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Evaluation of genetic variability in micropropagated propagules of ornamental pineapple [*Ananas comosus* var. *bracteatus* (Lindley) Coppens and Leal] using RAPD markers

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ABSTRACT. The objective of the present study was to evaluate the genetic variability in micropropagated plantlets of ornamental pineapple, after the fourth period of subculture. The basal culture medium consisted of MS salts, vitamins, 3% sucrose, liquid formulation, supplemented with 6-benzylaminopurine (BAP) at concentrations of 0.125, 0.25, 0.5, 1.0, and 2.0 mg/L. The addition of BAP influenced the occurrence of genetic variation revealed using random amplified polymorphic DNA (RAPD) markers. Of a total of 520 primers tested, 44 were selected and amplified; 402 monomorphic bands (97.2%) and 18 polymorphic bands (2.8%) resulted among regenerated plantlets. The polymorphic fragments were produced by 12 primers (OPA-01, OPA-20, OPB-01, OPB-19, OPC-19, OPF-13, OPL-17, OPM-13, OPP-16, OPT-07, OPV-19, and OPX-03). Among the primers that identified polymorphism, OPA-01, OPA-20, OPB-19, OPC-19, OPL-17, OPP-16, and OPX-3 each showed, one polymorphic band and OPF-13 amplified a maximum of three bands. In this study, the RAPD technique was effective in showing the occurrence of

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somaclonal variations that occur during the micropropagation process of ornamental pineapple cultivation in BAP-supplemented medium, and it is possible to detect the presence of genetic variation in early stages of plant development.

Key words: *Ananas comosus* var. *bracteatus*; Somaclonal variation; Genetic variation; *In vitro* culture; Mutation

INTRODUCTION

The ornamental pineapple (*Ananas comosus* var. *bracteatus* (Lindley) Coppens and Leal) is a monocotyledon plant from the Bromeliaceae family (Collins, 1960; Coppens and Leal, 2003) with great ornamental potential. The commercial propagation of this species is vegetative, using three types of propagules: crowns, suckers, and slips produced by the plant in the field. Each plant produces up to ten propagules per year, which limits its commercial cultivation (Correia et al., 2000). In addition, this kind of propagation may favor disease dissemination in the case of an unhealthy original plant. A strategy to avoid this limitation is to use *in vitro* culture for clonal propagation and fast growth of plants with important genotypes, theoretically, with the same genetic identity of the original material and with a high phytosanitary status. However, it has been observed that this technology may cause, in some situations, genetic modifications known as somaclonal variations (Larkin and Scowcroft, 1981) and also phenotypic modifications, called epigenetic variations (Evans and Bravo, 1986). These variations in micropropagated material, such as genetic and epigenetic modifications, need to be characterized. Genetic variations in plants from tissue culture have been cited in *Lycopersicon esculentum* Miller (Evans and Sharp, 1983), *Nicotiana alata* (Evans and Bravo, 1986), *Ananas comosus* (Das and Bhowmik, 1997; Gottardi et al., 2002; Feuser et al., 2003; Rodrigues et al., 2007), *Musa* sp (Martin et al., 2006) and *Curcubita pepo* (Leljak-Levanic et al., 2004), among others.

Recently, molecular markers have been utilized in the detection of variation or confirmation of genetic fidelity during micropropagation (Gupta et al., 1998; Tyagi et al., 2007). Among the polymerase chain reaction (PCR)-based markers frequently used, RAPD (random amplified polymorphic DNA) is considered to be efficient and cost effective. The technique only needs a few nanograms of DNA for a fast polymorphism analysis, does not require prior knowledge of DNA sequence, and does not involve radioactivity (Williams et al., 1990). RAPD markers have been employed to determine the clonal fidelity of micropropagated plants (Rani et al., 1995; Tang, 2001; Feuser, 2003). In the literature consulted, there is no knowledge about the genetic variability within and between plants derived from tissue culture of ornamental pineapple. The present study summarizes the application of RAPD markers to solve the problem mentioned.

