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In Vitro Mutation in *Rosa hybrida* cv. 'Pusa Gaurav' and Selection through RAPD and ISSR Markers

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ABSTRACT

Apical and axillary meristems of *Rosa hybrida* cv. 'Pusa Gaurav' were pretreated with various concentrations (0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0 µM) of oryzalin ($C_{12}H_{18}N_4O_6$) to induce variation *in vitro*. Depending on the concentration of oryzalin used, the mean survival rate of meristem culture decreased from 98.9% (5.0 µM, 0 h) to 9.85% (30 µM, 36 h), but the lethal dose (LD_{50}) was 20 µM oryzalin pretreated for 24 h. Oryzalin-treated microshoots were used to multiply shoots. The maximum rate of shoot multiplication occurred on Murashige and Skoog (MS) medium supplemented with 2 mg/l BAP (6-benzylaminopurine), 0.25 mg/l IAA (indole-3-acetic acid), and 50 mg/l Ads (adenine sulfate) and 20 µM oryzalin. The elongated shoots were rooted in half-strength MS medium supplemented with 0.25 mg/l IBA (indole-3-butyric acid) and about 65% rooted plants survived in the greenhouse. Molecular [Randomly amplified polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR)] analyses were used to determine the genetic variability of the meristems of oryzalin-treated plants. Six out of 20 RAPD primers and five out of 12 ISSR primers revealed polymorphisms (30.5 and 61.8%, respectively) among the *in vitro* plants indicating the efficiency of oryzalin to induce *in vitro* variability in hybrid rose and to detect variation through molecular markers. A comparative morphological analysis between the control and the mutant was done, which showed minor variations in some morphological characters (plant height, number of branches, foliage size, thorn density, flower diameter, flower depth, number of petals. Our findings will provide fundamental insight into an improved rose breeding program.

Keywords: hybrid rose, molecular marker, mutant, oryzalin
Abbreviations: Ads, adenine sulfate; **BAP**, 6-benzylaminopurine; **IAA**, indole-3-acetic acid; **ISSR**, Inter simple sequence repeat; **Kn**, kinetin; **LD_{50}**, lethal dose; **NAA**, 1-napthalene acetic acid; **RAPD**; randomly amplified polymorphic DNA

INTRODUCTION

Over the past few decades, breeders have faced many problems in rose improvement due to low sexual reproduction and poor germination of seed because of embryo abortion. Since the first report by Elliott in 1970 on shoot multiplication and rooting of rose many studies have been conducted using different concentrations of hormones for the initiation of callus, maintenance and regeneration of shoots and roots from the callus and directly from nodal segments, lateral and axillary buds and shoot tips (Rout *et al*. 1991; Vijya and Satyanarayan 1991; Rout *et al*. 1992; Chu *et al*. 1993; Syamal and Singh 1994; Kintzois *et al.* 1999; Dobois *et al*. 2000; Mohapatra and Rout 2005; reviewed in Khosh-Khui and Teixeira da Silva 2006). The application of tissue culture techniques for the regulation and commercial propagation of rose has been developed more recently. Among the rose cultivars, about 20% were developed on the basis of spontaneously derived sports (Krussmann 1974). This relatively high percentage may provide an opportunity to apply mutation induction techniques to rose breeding programs. Thus, induced mutation (both physical and chemical mutagens) in combination with *in vitro* culture could provide an opportunity to increase variability of an economically important cultivar and to obtain information about the potential of spontaneously arising sports in new variety. Induced mutation has been used to generate a wide range of mutants for enhanced response to abiotic and biotic stress (Jain 2005; Kumar *et al*. 2006). Oryzalin ($C_{12}H_{18}N_4O_6$) is a selective surface applied herbicide used as a chemical mutagen, inducing spindle inhibition. It has much more affinity for plant microtubules and is less hazardous to human health than other chemical mutagens like colchicine (Hugdahl and Morejohn 1993). Molecular marker techniques such as Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) have proven to be a powerful tool for estimating genetic variation in various plant groups (Williams *et al*. 1990; Pharmawati *et al*. 2004; Mohapatra *et al*. 2005). To our knowledge this is the first study on inducing mutations in meristem cultures of rose which was carried out to develop improved varieties through *in vitro* mutagenesis by using oryzalin as a chemical mutagen in hybrid rose and to evaluate genetic variability among the mutants by using molecular markers.

