. In other words, the tested model fit completely to the experimental results. In contrast, the predicted optimum models for number of leaves and number of roots had a 50% error, making them untrustworthy. Statistical analysis showed that the amount of IAA had a significant effect on number of shoots and number of leaves, whereas number of roots was significantly affected by a combination of IAA and BAP. Growth regulators were clearly important in shoots and root formation as shown by the increased number of shootsat high IAA concentrations observed here. This is similar to results reported in a study of *Dianthus caryophyllus* (Gutiérrez et al., 2010).

Somaclonal variation

The DNA isolated from the mother and daughter *B. purpurea* plantlets was good quality, with a band larger

Table 2. Number of shoots, leaves and roots formed in *Bletia purpurea* pseudobulbs after 45 days organogenic induction.

Design	Treatment	Response variables 45 days culture		
		Number		
		Shoots	Leaves	Roots
First design	1	2 ± 0.58	4 ± 0.58	2 ± 0.82
	2	3 ± 1.00	4 ± 0.57	2 ± 0.57
	3	2 ± 1.26	3 ± 0.00	2 ± 0.96
	4	3 ± 0.95	3 ±0.50	2 ± 0.96
	5	2 ± 2.21	3 ± 0.00	2 ± 1.08
	6	3 ± 2.38	3 ± 1.00	2 ± 2.00
	7	2 ± 0.96	4 ± 0.57	1 ± 0.50
Second design	8	2 ± 0.50	3 ± 0.00	1 ± 0.58
	9	1 ± 0.50	3 ± 1.00	0 ± 0.50
	10	2 ± 0.58	3 ± 0.50	1 ± 0.50

Table 3. Quadratic equations for each response variable based on regression analysis of all treatments in morphogenic induction of *Bletia purpurea* (Lam.).

Variable (Y)	Quadratic equation
Number of shoots	$Y = 2.09 + 0.75X_1 - 0.46X_2 - 0.20X_1^2 + 0.15X_2^2 + 1.54E\text{-}16X_1X_2$
Number of leaves	$Y = 3.45 - 0.39X_1 - 0.16X_2 + 0.17 X_1^2 + 0.01 X_2^2 + 3.99E\text{-}16 X_1X_2$
Number of roots	$Y = 1.19 - 0.046X_1 - 0.05X_2 + 0.08 X_1^2 + 0.08 X_2^2 + 4.59E\text{-}17 X_1X_2$

X_1: Indol acetic acid (IAA); X_2: 6-benzylaminopurine (BAP); X_1X_2: product of IAA and BAP concentration;X_1^2: IAA squared; and X_2^2: BAP concentration squared, pair of concentrations to be tested to estimate response.

than 12,000 bp isolated for all extracts (Figure 3), and concentrations of 5 to 10 µg. The A_{260}/A_{280} ratio ranged from1.85 to 1.90 and that for the A_{230}/A_{260} ranged from 1.81 to 1.89. These ranges suggest that the isolated DNA was free of proteins, polyphenolic and polysaccharide compounds, similar to results reported elsewhere (Li et al., 2007; Zambrano et al., 2002). Plant propagation via tissue culture has been reported to result in genetic instability due to the number of subcultures or the PGR concentrations used (Marulanda and Márquez, 2002). To determine whether this occurred in the present study, the plantlets from the optimum treatment in the experimental design validation were genetically evaluated by RAPD.

Each of the twenty oligonucleotides was used in PCR reactions and eighteen of these were amplified, resulting in 1 to 5 bands per oligonucleotide (51 total bands). Amplified fragment size ranged from 100-1500 bp, which coincides with previous reports (< 2000 bp) of analogous studies using this technique with other plant species. For example, in a study verifying DNA extract quality, Zambrano et al. (2002) amplified DNA extracts from *Saccharum* spp., *Musa* sp. and *Minihot esculenta*, with product size ranging from 200 to 1750 bp. Based on these results, the authors chose six oligonucleotides (OPJ-4, OPJ-7, OPJ-13, OPJ-15, OPJ-16 and OPJ-18) because they provided data on the number of bands and exhibited greater reproducibility. Molecular analysis of the six RAPD oligonucleotides identified 18 bands per individual (that is, three bands per oligonucleotide), with amplified band size ranging from 200 to 1000 bp.