MATERIAL AND METHODS

To evaluate genetic variability using RAPD, leaves were taken from *in vitro* cultures of *Ananas comosus* var. *bracteatus*. The cultures were established from axillary bud explants excised from the crown of one plant growing under field conditions. The starting medium was composed of MS salts (Murashige and Skoog, 1962), 3% sucrose, 0.7% agar and 100 mg/L myo-inositol, 0.1 mg/L thiamine-HCl, 0.05 mg/L pyridoxine-HCl, 0.05 mg/L nicotinic acid, 2.0 mg/L glycine, 1.0 mg/L 6-benzylaminopurine (BAP), 2.0 mg/L gibberellic acid (GA_3), and 0.01 mg/L α-naphthalene

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acetic acid. The cultures were illuminated 16 h per day with 32 μmol.m^{-2}.s^{-1} irradiance provided by fluorescent light and exposed to a temperature of 27 ± 2°C for 90 days. After this period, regenerated propagules of 1.5-2.0 cm in length were selected and added to flasks containing growth medium composed of MS salts, vitamins and 3% sucrose, supplemented with 0.125, 0.25, 0.5, 1.0, and 2.0 mg/L BAP, in liquid formulation. The pH of the medium was adjusted to 5.7 ± 0.1, before autoclaving. The cultures were incubated at 27°C with a 16-h photoperiod with exposure to 32 μmol.m^{-2}.s^{-1} irradiance. The propagules were subcultured monthly for 120 days. After the fourth subculture, 19 individuals for each BAP concentration were randomly selected for RAPD analysis, totaling 95 micropropagated propagules and one mother plant (control). The propagules were rooted and transferred to 3.0-cm diameter tubes containing PlantMax substrate, until they were transferred to a greenhouse where they were kept for evaluation of possible morphological changes. Every micropropagated plant characterized in this study was derived from just one axillary bud excised from the mother plant. Initially, a screening using 520 Operon Technologies Inc. primers was performed. To select the primers, the mother plant DNA and two individuals from *in vitro* culture were used. The primers that showed polymorphic bands and/or amplified a larger number of robust and well-defined bands were selected and afterwards evaluated to determine which ones were more informative with respect to polymorphism.

DNA extraction and RAPD analysis

DNA was extracted from fresh *in vitro* leaves of regenerated plants and the mother plant, as described in Ferreira and Grattapaglia (1998). The DNA quantification and quality evaluation was verified on 1% agarose gels stained with ethidium bromide and visualized under ultraviolet light. The DNA amplification reactions were run by PCR. Each reaction mixture contained: 3.0 μL 3.0 ng/μL genomic DNA, 4.92 μL autoclaved Milli-Q water, 1.30 μL 10X Taq DNA polymerase buffer, 1.04 μL dNTPs at 2.5 mM, 1.5 μL primer (Operon Technologies, USA) at 10 ng/μL and 0.2 μL Taq DNA polymerase enzyme, in a 13-μL total reaction. PCR amplification was performed in an automated thermal cycler (Perkin Elmer) programmed for 40 cycles , each consisting of an initial denaturation of 1 min at 92°C, followed by an annealing step of 1 min at 35°C and an extension step of 2 min at 72°C, followed by a final extension step at 72°C for 7 min. The amplified fragments were electrophoresed on 1.5% (w/v) agarose gels in 1X TBE buffer, visualized by staining with ethidium bromide and photographed on ultraviolet light using the Eagleye™ system (Stratagene®). The bands were identified visually by comparison to a 1-kb ladder (75 to 12,216 bp). The samples were scored based on presence or absence of the same size bands, where coding was 1 for presence on gel and 0 for absence. The entire analysis was repeated and only the reproducible bands were considered.

RESULTS AND DISCUSSION

Previously, a variety of experiments were conducted to select plant growth regulators to establish medium requirements for adventitious organ regeneration in ornamental pineapple tissue culture. BAP was effective for inducing *in vitro* shoot proliferation in this species (Santos, 2008). It seemed of interest to screen for the presence of somaclonal variation in regenerants obtained in medium supplemented with different BAP concentrations.

High-quality DNA was isolated from the ornamental pineapple leaves. Among the 520 RAPD OP-primers screened, 44 primers were selected based on the production of well-defined and scorable bands, which were used for fingerprint comparison between the

mother plant and micropopagated propagules. The data in Table 1 show the primers, primer sequences, number of bands produced and total number of amplicons revealed by RAPD analysis of micropropagated plantlets.

Table 1. Primers, primer sequences, and number of amplification bands produced by 44 RAPD primers in the evaluation of micropropagated propagules of *Ananas comosus* var. *bracteatus*, in culture medium supplemented with 6-benzylaminopurine, for 120 days.