MATERIALS AND METHODS

Material and *in vitro* culture

Cuttings (about 15 cm long from the apical end) of *Rosa hybrida* cv. 'Pusa Gaurav' were collected from the rose garden of the Regional Plant Resource Center, Bhubaneswar, India and brought to the laboratory in a beaker with distilled water. Nodal segments with internodes (~ 0.25 cm long) were used as the explant source. About 0.5-1.0 cm of internodes containing nodes were cut and washed thoroughly under tap with 2% (v/v) detergent (Labolene, Qualigen, India) for 15 min. This was followed by washing in running tap water for 15 min. Surface sterilization was made with 0.1% (w/v) mercuric chloride (Qualigen, India) for 15 min and subsequently washed with sterile distilled water three times. Twenty nodal explants were cultured in the test tubes (25 x 150 mm) containing MS (Murashige and Skoog 1962) medium supplemented with 6-benzylaminopurine (BAP), kinetin (Kn), adenine sulfate (Ads) (Sigma, USA) in different concentrations and combination with indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA) (Sigma). Each tube having one nodal explant

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and the basal end dipped into the medium. The media was solidified with 0.7% (w/v) agar-agar (Himedia, India). Operations from surface sterilization to inoculations were carried under sterile conditions in a laminar flow bench. Cultures were incubated at 25 ± 2°C under a 16-h photoperiod with light intensity of 3,000 lux. The subculture was made every 4 weeks after an initial culture of 8 weeks. Cultures were maintained at 50-60% RH. All the plant growth regulators were added prior to autoclaving. pH of the media was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCl before adding agar-agar. Media was sterilized for 15 min at 121°C (15 lb psi). After successful development of multiple shoots, the apical and axillary meristems were isolated aseptically and treated with different concentrations (0, 5.0, 10.0, 15.0, 20.0, 30.0 and 40.0 µM) of oryzalin (Duchefa Biochemie, Netherlands) for different time intervals (0, 1, 3, 6, 12, 18 and 24 h) with constant shaking at 100 rpm in a rotary shaker. The pretreated apical and axillary meristems were removed and soaked on aseptic blotting paper; subsequently they were transferred to glass Petri dishes containing basal MS medium supplemented with different concentrations of BAP (0.5-1.0 mg/l), IAA (0.01-0.05 mg/l), Ads (25-50 mg/l) and 20 µM oryzalin for growth). Statistical analysis (least significant difference (LSD, $p<0.05$)) was made by using IRRISTAT's (International Rice Research Institute, Philippines) software. Photoperiod was provided by cool and fluorescent light (3000 lux) for 16 h. After two weeks, treated explants were transferred to MS basal medium supplemented with 0.5 mg/l BAP, 0.01 mg/l IAA, and 25 mg/l Ads without oryzalin. After 4 weeks, the cultures were further transferred to MS medium supplemented with 1.0-2.0 mg/l BAP, 0.25 mg/l IAA and 50 mg/l Ads in order to maximize the number of multiple shoots. Both treated and untreated microshoots were separated from the cultures and transferred to half-strength MS medium supplemented with IAA or IBA (0, 0.1, 0.25 and 0.5 mg/l) with 2% (w/v) sucrose to induce rooting. The percentage rooting and days to rooting were recorded after two weeks' culture. The rooted plantlets were transferred to a greenhouse for acclimatization and watered at 2-day intervals. The rooted plantlets were grown successfully in 25 cm earthen pots until the flowering stage.