The RAPD technique generates results in which each band indicates a locus. A distinction between heterozygotic and homozygotic individuals cannot be made, but it does produce data on the presence or absence of genetic similarity between the tested individuals. The profiles produced in the present study demonstrated that the micropropagated plantlets were mono-merphic and analogue to their respective mother plants (Figure 4). Variability in plant DNA sequences has been monitored using RAPD, simple sequence repeats (SSR), ISSR and amplified fragment length polymorphism (AFLP) molecular markers. The lack of genetic variation observed here using RAPD has also been reported in *Curcuma longa* L. regenerated plantlets (Tyagi et al., 2007); micropropagation of two sugar cane varieties (Lal et al., 2008); micropropagation using rizomes of three *Vitis* spp. genotypes (Alizadeh and Singh, 2009); and clonal propagation of *Dendrobium second* Love from axillary shoots in the presence of thidiazuron (De Melo et al., 2006). In contrast, somaclonal variation has been

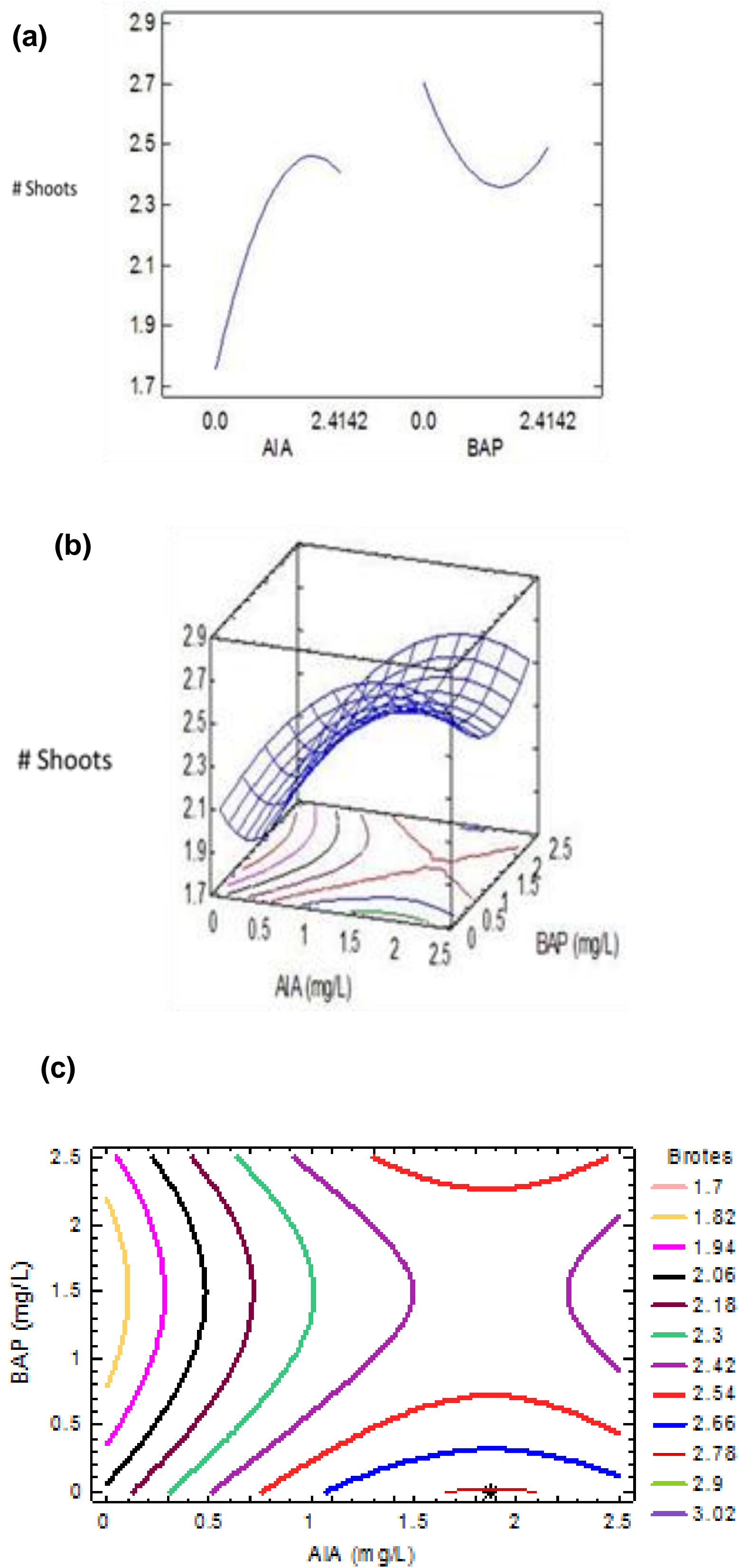

Figure 2. (a) Number of shoots as a function of IAA and BAP concentration (mg/l); b) stimated response surface; **c)** Surface edge for number of shoots response variable. * Indicates optimum values.

