Eriastrum densifolium[12], *Fragaria* sp.[13] and *Rheum* sp.[14]. Specific bands (Figure 2) in the range 200–300 bp, amplified by the primers OPX9, OPX10, OPF4, OPC2 were found only in multiple-flowering OP clones of Neelam and Banganapalli, while the same was absent in single-flowering clones and varieties. We hypothesized that these specific bands were responsible for genes associated with differential flowering behaviour of mangoes in the island ecosystem. A number of DNA-based techniques have been developed to identify genetic variability within species as well as identification of specific genes and relating DNA sequence to function[15]. This has led to the initiation of work on sequencing of the gene and utilizing the same in the mango breeding programme.

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Molecular marker-assisted selection of *in vitro* chemical mutagen-induced grapevine mutants

In vitro-induced mutagenesis using chemical mutagens like ethyl methane sulphonate (EMS) and ethidium bromide (EB) was achieved in grape (*Vitis vinifera* L.) cv. Pusa Seedless (PS). Both the mutagens were supplemented at 0.01–0.1% in the shoot elongation-cum-rooting half-strength MS medium containing 2.0 mg l^{-1} indole 3-butyric acid (IBA) and 200 mg l^{-1} activated charcoal. *In vitro* raised grapevines were aseptically cut into two-node microcuttings and were cultured onto mutagen-supplemented medium. The lethal dose (LD_{50}) on the basis of *in vitro* survival of microcuttings and their subsequent growth for EMS was adjudged as 0.04%, whereas it was 0.06% for EB. The putative mutants (vM_3) were hardened in a glasshouse and screened using RAPD–PCR analysis with 30 arbitrary decamer primers to isolate the solid mutants. Percentage of mutants identified after marker-assisted selection was higher in EMS (28.5) compared to EB (8.8) treatment. Identified solid mutants multiplied *in vitro* and were planted in the field for further evaluation of quality parameters after fruiting. The proposed technique can be utilized to generate solid mutants coupled with their early detection using molecular markers not only in different grape varieties, but also in vegetatively propagated perennial fruit crops.

Mutations are the ultimate source of variability in organisms. Variability caused by induced mutations is not essentially different from that caused by spontaneous mutations. The direct use of mutation is a valuable supplementary approach to plant breeding, particularly when it is desired to improve one or a few characters in an otherwise well-adapted variety/hybrid. Mutation-assisted breeding can play an important role in crop improvement either directly or by supplementing the conventional breeding. Studies on induced mutations in fruit crops have been performed particularly in apple, pear, peach, etc. and little in grape[1]. Irradiation has resulted in the development of some commercially important mutants in grape[2,3]. However, information on induction of somatic mutations is not sufficient. Combination of mutagenic treatments and *in vitro* technique is now possible in many seeds as well as vegetatively propagated crops. *In vitro* approach has certain advantages and may be an effective method[4] for obtaining desired mutations and can be increased by the use of cell and tissue culture. Furthermore, selection of appropriate mutagen and isolation of solid mutants are difficult, but are important steps in mutation breeding.

Grape is a highly heterozygous and vegetatively propagated fruit crop. It has a long juvenility period and takes a long time to come into flowering. Therefore, it takes more time to improve this fruit crop by conventional methods such as hybridization, recombination and selection. Yet, cultivar improvement efforts are impeded by the extensive labour and funding necessary to grow a large progeny in heterozygous woody perennials with

limited understanding of grapevine genetics. In addition, the lack of inbreed lines makes it difficult to perform genetic linkage analysis. A faster and more reliable breeding system would be of great value to aid in breeding and subsequent selection of the desired genotypes. Genetic markers are heritable entities that are associated with economically important traits and can be used as selection tools[5]. Marker-assisted selection provides a potential for increasing selection efficiency by allowing for early selection and reducing the plant population size. The present study on *in vitro* mutation induction in grape cv. PS was undertaken using two chemical mutagens in order to standardize the technique of *in vitro* induction of mutation followed by molecular marker-assisted selection.