Morphological/growth parameters

Morphological parameters namely plant height, foliage size, thorn density, number of branches, flower diameter and flower depth were measured every three months up to two years. Differences between various morphological parameters of control and mutant plants were compared by ANOVA using IRRISTAT (International Rice Research Institute, Philippines) software. LSD was determined at $p<0.05$.

DNA isolation and quantification

Leaves were collected from both control and treated microshoots and used for DNA analysis. Genomic DNA was extracted from young leaves using the *N*-cetyl-*N*,*N*,*N*-trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with modifications. Two grams of fresh leaf material were washed in distilled water and subsequently rinsed with 80% (v/v) ethanol and then ground in liquid nitrogen. Ten milliliters of preheated extraction buffer (4% (w/v) CTAB, 0.2% β-mercaptoethanol (v/v), 100 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.4 M NaCl) were then added per 2 g of leaf powder and incubated for two hours at 65°C. The DNA pellet was resuspended in 200 µl to 300 µl of Tris-EDTA buffer (10 mM). DNA quantification was performed by visualizing under UV light, after electrophoresis on a 0.8% (w/v) agarose gel. The resuspended DNA was then diluted in sterile distilled water to 5 ng/µl for use in amplification reactions.

PCR amplification for RAPD and ISSR assay

Twenty decamer primers corresponding to Kits A, B, D and N from Operon Technologies (Alameda, California, USA) and 12 synthesized ISSR primers (M/S Bangalore Genei, India) were initially screened to determine the suitability of each primer tested. Initial optimization of PCR was done for RAPD and ISSR assay, including concentration of template DNA, primers, $MgCl_2$, number of PCR cycles and above all annealing temperature. A 25 µl reaction mixture for RAPD and ISSR assay contained 20 ng template DNA, 100 mM of each dNTP, 15 ng of primer, 1X *Taq* buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% gelatin) and 0.5 U *Taq* DNA polymerase (M/S Bangalore Genei, India). $MgCl_2$ at 1.5 and 2.5 mM were used for RAPD and ISSR, respectively. Annealing temperature varied with the primer. DNA amplification was performed in a Thermo Cycler (PTC-100) (MJ Research, USA) programmed as follow: preliminary activation at 94°C for 2 min followed by 40 cycles of 20 sec at 94°C, 30 sec at annealing temperature and 1 min at 72°C, with a final extension at 72°C for 5 min. Amplification products were visualized in 1 and 1.5% agarose gels for RAPD and ISSR, respectively. Electrophoresis was made in 1X Tris acetate EDTA (TAE) buffer prestrained with ethidium bromide. A 100 bp ladder (M/S Bangalore Genei, India) was run in each gel as the size marker. Gel photographs were scanned through a Gel Documentation System (Gel Doc 2000, BioRad, USA). All PCR amplifications were repeated in triplicate to establish reproducibility of results.

RESULTS AND DISCUSSION

Significant differences were observed with regard to the survival rate of isolated meristems cultured at different concentrations of oryzalin (**Table 1**). Fifty percent lethality (LD_{50}) was observed when meristems were pretreated with 20 µM oryzalin for 24 h. Meristems treated with 30 µM oryzalin for 6 h, 25 µM for 18 h or 15 µM for 36 h also

Table 1 Effect of different concentrations of oryzalin on survival percentage in meristem of *Rosa hybrida* cv. 'Pusa Gaurav' after 2 weeks of culture on MS medium supplemented with 2 mg/l BA, 0.25 mg/l. IAA , 50 mg/l Ads and 3% sucrose.

