Genetics and Molecular Research

Mutagenic influences of colchicine on phenological and molecular diversity of *Calendula officinalis* L.

Y.I. El-Nashar[1,2] and M.H. Ammar[1,3]

[1]Plant Production Department, College of Food and Agriculture Sciences, King Saud University, King Saudi Arabia
[2]Ornamental Plants and Landscape Gardening Research Department, Horticultural Research Institute, Al-Montaza Garden, Giza, Egypt
[3]Rice Research and Training Center, Sakha, Kafr El-Sheikh, Egypt

Corresponding author: Y.I. El-Nashar
E-mail: yelnashar@ksu.edu.sa

Genet. Mol. Res. 15 (2): gmr.15027745
Received September 29, 2015
Accepted January 15, 2016
Published April 26, 2016
DOI http://dx.doi.org/10.4238/gmr.15027745

ABSTRACT. Six different colchicine concentrations: 0, 400, 800, 1200, 1600, and 2000 ppm, in combination with four soaking time treatments (1, 2, 3, and 4 h), were selected to assess the effects on germination, vegetative growth, and flower yield components in calendula plants. The molecular diversity among the treatments was assessed using ten SRAP marker combinations. Seed soaking in colchicine significantly enhanced both the fresh and the dry shoot and root masses, flowering date, number of flowers per plant, and flower diameter. At 1200-ppm colchicine combined with a 4-h soaking time, a superior effect on seed germination was observed, whereas 800 ppm for 4 h produced the highest number of flowers and the largest flower diameter. The earliest flowering time was found at 800 ppm combined with a short soaking time (1 h), while the 4-h soaking time with 800 ppm, is recommended for growing calendula outdoors, since it enhances flower development. At the molecular level, 752 fragments were successfully amplified

using the SRAP primers, with 280 genetic loci found throughout the calendula genome. The polymorphism percentage ranged from 79 to 100% and the polymorphic information content (PIC) values ranged between 0.85 and 0.97. The high number of detected loci and PIC values suggests a great power of SRAP markers in detecting mutant molecular diversity. Our results clearly show the existence of genetic variation among colchicine treated calendula plants and the clustering of the studied mutants was concordant with the colchicine concentration used.

Key words: Calendula; Colchicine; Mutation; Flowering yield; Molecular diversity; SRAP

INTRODUCTION

Mutation breeding is a recognized tool for enhancing the genetic variability in self-pollinated crops (Micke, 1988). The main aim of this breeding method is to enhance agronomic performance of locally adapted genotypes, in order to improve productivity and quality. Mutation induction results in a significant genetic variability platform and could lead to increased crop production (Novak and Brunner, 1992). Some mutations could induce novel parameters that did not exist previously, or that had been lost through long-term cultivation (Kharkwal and Shu, 2009).

Colchicine is a mutagen that prevents formation of microtubules and which is usually used for doubling the chromosome number. Thus, it is routinely utilized in polyploid plant formation. Colchicine effectively functions as a "mitotic poison," leading to noticeable mutagenic effects. Many reports highlight the mutagenic effects of colchicine on plant performance (Balkanjieva, 1980; Castro et al., 2003). Colchicine has been used to induce useful mutations in several economic ornamental plant species, such as *Datura*, *Portulaca*, *Petunia*, *Allium*, and *Cucurbita*. The resulting mutants generally produce larger inflorescences, fruits, and pollen grains, and shorter stems, (Pickens et al., 2006). Apart from the phenotypic traits, the mutagenic effects can be assessed more precisely using molecular markers. Molecular markers are considered essential tools in detecting genetic diversity among plant species (de Oliveira et al., 1996). Various molecular marker systems have been used, to detect molecular diversity. For instance, random amplified polymorphic DNA (RAPD) (Panwar et al., 2010), inter simple sequence repeat (Ansari et al., 2012), simple sequence repeat (SSR) (Panwar et al., 2010), and amplified fragment length polymorphism (AFLP) (Wang et al., 2005) markers. Shehata et al. (2009) used SSR markers to estimate the genetic distance in M_5 rice mutants. Sequence related amplified polymorphism (SRAP) is considered a simple and efficient tool with a higher throughput scale and higher reproducibility than RAPDs, and is easier to perform than AFLPs. The SRAP method can detect many co-dominant loci and can easily trap open reading frames (ORFs) (Li and Quiros, 2001). The main objective of the present study was to investigate the effects of six colchicine concentrations and four soaking durations on plant growth and flower yield components of calendula plants grown under open field conditions. In addition, we wanted to assess the genetic diversity resulting from various colchicine concentrations, using SRAP markers. This study highlights a significant improvement in flower yield quantity and quality, as affected by colchicine treatments. SRAP markers were employed, to confirm the existence of genetic variation at the molecular level, as a result of mutagen concentration and/or soaking time.

MATERIAL AND METHODS

Experimental details and treatments

Calendula seeds (Calypso orange cultivar, GOLDSMITH Seeds Company, CA, USA) were treated with various colchicine treatments and grown under open field conditions at the Department of Plant Production, Food and Agriculture Sciences College, King Saud University, Riyadh, Saudi Arabia. The experiment was conducted during the 2012/2013 season for the M_1-generation and during the 2013/2014 season for the M_2-generation.

Six different colchicine concentrations (0, 400, 800, 1200, 1600, and 2000 ppm) in combination with four seed soaking durations (1, 2, 3, and 4 h) were used. Following the treatments, the seeds were washed in distilled water and sown in plastic trays on September 19, 2012, for the M_1-generation. The total number of calendula seeds used in the experiment was 720 (24 treatments x 30 seeds for each treatment). Healthy and size uniform (40 days old) calendula seedlings were transplanted into 15 cm diameter plastic pots (one seedling/pot) containing sandy and clay soil (1:1 v/v). Bulked seeds of all selected and selfed M_1-plants from each mutagenic treatment were collected. Seeds obtained from the M_1-generation were re-planted for the M_2-generation on September 11, 2013.

Experimental layout

A split-plot layout with a randomized complete block design was used, to set up the experiment. The six colchicine concentrations were allocated to the main plots, whereas the four soaking durations (1, 2, 3, and 4 h) were arranged in the sub-plots. Each plot included three pots in each replicate, with a total of 720 pots. During harvest time, plant height, root length, and flower width were measured. Leaf number per plant was counted and leaf area was measured using a leaf area meter (LI-COR, Lincoln, NE, USA). The number of branches and flowers per plant were also counted. Leaf chlorophyll content was estimated using a Chlorophyll Content Meter CCM-200 (OPTI-SCINECE, Tyngsboro, Massachusetts, USA), which measures chlorophyll absorbance. This apparatus calculates values from the ratio of optical absorbance at 660 and 940 nm (Richardson et al., 2002). Flowering date and the fresh and dry weights of flowers were determined. Fresh and dry shoot and root masses were also recorded. Data were collected in the M_1- and M_2-generations of the two successive experimental seasons.