Primer	Sequence (5'-3')	Total number of bands	Polymorphic bands
OPA-01	CAG GCC CTT C	12	1
OPA-08	GTG ACG TAG G	12	
OPA-19	CAA ACG TCG G	11	
OPA-20	GTT GCG ATC C	7	1
OPB-01	GTT TCG CTC C	12	2
OPB-18	CCA CAG CAG T	10	
OPB-19	ACC CCC GAA G	9	1
OPC-19	GTT GCC AGC C	7	1
OPC-08	TGG ACC GGT G	8	
OPF-04	GGT GAT CAG G	7	
OPF-13	GGC TGC AGA A	14	3
OPG-19	GTC AGG GCA A	10	
OPI- 02	GGA GGA GAG G	15	
OPI-03	AAG GCG GCA G	9	
OPI-06	AAG GCG GCA G	10	
OPI-07	CAG CGA CAA G	12	
OPI-11	ACA TGC CGT G	10	
OPI-14	TGA CGG CGG T	8	
OPJ-13	CCA CAC TAC C	7	
OPK-17	CCC AGC TGT G	10	
OPL-08	AGC AGG TGG A	6	
OPL-17	AGC CTG AGC C	10	1
OPM-13	GGT GGT CAA G	12	2
OPN-16	AAG CGA CCT G	16	
OPO-07	CAG CAC TGA C	9	
OPO-10	TCA GAG CGC C	14	
OPP-16	CCA AGC TGC C	8	1
OPQ-05	CCG CGT CTT G	4	
POR-08	CCC GTT GCC T	6	
POR-09	TGA GCA CGA G	12	
OPR-10	CCA TTC CCC A	8	
OPR-14	CAG GAT TCC C	7	
OPR-16	CTC TGC GCG T	10	
OPR-17	CCG TAC GTA G	9	
OPS-04	CAC CCC CTT G	5	
OPT-07	GGC AGG CTG T	16	2
OPU-01	ACG GAC GTC A	12	
OPV-07	GAA GCC AGC C	7	
OPV-10	GGA CCT GCT G	8	
OPV-11	CTC GAC AGA G	5	
OPV-19	GGG TGT GCA G	12	2
OPX-03	TGG CGC AGT G	12	1
OPX-19	TGG CAA GGC A	12	
OPZ-08	GGG TGG GTA A	9	

The 44 primers produced 420 well-defined fragments; among them, 402 bands were monomorphic (97.2%) and 18 bands were polymorphic (2.8%) (Table 2). A total of 40,320 fragments (number of propagules x number of bands obtained) were produced with an aver-

age of 9.5 bands per primer. The length of the amplification products was between 298 and 5090 bp. The number of bands per primer varied from 4 (OPQ-05) to 16 (OPN-16 and OPT-07) (Table 1). The polymorphic fragments were produced by 12 primers (OPA-01, OPA-20, OPB-01, OPB-19, OPC-19, OPF-13, OPL-17, OPM-13, OPP-16, OPT-07, OPV-19, and OPX-03). Among the primers that identified polymorphisms, OPA-1, OPA-20, OPB-1 and OPB-20 produced one polymorphic band and OPF-13 amplified a maximum of 3 bands (Table 1). The polymorphism generated by OPC-19 was observed in 10 samples with genetic variation (38%). Three variants were detected by the primer OPL-17; OPF-13, OPP-16, and OPT-7 were each able to identify two independent variants. OPA-20, OPA-1, OPB-1, OPB-19, OPN-13, OPV-19, OPX-3 independently identified one somaclonal variant (Table 3). Examples of RAPD profile of mother plant and micropropagated plantlets are shown in Figure 1.

Table 2. Summary of random amplified polymorphic DNA (RAPD) products from micropropagation propagules and original plant of Ananas comosus var. bracteatus used in the present study.

Description	RAPD
Number of primers tested	520
Number of primers selected	44
Number of primers that showed polymorphisms	12
Total number of amplified bands	420
Total number of monomorphic amplicons	402
Total number of polymorphic amplicons	18
Percent of polymorphic bands	2.8%
Size of amplified bands	298-5090 bp
Average number of polymorphic bands per primer	1.5
Average number of bands per primer	9.5

Table 3. Presence (+) or absence (-) of polymorphic RAPD bands generated by primers in *Ananas comusus* var. *bracteatus* propagules cultured in media with different 6-benzylaminopurine (BAP) concentrations, after 120 days.