Oryzalin concentration (µM)	Percentage survival (Mean ± SD)[a]				
	Pretreatment (hours)				
	0	6	18	24	36
0	99.86 ± 0.04 d	92.35 ± 0.06 g	91.82 ± 0.06 g	85.30 ± 0.11 g	81.50 ± 0.12 g
5	98.86 ± 0.01 d	87.25 ± 0.13 f	86.50 ± 0.09 f	73.50 ± 0.67 f	67.30 ± 0.13 f
10	98.82 ± 0.06 cd	82.50 ± 0.11 e	78.25 ± 0.07 e	64.50 ± 0.12 e	59.35 ± 0.13 e
15	98.78 ± 0.08 c	79.30 ± 0.33 d	72.50 ± 0.12 d	62.50 ± 0.09 d	56.40 ± 0.09 d
20	98.60 ± 0.03 c	76.80 ± 0.03 c	63.11 ± 0.04 c	55.33 ± 0.12 c	37.30 ± 0.01 c
25	97.89 ± 0.08 b	69.35 ± 0.06 b	53.30 ± 0.09 b	41.39 ± 0.13 b	28.36 ± 0.06 b
30	97.68 ± 0.09 a	58.62 ± 0.11 a	38.68 ± 0.06 a	26.38 ± 0.08 a	9.85 ± 0.16 a

[a] 30 replicates per treatment, repeated in triplicate.
Mean having the same letter in a column were not significantly different by Duncan's Multiple Range test $P< 0.05$

Analysis of variance for effect of different concentrations of oryzalin on survival percentage in meristem of *Rosa hybrida* cv. 'Pusa Gaurav' after 2 weeks of culture on MS medium supplemented with 2 mg/l BA, 0.25 mg/l. IAA, 50 mg/l Ads and 3% sucrose.

Source of variation	df	Sum of Squares	Mean Squares	LSD *
Time of exposure (T)	4	31013.9	7753.47	0.067
Concentration (C)	6	18465.1	3077.52	0.079
T x C	24	8150.5	339.60	0.178
Error	70	0.8369	0.1195	

* Significant at $P < 0.05$ as determined by the F -test

Table 2 Effect of various growth medium with 20 µM oryzalin on shoot multiplication of *Rosa hydrida* cv. 'Pusa Gaurav' after 4 weeks of culture.

MS + growth regulators (mg/l)					Percentage shoot multiplication	Av. № of multiple shoots / explant	Av. shoot length (cm)
BAP	Ads	IAA	NAA	GA_3	(Mean ± S.D)[a]	(Mean ± S.D) [a]	(mean ± S.D) [a]
0	0	0	0	0	0	0	0
0.5	25	0.01	0	0	32.4 ± 2.7 a	1.70 ± 0.8 a	2.91 ± 0.8 a
1	50	0.01	0	0.5	41.6 ± 3.2 c	3.03 ± 1.1 b	3.13 ± 0.9 b
1.5	50	0.25	0	0.5	72.6 ± 4.3 h	4.12 ± 1.1 f	4.17 ± 0.6 f
2	50	0.25	0	0	78.4 ± 7.0 i	4.72 ± 0.5 h	4.12 ± 0.9 f
2	50	0	0.1	0	68.4 ± 3.7 g	3.84 ± 0.2 e	3.66 ± 0.8 d
2	50	0	0.25	0	66.4 ± 6.0 f	4.53 ± 0.7 g	3.56 ± 1.0 c
2	100	0	0.25	0.5	36.4 ± 6.0 b	3.67 ± 1.2 d	4.26 ± 1.0 g
0	50	0	0.25	0	56.8 ± 4.9 d	3.43 ± 0.9 c	3.76 ± 1.0 e
0	100	0	0.1	0	62.4 ± 5.4 e	3.50 ± 0.9 c	4.34 ± 0.9 h

[a] 30 replicates/treatment; repeated three times.
Mean having the same letter in a column were not significantly different by Duncan's Multiple Range test P< 0.05
Ads: adenine sulfate; BAP: 6-benzylaminopurine, GA_3: gibberellic acid; IAA: indole-3-acetic acid; NAA - 1-napthalene acetic acid

Analysis of variance for effect of various growth medium with 20 µM oryzalin on shoot multiplication of *Rosa hydrida* cv. 'Pusa Gaurav' after 4 weeks of culture.