Molecular analysis

Leaf samples of ten randomly selected plants from each concentration (0, 400, 800, 1200, 1600, and 2000 ppm) that had a 4 h soaking time were collected and pooled for molecular analysis. They were immediately dropped in liquid nitrogen and stored at -80°C until DNA isolation. The DNA isolation was carried out using a modified SDS protocol following Hoelzel (1998). Thirteen SRAP primer combinations were used, to estimate genetic diversity among the tested wheat genotypes. The SRAP primer combinations used are presented in Table 1. The SRAP-PCRs (polymerase chain reactions) were performed according to the protocol described by Alghamdi et al. (2014).

Table 1. List of SRAP primer combinations used in the current study.

Primer name	Forward 5'-3'	Primer name	Reverse 5'-3'
ME10	5-TGAGTCCAAACCGGAAG-3	EM4	5-GACTGCGTACGAATTACG
ME11	5-TGAGTCCAAACCGGTAA-3	EM5	5-GACTGCGTACGAATTACT
ME12	5-TGAGTCCAAACCGGTCC-3	EM6	5-GACTGCGTACGAATTAGC
ME16	5-GACTGCGTACGAATTCAC	EM7	5-GACTGCGTACGAATTATG

The PCR products were loaded on a 36-cm 16-capillary system of the 3130*xl* Genetic Analyzer **(**Applied Biosystems, Foster City, CA, USA) with the Big Dye Terminator v1.1 Cycle Sequencing Kit. The SRAP fragment analysis was performed using the Gene Mapper Analysis Software v. 3.7 Applied Biosystems (ABI). The threshold for allele calling was set at 200 relative florescence units, according to Wooten and Tolley-Jordan (2009). The fragment analysis was carried out for allele sizes in the range of 100-500 bp. The markers that showed single alleles across all studied samples were eliminated from the analysis. Data were analyzed using the Jaccard similarity coefficient (Jaccard, 1908). A dendrogram was constructed using the unweighted pair group method with arithmetic average [UPGMA] employing the SAHN (sequential, agglomerative, hierarchical, and nested clustering) method in the NTSYS-pc (v. 2.10) program (Rohlf, 2005).

Statistical analysis

The results were analyzed using Statistical Analysis System SAS v. 9.2, Institute, Cary, NC). Differences among means were tested using least significant difference (LSD) tests at the 0.05 level (Steel and Torrie, 1980). Correlation analyses were employed to examine relationships between the different concentrations and exposure times for the total chlorophyll content.

RESULTS

The analyses of variance (ANOVA) for the studied parameters are presented in Table 2. The results showed highly significant differences among mutagen concentrations (main plots), soaking time (sub-plots), as well as their interaction across all studied parameters, with the exception of root dry mass in the M_2-generation.

Germination percentage

Mean performance germination (%) and vegetative parameters of calendula as affected by colchicine concentrations and soaking time and their interactions during the M_1- and M_2-generations (2012/13-2013/14) are given in Table 3. The results indicate a germination improvement with elevated mutagenic concentrations up to 1200 ppm. At higher concentrations, the reverse was observed. The lowest germination percentages were detected at 1600 ppm with 4 h exposure time (38.32 and 39.52% for the M_1- and M_2-generations, respectively). The highest germination percentages were recorded at 1200 ppm (93.35 and 89.01%) with 4 h exposure time for M_1- and M_2-generations, respectively (Table 3).

Table 2. Analysis of variance for the studied calendula parameters as affected by various colchicine concentrations and soaking times during the M_1- and M_2-generations (2012/13 and 2013/14, respectively).

Source of variance	df	Mean squares									
		Seed germination		Plant height		No. of branches/plant		No. of leaves/plant		Leaf area/plant	
		M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
Block	2	25.19	231.33	0.34	10.53	3.01	0.68	23.72	186.06	752.92	5422.57
Conc.	5	1175.06**	999.07**	165.34**	11.99**	27.79**	8.76**	442.06**	194.02**	32,419.01**	15,458.65**
Error for main plot	10	3.69	30.75	7.30	3.81	4.99	1.73	42.19	115.61	1553.32	1918.92
Duration (Dur)	3	47.41**	24.63^{ns}	48.78**	8.83**	5.50**	4.65**	40.31^{ns}	4.54**	4232.44^{ns}	10,189.14**
Conc.* Dur.	15	474.54**	433.52**	22.96**	5.79**	3.03*	2.15*	57.20**	113.59*	5990.13**	3617.69*
Error for sub-main plot	36	5.79	28.67	6.54	1.22	1.11	0.98	17.0	61.75	1728.81	1696.52
		Shoot fresh mass/plant		Shoot dry mass/plant		Root length		Root fresh mass/plant		Root dry mass/plant	
		M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
Block	2	117.10	6.79	1.69	1.44	68.17	29.20	6.99	2.73	4.99	0.11
Conc.	5	378.55**	130.65**	6.17**	0.85**	117.11^{ns}	221.24**	8.90**	6.88**	133.78**	0.18^{ns}
Error for main plot	10	23.43	41.69	1.56	0.75	38.06	42.91	4.02	1.16	4.47	0.26
Duration (Dur)	3	30.17^{ns}	63.30**	0.47^{ns}	0.80*	245.32**	48.38^{ns}	3.8^{ns}	2.79^{ns}	118.98**	0.45^{ns}
Conc.* Dur.	15	66.04*	19.39*	3.59*	0.58*	166.19**	97.11*	6.67**	3.55*	116.82**	0.39^{ns}
Error for sub-main plot	36	18.95	10.81	0.58	0.26	60.30	24.14	2.27	2.02	4.41	0.24
		Flowering date		No. of flowers/plant		Flower diameter					
		M_1	M_2	M_1	M_2	M_1	M_2				
Block	2	854.91	87.88	6.76	0.43	0.06	0.31				
Conc.	5	299.10**	384.43**	49.38**	18.81**	1.34**	0.87*				
Error for main plot	10	19.95	36.78	7.58	5.26	0.47	0.50				
Duration (Dur)	3	85.09**	327.38**	13.98**	26.16**	0.11^{ns}	2.44**				
Conc.* Dur.	15	41.39**	140.31**	3.41*	9.52**	0.25*	1.27**				
Error for sub-main plot	36	4.27	41.96	2.02	1.46	0.13	0.28				

**Significant at 0.01 level of probability. *Significant at 0.05 level of probability. ^{ns}Not-significant.

Table 3. Germination (%) and vegetative parameters of calendula as affected by colchicine concentrations and soaking time during the M_1- and M_2-generations (2012/13 and 2013/14, respectively).