Micropropagated grape (*Vitis vinifera* L.) plantlets of variety PS were established using nodal segment as an explant collected from the 12-yr-old field-grown vines[6]. *In vitro* mass multiplication and maintenance of plantlets were done using the two-node repetitive microcuttings technique[7,8] on the shoot elongation-cum-rooting medium containing half-strength MS[9] medium supplemented with 2.0 mg l^{-1} IBA and 200 mg l^{-1} activated charcoal. Sucrose was added at 30 g l^{-1} along with medium solidification using 8 l^{-1} agar-agar (Qualigens, Mumbai).

The chemical mutagens, i.e. EMS and EB were added using micro-filter (0.22 µm; Minisart, Sartorius, USA) in sterilized shoot elongation-cum-rooting medium at 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1% levels. The *in vitro*-raised plantlets were excised aseptically into the two-node microcuttings, subcultured onto the mutagen-supplemented medium and marked as vegetative mutant one (vM_1) population. All the cultures were maintained in culture room (26 ± 2°C) under the 16/8 h light/dark regime (45 µmol $m^{-2} s^{-1}$). After *in vitro* proliferation, the surviving cultures were subcultured three weeks on the fresh shoot elongation-cum-rooting medium without any chemical mutagen. In this way, the putative mutants were multiplied up to the vM_3 population. All the putative mutants and control plantlets were then hardened and transferred to the field for further screening and study of the growth characteristics.

Total nucleic acid was extracted using 500 mg young leaves of control and putative mutant plants as described earlier[10]. DNA concentration for each sample was checked using VersaFluor™ Flurometer (BIO-RAD, USA). Isolated DNA was diluted in TE buffer having concentration up to 25 ng and kept under –20°C until analysis.

Amplification was performed according to the protocol described by Williams *et al.*[11], with few modifications. Thirty random decamer primers were employed for PCR amplification. Sequences of the primers that detected polymorphism are given in Table 1. The reaction volume was 25 µl, containing 2.5 µl of reaction buffer with 15 mM $MgCl_2$, 250 µM dNTPS, 1 unit of *Taq* DNA polymerase and 30 ng of each primer. This randomly amplified polymorphic DNA mixture contained approximately 25 ng template DNA. The total volume of the reaction mixture was adjusted using sterile deionized water. Amplification was performed in Biometra PCR thermocycler. Thermal cycles were programmed for initial denaturation at 94°C for 4 min. In each cycle, denaturation for 1 min at 94°C, annealing for 2 min at 32°C and extension for 2 min at 72°C was performed with the final extension after 40 cycles for 7 min. Following amplification, the samples were stored at 4°C prior to electrophoresis. Amplification was repeated thrice for each primer. The PCR product was separated on 1.2% agarose gel and photographed using Alphaimager™ documentation and analysis system with AlphaEase™ software. Per-

Table 1. Nucleotide sequences of primers that detected polymorphism

Primer code	Sequence
P1	5′-CAGGCCCTTC-3′
P2	5′-GTGCCTAACC-3′
P3	5′-GAAACGGGTG-3′
P4	5′-TGGTGACTGA-3′
P5	5′-TGCCGAGCTG-3′
P6	5 -GGTGCACGTT-3′
P7	5′-GGGTAAAGCC-3′

Table 2. Percentage of mutants identified after molecular marker-assisted selection of *in vitro* chemical-induced mutants in Pusa Seedless grape cultivar

	Mutant		
Mutagen	Putative	Solid	Percentage
Ethyl methane sulphonate	28	8	28.5
Ethidium bromide	34	3	8.8