Primer	Polymorphic regenerants																							
	M	5	7	15	16	26	27	28	38	39	50	51	52	53	54	58	63	64	66	74	75	78	86	93	95
OPA-01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
OPA-20	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPB-01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
OPB-01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
OPB-19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
OPC-19	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-
OPF-13	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPF-13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
OPF-13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
OPL-17	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-
OPM-13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
OPM-13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
OPP-16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
OPT-07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
OPT-07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
OPV-19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
OPV-19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
OPX-03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

M: mother plant; propagules 5, 7, 15, and 16 (0.125 mg/L BAP); propagules 26, 27, 28, 38, and 39 (0.25 mg/L BAP); propagules 50, 51, 52, 53, 54, and 58 (0.5 mg/L BAP); propagules 63, 64, 66, 74, and 75 (1.0 mg/L BAP); propagules 78, 86, 93, and 95 (2.0 mg/L BAP).

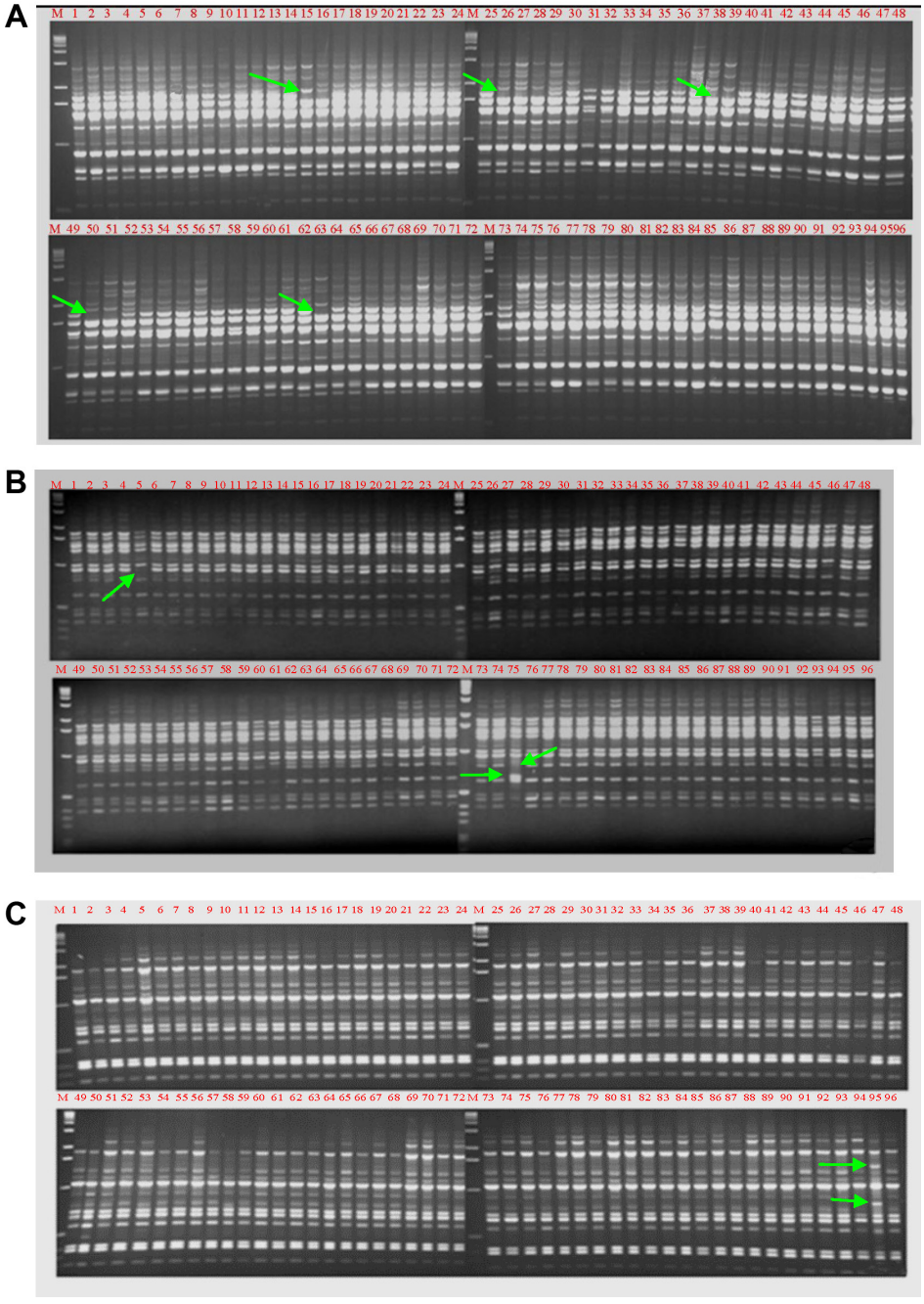

Figure 1. Molecular pattern of micropropagated plantlets revealed by RAPD on 1.5% agarose gels, with the primers: **A.** OPC-19; **B.** OPF-13, and **C.** OPM-13. The arrows indicate polymorphic markers. The propagules were maintained in basal medium, liquid formulation, supplemented with BAP, subcultured every 30 days, for 120 days. M = mother plant. Lanes 1-96 = propagules regenerated *in vitro* in medium containing BAP.

The percentage of polymorphism detected in *Ananas comosus* var. *bracteatus* in BAP-containing medium was estimated to be 21 to 42%, confirming the occurrence of variation during the micropropagation process. The variation of the electrophoretic pattern found in genomic DNA of regenerated propagules, after 120 days of culture, was probably due to the medium supplementation with BAP, according to a contingency test that showed a significant chi-square value (Table 4). This finding is consistent with those of Evans et al. (1984), Rani et al. (1995), Salvi et al. (2001), Feuser et al. (2003), and Costa and Zaffari (2005). In *Ananas bracteatus* cv. *striatus*, phenotypic variations of 52% albino plants and 20.5% green plants were described by Costa and Zaffari (2005). In commercial pineapple, Feuser et al. (2003) observed a more reduced rate