Colchicine conc. (ppm)	Time duration (h)	Seed germination (%)		Plant height (cm)		No. of branches/plant		No. of leaves/plant		Leaf area/ plant (cm^2)	
		M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
Control (water)	1	42.52 ± 1.59	43.81 ± 2.69	11.70 ± 0.67	13.57 ± 2.07	4.01 ± 1.00	4.33 ± 0.57	22.33 ± 4.69	23.02 ± 3.23	127.62 ± 4.31	156.31 ± 1.98
	2	40.64 ± 1.65	45.07 ± 2.69	11.84 ± 0.66	13.61 ± 2.05	3.87 ± 1.00	4.41 ± 0.58	22.28 ± 4.64	23.08 ± 3.29	126.51 ± 5.30	149.36 ± 2.31
	3	47.60 ± 1.64	46.47 ± 2.67	10.98 ± 0.66	13.58 ± 2.05	3.98 ± 1.01	4.38 ± 0.58	22.30 ± 4.68	23.05 ± 3.27	127.37 ± 4.28	164.33 ± 2.15
	4	49.97 ± 1.66	50.40 ± 2.65	12.52 ± 0.65	13.42 ± 2.08	3.84 ± 1.02	4.26 ± 0.57	22.38 ± 4.67	22.87 ± 3.27	127.49 ± 5.31	158.29 ± 3.04
400	1	43.69 ± 2.24	61.02 ± 3.06	12.72 ± 1.80	14.13 ± 1.25	5.06 ± 2.00	5.29 ± 1.15	19.12 ± 2.64	31.63 ± 3.29	111.29 ± 6.09	166.66 ± 3.97
	2	48.34 ± 1.86	52.82 ± 3.39	15.06 ± 0.79	15.11 ± 0.76	6.11 ± 1.73	5.35 ± 0.58	26.01 ± 2.00	35.01 ± 4.54	160.05 ± 2.04	173.36 ± 2.36
	3	63.32 ± 1.73	67.72 ± 3.21	17.65 ± 3.12	15.23 ± 0.81	6.05 ± 3.00	5.00 ± 1.15	31.60 ± 5.08	45.56 ± 2.93	224.22 ± 7.89	204.00 ± 3.12
	4	41.67 ± 1.66	51.23 ± 4.13	26.43 ± 1.94	17.43 ± 0.59	7.10 ± 1.00	6.67 ± 1.00	35.66 ± 4.86	51.01 ± 2.51	260.06 ± 6.33	272.62 ± 4.25
800	1	54.37 ± 3.96	50.57 ± 2.53	21.07 ± 4.71	15.67 ± 0.67	5.67 ± 0.58	4.66 ± 0.57	32.29 ± 2.08	44.51 ± 5.12	211.92 ± 6.45	190.65 ± 2.43
	2	58.41 ± 1.81	59.65 ± 1.07	20.83 ± 0.75	15.02 ± 0.82	5.04 ± 1.73	4.32 ± 1.15	23.33 ± 4.13	37.33 ± 1.53	172.15 ± 5.65	136.57 ± 3.15
	3	40.0 0 ± 1.69	41.36 ± 3.42	17.73 ± 1.60	13.23 ± 0.42	4.65 ± 2.52	3.67 ± 0.60	22.36 ± 4.51	31.68 ± 6.55	155.17 ± 6.41	115.01 ± 4.16
	4	4 5.65 ± 3.23	46.71 ± 1.53	18.23 ± 2.10	13.93 ± 0.84	5.06 ± 1.73	5.31 ± 1.53	27.66 ± 3.04	39.00 ± 4.88	192.06 ± 6.63	149.05 ± 4.47
1200	1	51.15 ± 0.97	50.58 ± 0.97	19.87 ± 2.05	16.33 ± 1.76	5.33 ± 1.53	6.65 ± 1.73	23.28 ± 3.05	40.31 ± 6.77	241.42 ± 4.45	148.49 ± 3.17
	2	67.21 ± 1.01	63 15 ± 3.54	17.53 ± 0.86	15.66 ± 2.40	4.15 ± 1.00	6.02 ± 1.15	24.34 ± 3.16	47.63 ± 6.53	138.30 ± 4.24	256.63 ± 5.31
	3	61.49 ± 1.62	69.83 ± 1.93	15.47 ± 2.14	15.90 ± 2.01	3.66 ± 1.53	5.33 ± 0.61	17.59 ± 3.21	38.35 ± 4.93	194.23 ± 5.48	179.83 ± 3.31
	4	93.35 ± 0.56	89.01 ± 2.08	13.27 ± 1.60	15.43 ± 1.55	2.67 ± 1.52	6.30 ± 0.57	17.67 ± 3.79	50.33 ± 0.58	126.59 ± 3.25	234.11 ± 4.96
1600	1	53.36 ± 1.68	65.86 ± 4.24	20.13 ± 3.82	15.76 ± 1.47	4.02 ± 1.01	5.68 ± 2.08	22.01 ± 3.61	46.59 ± 6.85	182.53 ± 5.89	268.96 ± 5.26
	2	56.67 ± 1.65	60.59 ± 3.39	13.73 ± 2.78	15.70 ± 0.44	4.06 ± 1.00	4.32 ± 0.58	19.30 ± 4.13	42.00 ± 4.54	120.82 ± 3.10	264.55 ± 2.21
	3	61.64 ± 1.63	56.02 ± 2.48	13.67 ± 1.72	14.77 ± 1.99	4.03 ± 1.02	3.35 ± 1.15	18.64 ± 5.50	39.02 ± 6.00	73.27 ± 5.88	221.34 ± 5.16
	4	38.32 ± 1.75	39.58 ± 2.81	10.32 ± 1.21	13.40 ± 0.89	1.34 ± 0.05	2.65 ± 1.14	11.69 ± 0.58	35.30 ± 4.57	60.81 ± 2.95	208.57 ± 5.99
2000	1	70.78 ± 2.38	69.62 ± 3.48	12.03 ± 2.59	13.83 ± 1.76	3.24 ± 1.73	5.34 ± 0.49	13.66 ± 4.93	39.58 ± 6.89	61.98 ± 4.64	222.42 ± 4.86
	2	62.07 ± 1.14	60.03 ± 2.44	10.01 ± 1.71	13.43 ± 1.45	1.33 ± 0.06	3.67 ± 1.52	11.01 ± 4.35	39.03 ± 4.09	61.87 ± 4.49	172.02 ± 7.01
	3	44.96 ± 1.63	46.81 ± 3.62	10.70 ± 1.71	13.77 ± 0.98	1.21 ± 0.02	3.00 ± 1.00	11.29 ± 1.52	36.67 ± 5.69	58.22 ± 2.08	170.13 ± 2.30
	4	52.19 ± 0.94	52.79 ± 1.98	9.80 ± 0.79	9.30 ± 0.78	1.02 ± 0.58	2.36 ± 1.16	9.32 ± 1.53	21.66 ± 3.51	50.31 ± 4.19	97.89 ± 6.39
$LSD_{0.05}$ for Conc.*Dura.		4.45	6.07	4.45	2.23	2.41	1.83	7.57	15.22	59.83	66.17