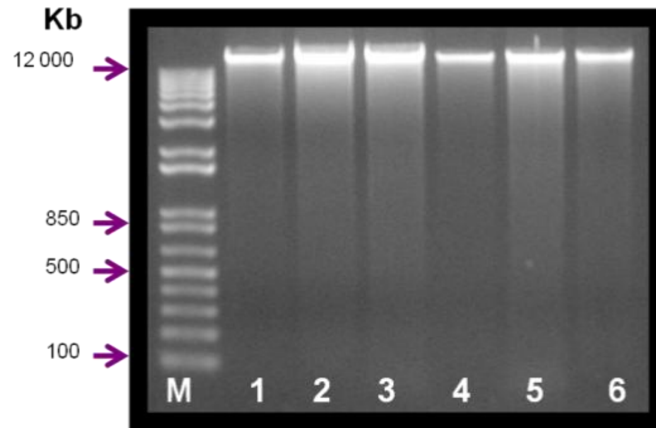

Figure 3. Agarose gel of *Bletia purpurea* (Lam.) extracts produced with the CTAB method. DNAsamples (3 µL) were fractionated by electrophoresis in 1% native agarose gel and stained with 0.5 µg ml^{-1}ethidium bromide. M: DNA ladder (1 Kb plus DNA Ladder, Invitrogen); rows 1, 3 and 5 correspond to mother plantlet extracts, rows 2, 4 and 6 correspond to daughter plantlet extracts.

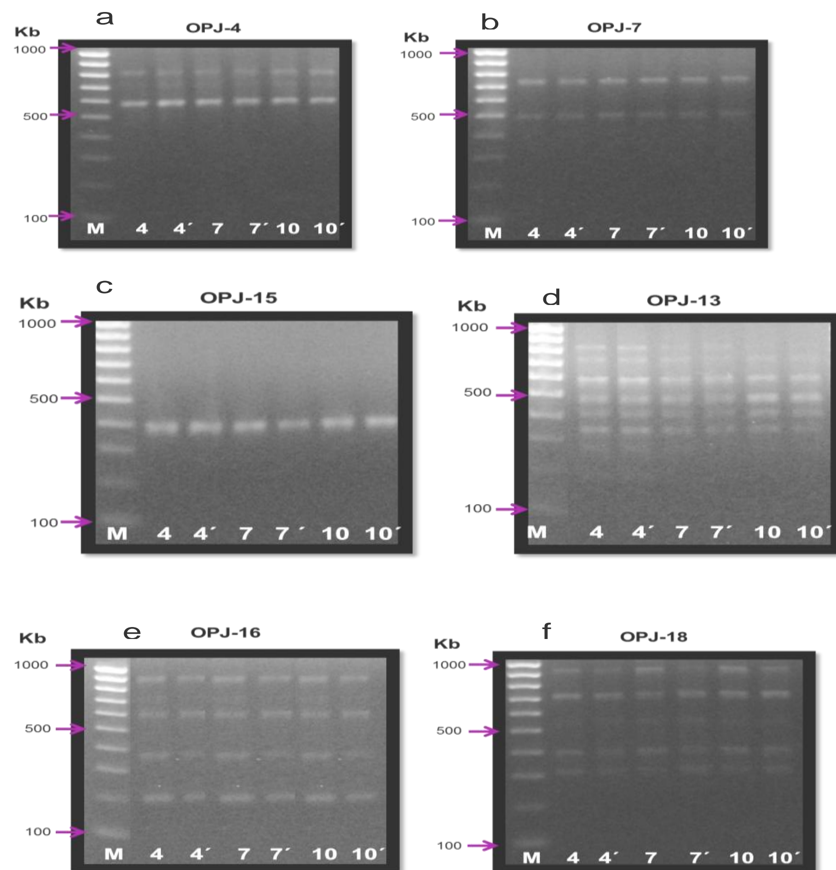

Figure 4. RAPD analysis, using oligonucleotides a) OPJ-4, b) OPJ-7, c) OPJ-13 d) OPJ-16, d) OPJ-16 and e) OPJ-18 of mother plantlets, and daughter plantlets produced by organogenic induction of *B. purpurea* pseudobulbs. Lane M = 1 kb molecular weight marker (Invitrogen). Lines with numbers 4, 7 and 10, represent the assigned code to mother plant source of explants; lines with numbers 4', 7' and 10´, represent the respective daughter plant.

documented in plantlets regenerated by *in vitro* induction of adventitious shoots from pineapple leaves (Soneji et al*.,* 2002). In another case, small variations were detected in chrysanthemum plantlets under different proliferation conditions and using regeneration mediated by callus formation (Martin et al., 2002). Finally, genetic variations were reported in somaclons from protocorm type bodies in *Phalaenopsis* True Lady (Chen et al., 1998).

Conclusion

The response surface methodology was applied to optimize IAA and BAP concentrations in direct organogenic induction of *B. purpurea* (Lam.) pseudobulb explants. Optimum concentration for shoot production was 1.8 mg/l IAA and the resulting mathematical model can predict response behavior without errors. Molecular analysis using RAPD showed no changes in band patterns in a comparison of mother and daughter plantlets. Therefore, the present propagation protocol can be applied without risk of genetic instability in regeneration of the first generation.

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