		% of shoot multiplication		Av. № of multiple shoots/explant		Av. Shoot length	
Source of variation	df	Mean squares	LSD*	Mean squares	LSD*	Mean squares	LSD*
Replication	2	0.115	0.295	0.024	0.052	0.003	0.038
Treatment	8	830.58	0.511	2.404	0.091	0.735	0.066
Error	16	0.0872	-	0.002	-	0.001	

* Significant at P < 0.05 as determined by the F-test

Table 3 Comparison of the morphological characteristics of control (untreated) and mutant (treated) plants of *Rosa hybrida* cv. 'Pusa Gaurav' after 10 months' transfer to a greenhouse.

Treatments	Morphological characteristics						
	Av. height of the plant (cm) (Mean ± SE)	Av. foliage size (cm) (Mean ± SE)	Av. thorn density / plant (Mean ± SE)	Av. № of branches / plant (Mean ± SE)	Av. flower diameter / flower (cm) (Mean ± SE)	Av. flower depth /flower (cm) (Mean ± SE)	Av. № of petals/flower (Mean ± SE)
Control (untreated plant) (Mean ± SD) [a]	69.3 ± 0.4 b	5.7 ± 0.4 a	16.0 ± 0.2 b	17.0 ± 0.4 b	5.50 ± 0.12 a	2.30 ± 0.2 a	21.0 ± 0.6 a
Select as mutant (treated plant) (Mean ± SD) [a]	59.7 ± 0.4 a	5.68 ± 0.1 a	13.90 ± 0.9 a	14.50 ± 0.2 a	5.28 ± 0.1 a	3.02 ± 0.1 b	25.03 ± 0.1b

[a] Sixty plants evaluated after 2 months of transfer.
Mean having the same letter in a column were not significantly different by Duncan's Multiple Range test P< 0.05

Analysis of variance for comparison of morphological characteristics of control (untreated) and mutant (treated) plants of *Rosa hybrida* cv. 'Pusa Gaurav' after 10 months' transfer to a greenhouse.

Source of variation	df	Av. height of the plant		Av. foliage size		Av. thorn density / plant		Av. № of branches / plant		Av. flower diameter / flower (cm)		Av. flower depth /flower (cm)		Av. № of petals/flower	
		M.S	LSD*	M.S	LSD*	M.S	LSD*	M.S	LSD*	M.S	LSD*	M.S	LSD*	M.S	LSD*
Replication	2	0.240	1.20	0.073	1.41	0.036	0.578	0.066	1.689	0.029	0.374	0.024	0.420	0.170	1.991
Treatment	1	138.24	0.983	0.0005	1.155	6.615	0.472	9.375	1.379	0.863	0.305	0.777	0.342	24.36	1.625
Error	2	0.080	-	0.111	-	0.018	-	0.158	-	0.008	-	0.010	-	0.220	-

M.S – Means Squares; * Significant at P < 0.05 as determined by the F-test.