Vegetative parameters

At lower colchicine concentrations, 400 and 800 ppm, plant height increased, whereas negative effects were observed at higher concentrations and longer soaking times. The shortest calendula plants were obtained at the 2000 ppm colchicine concentration with 4 h soaking duration, for both M_1- (9.80 cm) and M_2- (9.30 cm) generations (Table 3). The increases in colchicine concentration with time led to a decrease in number of branches per plant. The highest concentration of colchicine (2000 ppm), combined with a 4 h exposure time, resulted in the lowest number of branches (Table 3). The colchicine concentration at 400 ppm combined with an exposure time less than 4 h, significantly increased the observed number of branches per plant. Under higher colchicine concentrations (1200, 1600, and 2000 ppm), the number of branches decreased with increasing soaking time.

Significant differences were detected among calendula M_1- and M_2-generations in the number of leaves per plant. At lower colchicine concentrations (400 ppm) an increase in number of leaves per plant was observed with increasing soaking time. The highest leaf number (35.66 and 51.01) was detected at 400 ppm with a 4 h soaking time (Table 2). However, at the highest colchicine concentrations (1600 and 2000 ppm), a decrease in leaf number per plant was observed, regardless of exposure time.

The results shown in Table 3 indicate clear differences in leaf area under the studied treatments. The lower colchicine concentration 400 ppm, combined with a soaking time of 4 h, resulted in the largest leaf areas (260.06 and 272.62 cm^2) for the M_1- and M_2-generations, respectively. In contrast, the highest colchicine concentration (2000 ppm), combined with the longest soaking time (4 h), generated the smallest leaf area of calendula plants (Table 3).

Significant differences were detected between the M_1- and M_2-generations in fresh and dry shoot mass (Table 4). The lowest colchicine concentration (400 ppm) resulted in an increased fresh and dry shoot mass, with increasing soaking times. The highest concentration of colchicine (2000 ppm) resulted in a decrease in fresh and dry shoot mass, regardless of soaking duration. The root length, in both the M_1- and M_2-generations, decreased with increasing colchicine dose. The data presented in Table 4 suggests that root length increased with increasing soaking time at the lowest colchicine concentration (400 ppm), whereas the opposite was observed at the 2000 ppm dose.

The results obtained from the M_1- and M_2-generations indicate significant mutagen effects on fresh root mass. In the M_1-generation, the lowest colchicine concentration (400 ppm) combined with prolonged soaking time resulted in an increased fresh root mass, particularly at the 3 and 4 h soaking durations. Positive effects on fresh root mass at that same concentration were also observed in the M_2-generation. On the other hand, prolonged soaking times at the highest colchicine concentration (2000 ppm) had negative effects in both the M_1- and M_2-generations for this trait (Table 4).

Significant differences in calendula dry root mass were detected in the M_1-generation. The lower colchicine concentration (400 ppm) resulted in an increase in dry root mass with increasing soaking time. On the other hand, at the highest colchicine concentration (2000 ppm), dry root mass decreased with increasing soaking duration. No significant differences were detected among the M_2-generation in calendula dry root mass (Table 4).

The chlorophyll content is essential in plant life, due its role in the photosynthesis. There were no significant differences in total chlorophyll content in plants treated with different colchicine concentrations or at varying times in neither generation (Figure 1).

Table 4. Shoot and root studied parameters of calendula as affected by colchicine concentrations and soaking time during the M_1- and M_2-generations (2012/13 and 2013/14, respectively).