of somaclonal variation among plantlets regenerated from *in vitro* culture using either stationary liquid medium or a temporal immersion system. These authors utilized 10 RAPD primers and detected 7.5 and 5.0% of somaclonal variants for the stationary and temporal immersion systems, respectively. However, in this latter study, the authors did not state the period of culture in which the samples were taken for RAPD analysis. The culture period directly influences the appearance of somaclonal variation (Skirvin et al., 1994), and the range of variation of 1 to 3% is expected in the process of micropropagation. Some authors have indicated that the treatment used in tissue culture, with high growth rate, may increase the variant numbers (Bairu et al., 2006). The regeneration systems from organized meristems, such as shoot tip and axillary buds, are considered to be the most efficient methods to guarantee genetic integrity of the micropropagated material. The regeneration methods from leave explants (Kawiak and Lojkowska, 2004) and callus (Skirvin et al., 1994) are considered to be less stable permitting the occurrence of genetic variation. In *Drosera binata*, plantlets regenerated through shoot tip preserve the genetic integrity of micropropagated plants. In *Curcuma longa*, rhizome bud explants used to establish cultures show genetic homogeneity in the regenerated propagules, when comparing them with the mother plant (Tyagi et al., 2007). However, plants regenerated from leaf base callus have shown variation at the DNA level during *in vitro* culture (Salvi et al., 2001; Tyagi et al., 2007).

Table 4. Chi-square values (χ^2) and probability (P) of contingency test in the determination of genetic variability of propagules and original plant of *Ananas comosus* var. *bracteatus* as a result of the micropropagation process.

BAP concentration (mg/L)	Normal plants	Variant plants	χ^2	P
0.125	15	4	10.3	0.001-0.005
0.25	15	4	10.3	0.001-0.005
0.5	11	8	55.0	<0.001
1.0	14	5	18.1	<0.001
2.0	14	5	18.1	<0.001

The propagules were maintained *in vitro* in liquid medium supplemented with 6-benzylaminopurine (BAP), subcultured every 30 days and evaluated by RAPD after 120 days.

In this study, RAPD was effective in showing the variations that may occur as a result of mutations during the micropropagation process of *Ananas comosus* var. *bracteatus* in BAP-supplemented medium, and that could be useful in detecting the presence of genetic variation in the initial stages of plant development. Also, these findings could be applied in the breeding of this species. The regenerated plants are currently growing in a greenhouse, and later they will be transferred to the field and screened for morphological variation.

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