showed 50% survival, but after transfer to fresh medium the survival of meristems was low (20-30%) (data not shown). The survival rate of meristems decreased as the concentration of oryzalin in the medium increased, irrespective of the pretreatment period. Since DNA synthesis is not blocked by oryzalin meristematic cells can go through several cycles of endoreduplication (Grandjean *et al*. 2004). The exposure of meristems to oryzalin for 24 h would be sufficient for these cells to go through a second spindle-inhibited mitosis, to produce octoploid cells in which further reduplication might occur within a 48-h exposure. The poor viability of treated shoots due to high ploidy levels adversely affected growth of *Rosa* (Allum *et al*. 2007). Kermani *et al*. (2003) reported that oryzalin induced chromosome doubling in rose and this had an effect on plant morphology and pollen viability. They reported that the leaves of all chromosome-doubled plants were thicker and darker green than those of original undoubled plants tetraploids had significantly greater leaf breadth/length ratios than their diploid progenitors and internode length was increased in all diploid to tetraploid conversions while the number of petals was double that of its diploid progenitor. Diploid forms of 'Pink surprise' and 'Mermaid' produced no viable pollen but the tetraploid forms produced 3.8 to 11.2% viable pollen, respectively. Allum *et al*. (2007) reported that the concentration of oryzalin has a profound effect on chromosome doubling in rose. The frequency of survival depends on the exposure time. In this study thirty meristems were initially pretreated with 20 µM oryzalin for 24 h and subsequently transferred to MS basal medium supplemented with different concentrations of BAP (0.5-2 mg/l), IAA (0.01-0.25 mg/l), Ads (25-50 mg/l) and 20 µM oryzalin (**Table 2, Fig. 1A**). The percentage shoot multiplication ranged from 32.4 ± 0.5 to 78.4 ± 1.3 and the maximum percentage shoot multiplication was observed in MS medium supplemented with 2.0 mg/l BA, 0.25 mg/l IAA, 50 mg/l Ads and 20 µM oryzalin (**Fig. 1B**). The average length of shoots ranged from 2.91 ± 0.16 to 4.34 ± 0.18 cm (**Table 2**). *In vitro*-grown microshoots were transferred to various rooting media to induce rooting. About 80% of microshoots rooted on half-strength MS medium supplemented with 0.25 mg/l IBA and 2% sucrose within 12 days of culture (**Figs. 1C, 1D**). About 65% of the rooted plants, after transfer to a greenhouse, survived and flowered (**Fig. 1E**). Morphological analysis was done as per the Indian Council of Agriculture Research (ICAR) guidelines. There was a significant difference between treated and untreated plants, especially with respect to the average height of the plant, size of the leaves, thorn density, flower depth and number of petals (**Table 3, Figs. 2A-C**). Some variations were also found in petal colors (**Fig. 2B**).

Six out of twenty RAPD primers and five out twelve ISSR primers showed polymorphism, 30.5 and 61.8% in RAPD and ISSR, respectively (**Table 4**). Some new bands appeared within the 500-2800 bp range and a few bands

Fig. 1 *In vitro* mutagenesis of *R. hybrida* cv. 'Pusa Gaurav'. (**A**) Oryzalin treated isolated meristems cultured on MS medium supplemented with 0.5 mg/l BAP, 0.25 mg/l IAA, 50 mg/l Ads and 20 µM oryzalin after 2 weeks of culture. (**B**) Development of multiple shoots on MS medium supplemented with 2.0 mg/l BAP, 0.25 mg/l IAA, 50 mg/l Ads and 20 µM oryzalin after 4 weeks of culture. (**C, D**) Induction of roots from multiple shoots on ½MS medium supplemented with 0.25 mg/l IBA and 2% sucrose after 12 days of culture. (**E**) Rooted plantlets established in pots and bearing flowers.

Fig. 2 Morphological variation in plants (**A**), flower (**B**) and leaf (**C**) *in vitro* raised plants of *R. hybrida* cv. 'Pusa Gaurav' after 2 months of transfer to greenhouse conditions.

Table 4 Analysis of polymorphism of untreated and mutant microshoots of *Rosa hybrida* cv. 'Pusa Gaurav' using RAPD and ISSR primers.

Primer	Primer sequence (5′-3′)	Total № of bands	Monomorphioc bands	Polymorphic bands	% polymorphisim	Band range (Kbp)
RAPD						
OPA-02	TGCCGAGCTG	8	7	1	13.3	0.5-2.0
OPD-08	GTGTGCCCCA	6	5	1	16.6	0.4-1.9
OPD-11	AGCGCCATTG	9	8	1	11.1	0.5-2.2
OPN-2	ACCAGGGGCA	7	4	3	42.8	0.8-2.8
OPN-7	CAGCCCAGAG	7	4	3	42.8	0.7-2.3
OPN-8	ACCTCAGCTC	9	7	2	28.5	0.4-2.1
ISSR						
IG-01	AGGGCTGGAGGAGGGC	8	1	7	87.5	0.5-1.9
IG-03	GAGGGTGGAGGATCT	5	2	3	40.0	1.2-2.1
IG-07	ACAGACAGACAGACAG	7	3	4	57.1	0.6-1.9
IG-16	CAG(CA) 7	8	4	4	50.0	0.4 -2.2
IG-22	(GA)8	6	3	3	50.0	0.4 -2.0