Colchicine conc. (ppm)	Time duration (h)	Shoot fresh mass/ plant (g)		Shoot dry mass/ plant (g)		Root length (cm)		Root fresh mass/ plant (g)		Root dry mass/ plant (g)	
		M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
Control (water)	1	16.62 ± 4.54	15.80 ± 2.84	2.86 ± 1.05	2.19 ± 0.82	30.47 ± 3.75	25.10 ± 3.61	4.82 ± 1.97	2.90 ± 0.19	1.22 ± 0.53	1.10 ± 0.07
	2	16.43 ± 4.55	15.83 ± 2.82	2.79 ± 1.03	2.29 ± 0.82	30.39 ± 3.76	24.93 ± 3.59	4.78 ± 1.96	2.43 ± 0.15	1.19 ± 0.54	1.15 ± 0.09
	3	16.59 ± 4.50	15.74 ± 2.79	2.81 ± 1.06	2.26 ± 0.79	30.68 ± 3.78	25.16 ± 3.55	4.81 ± 1.96	2.83 ± 0.12	1.20 ± 0.52	1.19 ± 0.11
	4	16.51 ± 4.55	15.85 ± 2.80	2.84 ± 1.05	2.20 ± 0.83	30.53 ± 3.75	25.07 ± 3.60	4.85 ± 1.95	2.98 ± 0.15	1.24 ± 0.53	1.21 ± 0.19
400	1	20.78 ± 5.19	16.10 ± 2.08	3.51 ± 1.14	2.76 ± 0.23	32.26 ± 2.32	15.11 ± 0.56	6.27 ± 0.66	3.02 ± 0.56	1.58 ± 2.03	0.84 ± 0.39
	2	22.24 ± 2.95	16.83 ± 1.99	3.69 ± 0.59	2.98 ± 1.15	36.83 ± 4.17	18.53 ± 4.85	5.17 ± 3.19	3.10 ± 1.21	1.78 ± 0.63	1.41 ± 0.26
	3	26.05 ± 6.26	18.46 ± 2.12	3.83 ± 1.71	3.30 ± 0.76	41.89 ± 5.75	18.80 ± 4.06	7.48 ± 2.10	3.11 ± 0.91	2.08 ± 0.46	1.14 ± 0.50
	4	26.56 ± 1.79	22.11 ± 2.81	4.20 ± 0.20	3.39 ± 0.15	25.57 ± 3.10	23.00 ± 4.58	6.79 ± 1.95	3.31 ± 1.21	2.13 ± 0.37	1.37 ± 1.04
800	1	21.76 ± 3.08	11.50 ± 2.03	3.49 ± 0.12	2.17 ± 0.72	25.94 ± 6.23	19.81 ± 4.10	4.39 ± 0.33	3.70 ± 1.91	1.48 ± 0.80	1.45 ± 0.92
	2	18.90 ± 2.02	13.77 ± 1.08	2.80 ± 0.44	2.43 ± 0.27	36.43 ± 6.79	20.21 ± 5.36	3.41 ± 0.41	4.80 ± 1.97	0.95 ± 0.13	1.61 ± 0.45
	3	19.28 ± 3.46	10.63 ± 2.08	2.65 ± 0.54	2.03 ± 0.11	23.93 ± 3.86	23.20 ± 4.22	3.58 ± 0.31	2.63 ± 0.78	0.81 ± 0.06	1.02 ± 0.28
	4	22.87 ± 5.54	13.87 ± 1.07	3.63 ± 1.09	3.08 ± 0.94	43.57 ± 6.23	20.13 ± 3.16	4.55 ± 0.78	6.97 ± 2.74	1.44 ± 0.32	1.55 ± 0.46
1200	1	24.11 ± 4.04	13.52 ± 2.58	3.27 ± 0.43	2.56 ± 0.56	28.97 ± 4.05	26.97 ± 4.15	4.50 ± 1.01	3.87 ± 1.29	1.09 ± 0.27	1.20 ± 0.63
	2	21.10 ± 2.86	16.67 ± 2.05	3.64 ± 1.21	3.01 ± 0.62	35.17 ± 4.06	32.37 ± 2.18	6.92 ± 2.68	6.32 ± 2.77	1.99 ± 1.07	1.82 ± 0.79
	3	18.82 ± 3.34	15.97 ± 1.83	2.63 ± 0.52	2.15 ± 0.19	31.74 ± 4.18	30.53 ± 5.42	4.53 ± 1.11	4.23 ± 2.19	1.10 ± 0.36	1.38 ± 0.67
	4	16.41 ± 3.22	17.51 ± 1.71	3.07 ± 0.81	2.18 ± 0.14	28.02 ± 3.87	27.97 ± 0.41	5.89 ± 1.60	3.93 ± 1.04	1.89 ± 0.53	1.24 ± 0.20
1600	1	19.20 ± 3.52	18.05 ± 1.13	2.45 ± 0.57	2.44 ± 0.80	26.70 ± 4.21	32.26 ± 6.84	3.12 ± 1.03	3.90 ± 1.21	0.82 ± 0.24	1.22 ± 0.42
	2	11.57 ± 1.81	17.30 ± 2.85	2.30 ± 0.53	2.16 ± 0.68	29.40 ± 4.75	34.20 ± 2.17	4.36 ± 1.14	3.73 ± 0.85	1.13 ± 0.48	1.48 ± 0.29
	3	15.22 ± 5.78	15.71 ± 1.79	2.50 ± 1.15	2.38 ± 0.94	21.41 ± 3.32	29.26 ± 2.02	4.23 ± 1.90	4.97 ± 1.70	1.23 ± 0.49	1.97 ± 0.68
	4	8.36 ± 2.50	11.47 ± 1.72	1.31 ± 0.41	2.18 ± 0.08	29.13 ± 4.82	20.77 ± 3.47	2.53 ± 0.76	2.23 ± 0.60	0.64 ± 0.16	0.88 ± 0.04
2000	1	11.83 ± 4.98	14.93 ± 2.76	2.71 ± 1.41	2.15 ± 0.61	25.53 ± 4.15	26.73 ± 2.21	5.83 ± 2.09	3.73 ± 0.62	1.74 ± 0.94	0.84 ± 0.20
	2	7.78 ± 4.70	11.10 ± 0.97	1.66 ± 0.75	1.89 ± 0.53	24.53 ± 5.52	26.57 ± 1.13	3.29 ± 1.38	3.57 ± 0.78	1.01 ± 0.44	1.64 ± 0.11
	3	7.15 ± 2.15	8.83 ± 0.65	1.55 ± 0.18	1.76 ± 0.45	23.21 ± 6.81	25.16 ± 5.75	2.82 ± 1.03	3.13 ± 1.02	0.83 ± 0.14	1.48 ± 0.44
	4	6.57 ± 4.11	8.07 ± 1.04	1.53 ± 1.38	1.26 ± 0.19	23.77 ± 6.45	16.50 ± 5.49	1.99 ± 2.62	2.00 ± 1.54	0.78 ± 0.92	1.19 ± 0.53
$LSD_{0.05}$ for Conc.*Dura.		7.90	6.32	1.46	1.01	9.14	8.91	2.81	2.41	0.86	ns