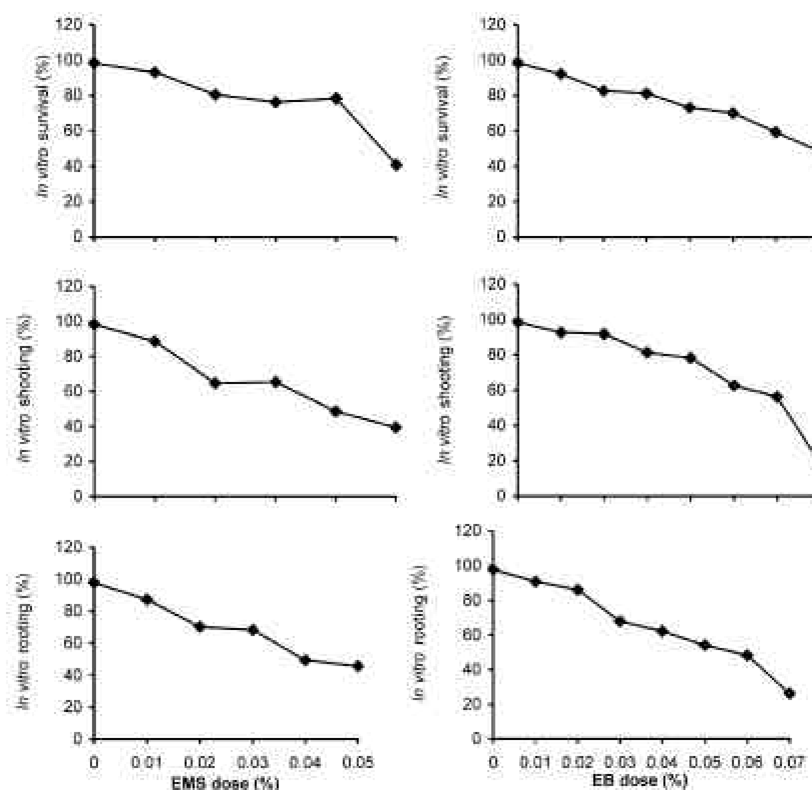

Figure 1. Effect of chemical-induced *in vitro* mutagenesis in tissue cultured Pusa Seedless grapevine.

Table 3. Amplification product and primers used for characterization of ethyl methane sulphonate and ethidium bromide induced *in vitro*-developed mutants of Pusa Seedless grape

	RAPD marker*														
Mutant code	$P1_{2240}$	$P1_{700}$	$P1_{470}$	$P2_{980}$	$P3_{900}$	$P4_{1420}$	$P4_{1000}$	$P4_{730}$	$P4_{550}$	$P5_{1600}$	$P5_{1400}$	$P5_{760}$	$P6_{2150}$	$P6_{1000}$	$P7_{750}$
Control	+	–	–	–	–	+	–	–	+	–	+	+	+	+	–
PS-EMS-1	+	–	–	–	–	+	–	–	–	+	–	+	+	+	–
PS-EMS-2	+	+	–	–	–	+	–	–	–	–	+	+	+	+	–
PS-EMS-3	+	–	–	–	–	+	–	+	–	–	+	+	+	+	–
PS-EMS-4	+	–	–	–	–	+	–	–	–	–	+	+	+	+	–
PS-EMS-5	+	–	+	–	–	+	–	–	–	–	+	+	+	–	–
PS-EMS-6	+	–	–	–	+	+	–	–	–	–	+	+	+	+	–
PS-EMS-7	+	–	–	–	–	–	+	–	–	–	+	+	+	+	–
PS-EMS-8	+	–	–	–	–	+	–	–	–	–	+	–	+	+	–
PS-EB-1	+	–	–	–	–	+	–	–	+	–	+	+	+	+	–
PS-EB-2	+	–	–	+	–	+	–	–	+	–	+	+	+	+	+
PS-EB-3	+	–	–	–	–	+	–	–	+	–	+	+	+	+	–
PS-EB-4	+	–	–	–	–	+	–	–	+	–	+	+	+	+	–
PS-EB-5	+	–	–	+	–	+	–	–	+	–	+	+	+	+	–
PS-EB-6	+	–	–	–	–	+	–	–	+	–	+	+	+	+	+
PS-EB-7	+	–	–	–	–	+	–	–	+	–	+	+	+	+	–
PS-EB-8	+	–	–	–	–	+	–	–	+	–	+	+	+	+	–

*Number and letter preceding the subscript (size of RAPD marker in bp) refer to the primer used to generate the marker.
+, Presence of band; –, Absence of band.

centage of mutants identified after marker-assisted selection is given in Table 2.

Bands on RAPD gels were scored as either present (1) or absent (0) for the genotypes studied. Common band analysis was conducted using the computer program SPSS to determine the genetic distance values between them. The figures for genetic distances were then used as input data for cluster analysis to generate a dendrogram.