*Kbp: Kilo base pair

disappeared between 950 and 2000 bp (**Figs. 3A-D**). These types of polymorphisms are found due to alterations in the number of primer binding sites during mutagenesis. Polymorphisms in RAPD primers were also observed in soybean after colchicine treatment due to an alteration in the number of primer binding sites (Hofman *et al.* 2004). Genetic variation was also detected by RAPD analysis in potato by using colchicine (Teparkum and Veilleux 1998). Polymorphic amplification products, which represent one allele per locus, can result from changes in either the sequence of the primer-binding site, such as point mutations, or from changes altering the size or preventing successful amplification of a target DNA such as insertions, deletions, and inversions (Rani *et al*. 1995). Since RAPD markers are resolved on low-resolution agarose gels, point mutations caused by an alkylating agent occurring within the amplified region will go undetected. Mutations resulting in polymorphisms are those occurring on primer binding sites, leading to an increase or decrease in the total number of primer binding sites, and consequently the number of amplified fragments. These polymorphisms indicate the presence of genetic differences in meristems of *R. hybrida* cv. 'Pusa Gauarav' treated with various concentrations of oryzalin.

Our study showed the efficiency of oryzalin in inducing *in vitro* mutation in hybrid rose. RAPD and ISSR markers are useful to detect polymorphisms in meristem cultures of hybrid roses subjected to chemical mutagenesis as they provide a sufficient numbers of variables. This approach can then be used to rapidly screen large numbers of meristems in efforts to detect mutants together with *in vitro* techniques. Mutation-assisted breeding can contribute greatly for genetic improvement of rose.

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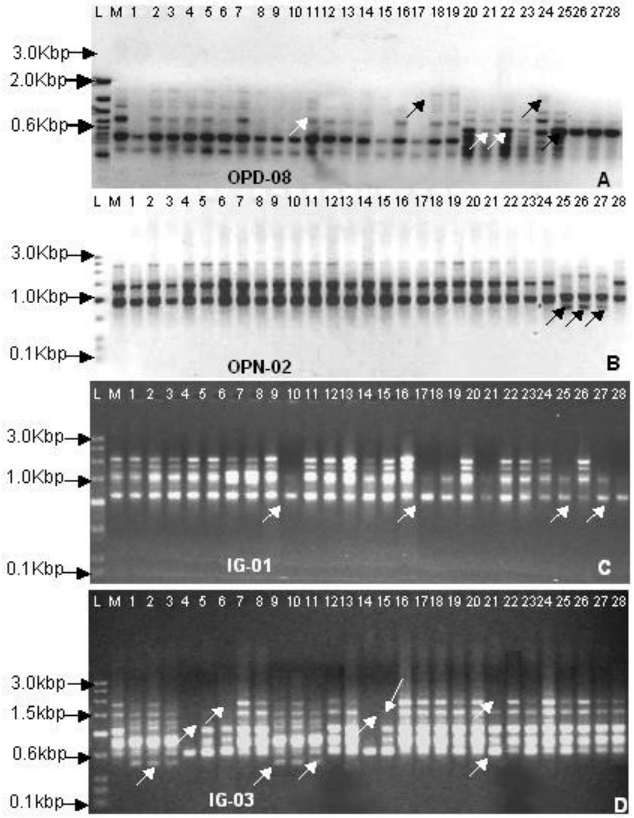

Fig. 3A-D DNA profiles of *in vitro*-raised plants by using different primers (**A**) OPD-08 (5′-GTGTGCCCCA-3′), (**B**) OPN-02 (5′-ACCAGGGG CA-3′), (**C**) IG-01 (5′-AGGGCTGGAGGAGGGC-3′) and (**D**) IG-03 (5′-GAGGGTGGAGGATCT-3′). L, DNA size marker, M, control (untreated), 1-28 mutant plants (20 µM oryzalin-treated) of *Rosa hybrida* cv. 'Pusa Gaurav'.

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