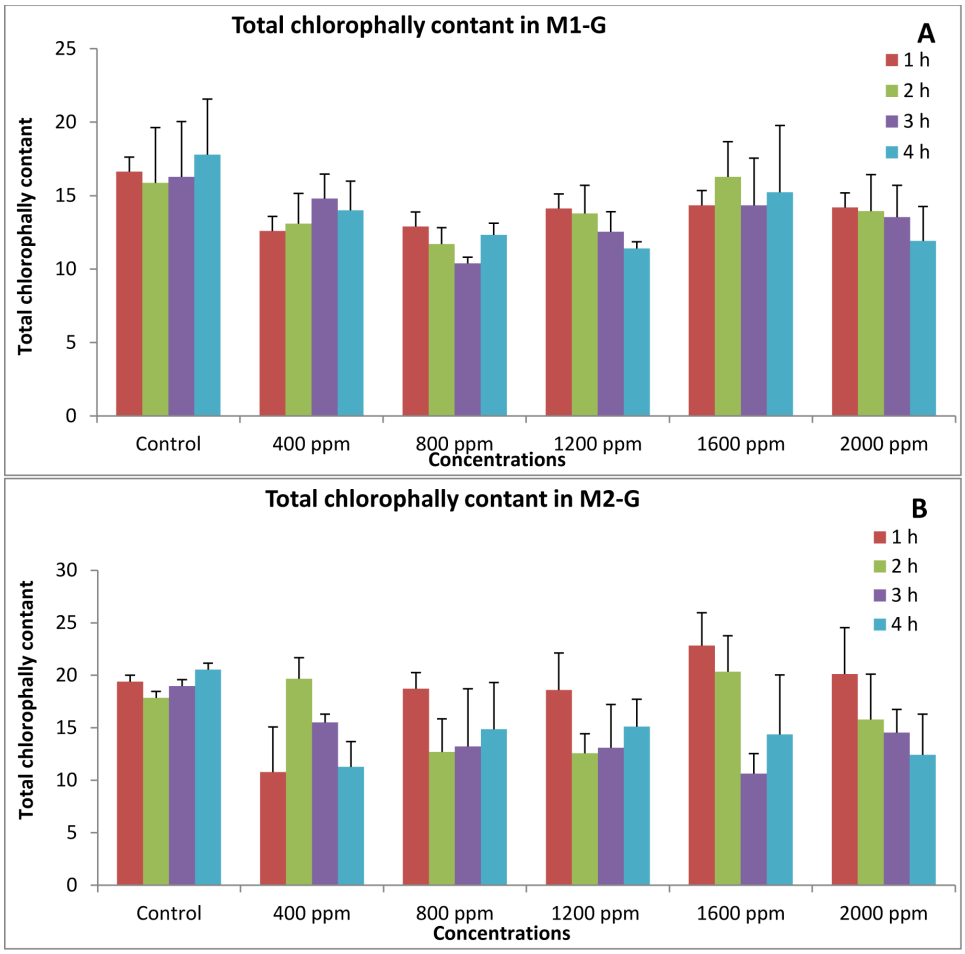

Figure 1. Effects of the interaction between colchicine concentration and soaking duration on total chlorophyll content in the calendula M_1- (**A**) and M_2-generation (**B**).

Flower yield components

Days to flowering in both generations varied with different mutagenic concentrations (Table 5). The trait values ranged from 102.33 to 123.22 days in the M_1-generation and from 90.29 to 119.32 days in the M_2-generation. With increasing mutagen concentration, the number of flowers decreased. In both the M_1- and M_2-generations, the number of flowers was lowest at 2000 ppm with 4 h soaking time, whereas the highest number of flowers were observed at concentrations below 800 ppm combined with 4 h soaking time (8.66 and 11.32 for the M_1- and M_2-generations, respectively). The mutagen is known to alter flower diameter. In the M_1- and M_2-generations, the diameter range exceeded the control treatment in most of the treatments, except for at 400 ppm combined with a 4-h soaking duration. The flower diameter ranged from 3.60 to 7.03 cm (Table 5).

Table 5. Studied calendula flower parameters as affected by colchicine concentrations and soaking time during the M_1- and M_2-generations (2012/13 and 2013/14, respectively).

Colchicine conc. (ppm)	Time duration (h)	Flowering date (days)		No. of flowers/plant		Flower diameter (cm)	
		M_1	M_2	M_1	M_2	M_1	M_2
Control (water)	1	110.17 ± 3.61	107.02 ± 3.29	4.67 ± 2.31	4.03 ± 1.01	5.63 ± 0.68	5.06 ± 0.36
	2	109.88 ± 3.64	105.81 ± 3.26	4.58 ± 2.30	4.00 ± 1.02	5.58 ± 0.67	5.01 ± 0.38
	3	109.98 ± 3.61	106.32 ± 3.24	4.62 ± 2.34	3.96 ± 1.01	5.56 ± 0.68	4.92 ± 0.36
	4	111.08 ± 3.62	106.97 ± 3.30	4.38 ± 2.31	3.89 ± 1.00	5.61 ± 0.67	4.98 ± 0.36
400	1	108.89 ± 4.93	96.00 ± 3.01	5.03 ± 1.73	3.67 ± 1.15	6.33 ± 0.34	5.52 ± 0.50
	2	110.10 ± 4.24	96.05 ± 3.00	8.65 ± 2.00	8.76 ± 1.51	6.81 ± 0.34	6.01 ± 0.75
	3	110.00 ± 4.44	95.33 ± 3.51	8.01 ± 3.06	3.05 ± 1.00	6.73 ± 0.60	5.46 ± 0.49
	4	107.33 ± 3.25	95.70 ± 2.51	6.38 ± 2.31	6.34 ± 1.15	6.40 ± 0.43	3.60 ± 0.66
800	1	102.33 ± 4.48	90.29 ± 3.79	6.00 ± 2.08	5.36 ± 1.53	5.90 ± 0.23	5.47 ± 0.35
	2	114.33 ± 3.25	91.64 ± 2.52	6.05 ± 3.00	5.01 ± 1.00	6.41 ± 0.43	5.32 ± 1.03
	3	115.44 ± 4.68	101.66 ± 3.03	5.03 ± 1.73	5.66 ± 0.58	6.69 ± 0.37	5.46 ± 0.40
	4	110.01 ± 4.58	111.02 ± 2.65	8.66 ± 3.00	11.32 ± 1.00	7.03 ± 0.15	6.63 ± 0.63
1200	1	115.33 ± 5.66	98.59 ± 5.28	4.68 ± 1.53	6.29 ± 2.52	6.25 ± 0.28	6.48 ± 0.15
	2	121.17 ± 5.43	99.28 ± 2.08	5.67 ± 1.15	8.02 ± 3.04	6.59 ± 0.21	5.70 ± 0.26
	3	118.78 ± 4.94	91.33 ± 3.86	4.04 ± 1.00	7.33 ± 1.53	6.37 ± 0.24	4.43 ± 0.76
	4	119.89 ± 5.60	94.64 ± 4.39	2.63 ± 1.15	6.00 ± 1.01	6.09 ± 0.31	5.30 ± 0.95
1600	1	106.28 ± 5.29	112.03 ± 4.56	4.66 ± 1.01	5.33 ± 1.52	6.53 ± 0.14	5.63 ± 0.25
	2	119.44 ± 5.44	93.30 ± 2.51	3.32 ± 1.16	6.28 ± 0.57	6.07 ± 0.37	6.20 ± 0.26
	3	117.89 ± 4.75	111.00 ± 5.52	4.00 ± 0.58	4.30 ± 1.53	5.98 ± 0.20	5.13 ± 0.90
	4	122.22 ± 6.83	118.12 ± 4.33	1.07 ± 0.05	4.41 ± 1.16	6.48 ± 1.04	4.57 ± 0.42
2000	1	121.89 ± 6.97	98.67 ± 3.06	3.01 ± 2.00	7.66 ± 1.01	6.54 ± 0.11	6.10 ± 0.62
	2	119.02 ± 5.53	91.05 ± 3.19	1.06 ± 0.04	5.27 ± 1.52	6.15 ± 0.04	5.87 ± 0.71
	3	118.78 ± 4.26	115.02 ± 3.58	1.04 ± 0.03	5.04 ± 1.53	6.50 ± 0.25	5.33 ± 0.15
	4	123.22 ± 5.53	119.32 ± 3.00	1.00 ± 0.03	1.65 ± 0.56	6.19 ± 0.27	5.11 ± 0.72
$LSD_{0.05}$ for Conc.*Dura.		11.32	11.04	2.89	2.54	0.65	0.98