In vitro micropropagation studies were conducted as reported earlier by Singh and coworkers[7,8]. The cultures were established from field-grown vines using nodal segment on MS medium supplemented with 4.0 mg l^{-1} 6-benzylaminopurine + 0.2 mg l^{-1} naphthaleneacetic acid. The axillary shoots were excised and subcultured onto the shoot elongation-cum-rooting medium containing half-strength MS basal medium supplemented with 2.0 mg l^{-1} IBA and 200 mg l^{-1} activated charcoal. Three-week period was found optimum for shifting the rooted plants for *in vitro* hardening. However, prolonging the culture period to six weeks produced a long curling vine, which could then be excised into two-node microcuttings for further multiplication through regular subcultures. The two-node microcuttings used for multiplication of cultures showed a distinct polarity, since the upper node gave rise to shoot while rooting took place from the lower node, thereby avoiding competition in the above two processes. Furthermore, intervening callus phase was not observed during any of the multiplication stages.

Variable responses were observed when EMS and EB were added to the medium. On the basis of *in vitro* survival of microcuttings and their subsequent *in vitro* growth response, the LD_{50} value for EMS treatments was recorded as 0.04% and for EB it was 0.06% (Figure l). As the concentration of mutagenic agent in the medium increased, reduction in sprouting of shoot and root was observed. Complete mortality of *in vitro* microcuttings was noted in and above the 0.06% EMS and 0.08% EB supplemented medium. Various types of abnormality symptoms like curling/scorching of leaves, albino leaves, stunted root/shoot growth, chimera formation, etc. were observed (data not shown). After hardening of the vM_3 population, the *in vitro* putative mutant survival was higher in EB (34) than in EMS (28). However, after screening by RAPD markers three solid mutants were obtained in EB treatment and eight in EMS treatment. Table 2 shows that the percentage of solid mutant (28.5) was higher with EMS than with EB (8.8). Kuksova *et al.*[12] reported *in vitro* mutagenesis in grape using colchicine and gamma irradiation. They observed that the effect of colchicine treatment was less than gamma irradiation with respect to polyploidy plant formation. Till date, there is no report available on the use of EMS and EB for *in vitro* induction of mutation in grape. However, several reports on different crops have shown the efficacy of EMS and EB for *in vitro* mutagenesis in *Capsicum annum* L.[13], *Solanum surattense* Brum.f.[14] and *Brassica juncea* L.[15].

Although breeding of improved varieties through induced mutations is an established technology, many drawbacks limit the use of physical/chemical-induced mutagenesis. Furthermore, selection of a desired mutant is a tedious process[16]. Using morphological characters, early selection of solid mutants is difficult from a large putative mutant population. Isozyme markers are known to suffer from several ontogenic variations, which are limited in number. Furthermore, DNA sequence changes that do not alter the amino acid sequence of polypeptides cannot be detected by isozyme analysis. Restriction fragment length polymorphism (RFLP) involves complicated procedures and the search for probe enzyme combinations, which detect fragments altered by mutation, cannot be easily achieved. Therefore, it is not surprising that neither isozymes nor RFLP analysis is useful to generate sufficient polymorphism. In RAPD assay, the nature of the fragments that are amplified is highly dependent on the primer sequence and on the genomic

Table 4. Proximity matrix based on RAPD of *in vitro* ethyl methane sulphonate-induced mutants in Pusa Seedless grape

Mutant code	1	2	3	4	5	6	7	8	9
Control	1.00								
PS-EMS-1	0.68	1.00							
PS-EMS-2	0.79	0.68	1.00						
PS-EMS-3	0.79	0.68	0.79	1.00					
PS-EMS-4	0.90	0.77	0.90	0.90	1.00				
PS-EMS-5	0.68	0.55	0.68	0.68	0.77	1.00			
PS-EMS-6	0.79	0.68	0.79	0.79	0.90	0.68	1.00		
PS-EMS-7	0.68	0.55	0.68	0.68	0.77	0.55	0.68	1.00	
PS-EMS-8	0.79	0.65	0.79	0.79	0.88	0.65	0.79	0.65	1.00

Table 5. Proximity matrix based on RAPD of *in vitro* ethidium bromide-induced mutants in Pusa Seedless grape