Molecular analysis

Of 15 tested SRAP primer combinations, ten gave reproducible patterns and were subsequently used for the molecular analysis. A summary of the SRAP primer combinations is presented in Table 6. The ten primers successfully amplified a total of 752 fragments, across the six tested samples, with an average of 75.2 amplified fragments/primer combination. The number of genetic loci detected by the SRAP markers ranged from eight loci in primer ME12 x EM7, to 37 loci in primer ME11 x EM6, with a total of 280 loci across primers. Out of these, 262 loci were polymorphic with a polymorphism percentage that ranged from 79% in the ME10 x EM6 combination, to 100% in the case of the ME11 x EM5, ME12 x EM7, and ME16 x EM4 primer combinations. The polymorphic information content (PIC) values ranged from 0.85 for ME12 x EM7 to 0.97 for the ME11 x EM5 primer combination, with an overall PIC value of 0.95. Based on the SRAP results, the UPGMA method was used to construct a dendrogram that clustered the six used concentrations based on their Jaccard similarity coefficients (Jaccard, 1908). The Jaccard similarity coefficients are presented in Table 7 and the SRAP based dendrogram explaining the genetic relationships is presented in Figure 2. The results revealed that the clustering agreed well with the phenological responses detected among the colchicine treatments for most of the studied parameters. At a similarity level of 53%, the six concentrations were divided into two main groups, the first group, A, contained the 0, 400, 800, and 1200 ppm treatments that gave positive phenological responses in general, whereas group B contained the higher concentrations, 1600 and 2000 ppm, that resulted in negative impacts on the vegetative and flower parameters. The detected similarity level ranged from 53 to 78%. The most similar pair of mutagen treatments was 1600 and 2000 ppm, followed by the control and 400 ppm with a similarity of 78 and 70%, respectively.

Table 6. Summary of SRAP primer combinations found in calendula as affected by different concentrations of colchicine.

Primer combination	Total No. of fragments	Total No. of loci	Polymorphic loci	% Polymorphism	PIC value
ME10 x EM5	91	32	31	97	0.96
ME10 x EM6	95	28	22	79	0.95
ME10 x EM7	60	24	23	96	0.95
ME11 x EM5	110	36	36	100	0.97
ME11 x EM6	104	37	34	92	0.96
ME11 x EM7	106	31	27	87	0.96
ME12 x EM5	64	28	26	93	0.95
ME12 x EM6	51	33	32	97	0.95
ME12 x EM7	25	8	8	100	0.85
ME16 x EM4	46	23	23	100	0.95
Total	752	280	262		
Average	75.2	28	26.2	94	0.95

Table 7. Similarity coefficients among various concentrations of colchicine as revealed by SRAP data.

	Control	400 ppm	800 ppm	1200 ppm	1600 ppm	2000 ppm
Control	1.00					
400 ppm	0.70	1.00				
800 ppm	0.65	0.69	1.00			
1200 ppm	0.61	0.66	0.62	1.00		
1600 ppm	0.54	0.56	0.64	0.53	1.00	
2000 ppm	0.45	0.54	0.50	0.45	0.78	1.00

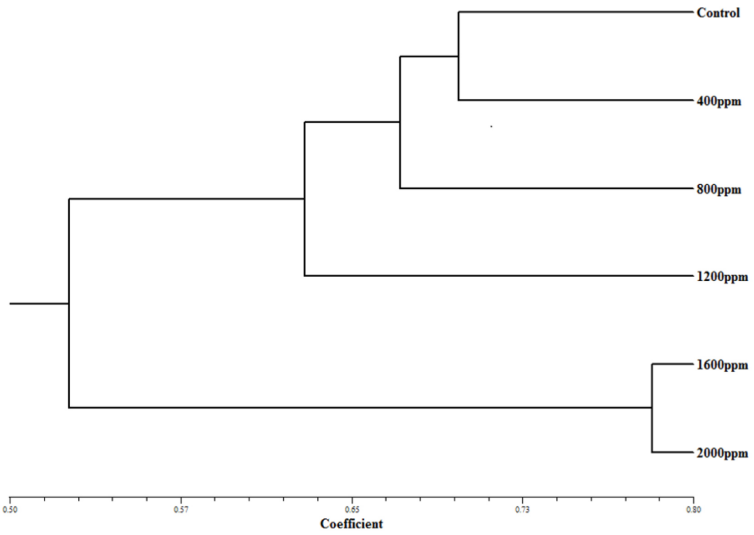

Figure 2. Dendrogram explaining the genetic relationships among mutants derived from various colchicine treatments as revealed by SRAP data using the UPGMA method.

DISCUSSION

Chemical mutagenesis plays a vital role in the improvement of ornamental plants. The use of chemical mutagens in crop improvement has been adopted in many crops. This has

helped to initiate large-scale mutation breeding experiments for various practical applications (Biswas and Datta, 1988; Chopra, 2005). Significant mutagenic effects on seed germination were found to be due to meristematic tissues in the germinating seeds. This could result in severe physiological disturbances and acute chromosomal aberrations (Singh et al., 1997). Ananthaswamy et al. (1971) reported that chromosomal aberrations induce catalase and lipase enzymatic activity. They also affect the hormonal activity that leads to reduced wheat seed germination as suggested by Lewis et al. 2002. Reduced calendula plant growth at higher colchicine doses may be attributed to (i) sudden changes in the metabolic status of the seeds at certain levels of the mutagen, (ii) inhibition of auxin synthesis or a gradual drop in the auxin levels, (iii) growth inhibitor destruction, (iv) an increase in growth promoters, and (v) a decline in the assimilation mechanism (Roychowdhury and Tah, 2011).