Mutant code	1	2	3	4	5	6	7	8	9
Control	1.00								
PS-EB-1	1.00	1.00							
PS-EB-2	0.82	0.82	1.00						
PS-EB-3	1.00	1.00	0.82	1.00					
PS-EB-4	1.00	1.00	0.82	1.00	1.00				
PS-EB-5	0.90	0.90	0.91	0.90	0.90	1.00			
PS-EB-6	0.90	0.90	0.91	0.90	0.90	0.81	1.00		
PS-EB-7	1.00	1.00	0.82	1.00	1.00	0.90	0.90	1.00	
PS-EB-8	1.00	1.00	0.82	1.00	1.00	0.90	0.90	1.00	1.00

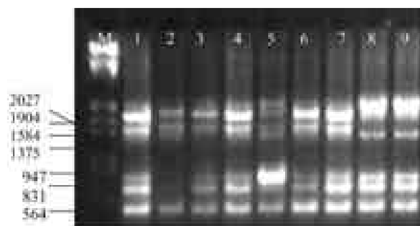

Figure 2. RAPD pattern obtained with P4 primer in EMS-induced mutants of Pusa Seedless grape. M, DNA size marker (*Eco*RI/*Hin*dIII digested-*1*DNA in bp); lane 1, Mother plant and lanes 2–9, Solid mutants.

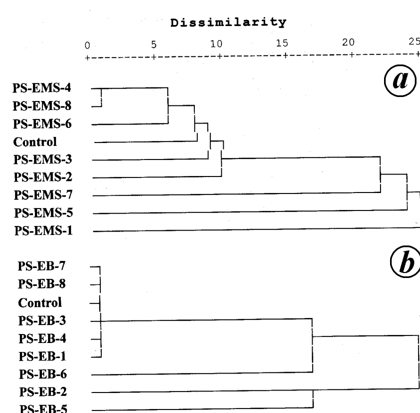

Figure 3. Dendrogram illustrating clustering of amplification pattern of *in vitro* (***a***) ethyl methane sulphonate and (***b***) ethidium bromide-induced mutants of Pusa Seedless grape.

DNA sequence being assayed. Primers differing by a single nucleotide generate quite different profiles. Thus, this technique may detect single-base changes in genomic DNA if sufficient primers are assayed. In the present study, the polymorphic RAPD primers were effectively used for selection of solid mutants in grape cv. PS. Marker-assisted selection provides a potential for increasing selection efficiency by allowing earlier selection and also reducing the plant population size. Furthermore, knowledge of trait-specific molecular marker technology will improve the precision in selection of a desired mutant. The effect of EMS and EB mutagens for *in vitro* mutagenesis of grape plantlets was prominent when tested with RAPD analysis. Seven primers (Table 1) out of thirty were able to identify solid mutants. These selected RAPD primers generated fifteen RAPD markers (Table 3). The size of the amplification product ranged from 400 to 2500 bp (Figure 2). Putative mutants which showed similar banding pattern with mother plants were discarded from the population.

The proximity matrix showed that PS-EMS-l, PS-EMS-5 and PS-EMS-7 mutants were more distinct from the control and other mutants; whereas PS-EMS-2, PS-EMS-3, PS-EMS-4, PS-EMS-6 and PS-EMS-8 were quite close to each other and with the control (Table 4; Figure 3). EB was found less effective when compared to EMS. Furthermore, only three solid mutants, i.e. PS-EB-2, PS-EB-5 and PS-EB-6 were found due to EB treatments (Table 5). However, others showed similarity with the control. Several workers have showed that RAPD markers, which can quickly detect a large number of genetic polymorphisms, have led to the creation of genetic maps in a number of woody fruit crops[17] and detection of mutations in soybean[18], Brazilian rice[19], citrus[20], pear[21] and cherry[22], including changes due to DNA damage[23]. The solid mutants identified based on RAPD analysis were transplanted in field for evaluation of horticultural traits. Breeding of improved fruit varieties through mutagen-induced mutations is a tried, tested and accepted technology. It has been mainly used to change one or two traits in an otherwise improved variety. However, most of the commercial mutants in horticultural crops have been limited to only ornamentals. Hence, there is need to adopt and incorporate induced mutations in combination with *in vitro* techniques and marker-assisted selection much more widely to breed new varieties, and their rapid release and spread to enhance horticultural production in India.

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