Artificial induction of mutations by colchicine leads to an alteration of the plant genome through an increased cellular division rate and an expansion of the meristematic regions, probably through alterations of the signaling pathway (Uno et al., 2001). In the present study, we found that colchicine induced taller mutants compared with the control treatment. This agrees with the results found by Nura et al. (2013); they reported significant increases in plant height of colchicine treated sesame plants. In contrast, the findings of Maluszynski et al. (2001) showed a noticeable decrease in the height of rice plant, as a result of induced mutation. The colchicine may have influenced cytokinin activity, which is essential for plant development (Deikman and Ulrich, 1995).

Colchicine treatments also resulted in an increase in the number of leaves per plant and in leaf area. This is in accordance with the findings of Nura et al. (2011), who found an increase in leaf number and area among jute plant mutants. An increase in leaf number provides an increase in the surface for gaseous exchange that has a considerable effect on the photosynthesis, as reported by Lockhart et al. (1996).

Lower colchicine concentration led to an increase in the number of flowers. However, elevated colchicine levels led to a reduction in the number of flowers in the M_1-generation, which agrees with the findings in *Vigna mungo* (Mahna et al., 1989). The emergence of late and early flowering calendula mutants in the M_1-generation found in our study was similar to that of Archana et al. (2004) and Roychowdhury and Tah (2011). The strong mutagenic effect on this trait is probably due to the tendency of the mutagen to alter gene(s) responsible for inducing flowering, by altering plant response to environmental signals (Lewis et al., 2002).

Among the various treatments tested, the most positive results in terms of plant growth and development promotion were obtained at 400 ppm colchicine concentration combined with a 3 h soaking time. This treatment resulted in the best values for most studied traits, compared to all other treatments.

A genetic alteration due to colchicine treatment was reported by Nassar et al. (2008) who studied anatomical alterations in the cassava plant. They found that the tetraploid type showed more prismatic and druse crystals in the cortical parenchyma, and its pericycle fibers had thicker walls. In addition, the secondary xylem of the tetraploid types was wider than the diploid ones, having thinner walls and less starch. Furthermore, Hu et al. (2015) studied the root tip chromosome karyotype of hyacinth cultivars. The basic chromosome number of the hyacinth cultivar was eight, and the number of chromosomes in the diploid, triploid, tetraploid, and aneuploid cultivars were 16, 23, 24, 31, and 32, respectively. In a similar study, Wu et al. (2015) studied the induction and identification of *Stylosanthes guianensis* tetraploids. Souza et al. (2015) observed the genetic alteration and meiotic behavior of *Brachiaria decumbens* hybrids.

Since the cytological, cellular, and anatomical behavior of colchicine in well established in many plant species, we used a SRAP marker system to study the genetic changes at the molecular level. The molecular data analysis, using SRAP markers, showed the existence of significant genetic diversity across calendula plants exposed to various concentrations of colchicine. The SRAP markers had a high resolution power and could discriminate the positive effects of colchicine at the lower concentrations, from the lethal concentrations that caused serious genetic errors leading to a reduction in growth and the flower parameters. The lower concentrations of colchicine showed positive effects on calendula phenology and flower yield. The high number of genetic loci detected and the high PIC values for the SRAP markers reveals the power of this technique in mutant discrimination and assessing molecular diversity. One advantage of SRAP markers is that they target ORFs (Li and Quiros, 2001). Thus, the markers obtained are in functional regions, which explains why they agreed with the phenological performance. Our results highlight the usefulness of molecular markers in detecting the mutagenic effects of colchicine on calendula and could significantly aid in the detection of useful mutants with improved flower yield and quality.

CONCLUSION

This study highlights a significant improvement of flower yield quantity and quality, as affected by colchicine treatments. SRAP markers were employed to confirm the existence of genetic variation at the molecular level as a result of the mutagen concentration and/or soaking time. It is clear that the colchicine mutagenic effects enhanced seed germination at a concentration of 1200 ppm combined with a 4 h soaking time. At 400 ppm colchicine concentration with a 4 h soaking time, enhanced plant performance was observed in both the studied generations. In contrast, at 2000 ppm colchicine concentration with a 4 h soaking time adverse effects on plant growth and flowering parameters were generally displayed in both generations. Flower yield and time could be significantly improved in calendula, through treatment with 800 ppm colchicine combined with prolonged seed soaking. The molecular analyses highlight the usefulness of SRAP molecular markers in detecting the mutagenic effects of colchicine as well as in detecting genetic diversity at the molecular level.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors wish to thank the Agricultural Research Center, the College of Food and Agricultural Sciences, and the Deanship of Scientific Research, King Saud University, Saudi Arabia, for supporting this work.

REFERENCES

Alghamdi SS, Khan AM, Ammar MH, El-Harty EH, et al. (2014). Phenological, nutritional and molecular diversity assessment among 35 introduced lentil (*Lens culinaris* Medik.) genotypes grown in Saudi Arabia. *Int. J. Mol. Sci.* 15: 277-295. http://dx.doi.org/10.3390/ijms15010277

Ananthaswamy HN, Vakil UK and Sreenivasan A (1971). Biochemical and physiological changes in gamma-irradiated wheat during germination. *Radiat. Bot.* 11: 1-12. http://dx.doi.org/10.1016/S0033-7560(71)91257-9

Ansari SA, Narayanan C, Wali SA, Kumar R, et al. (2012). ISSR markers for analysis of molecular diversity and genetic structure of Indian teak (*Tectona grandis* L.f.) populations. *Ann. For. Res* 55: 11-23.

Archana P, Taware SP and Raut VM (2004). Induced variation in quantitative traits due to physical (γ rays), chemical (EMS) and combined mutagen treatment in soybean. *Mutat. Res.* 334: 49-55.

Balkanjieva J (1980). Influence of genotype on mutagenic variability in (*H. vulgaris*) following colchicine treatment. *Barley Genet. Newslett*. (10/11): 7-10.

Biswas AK and Datta AK (1988). Induced mutation in two *Trigonella* species. *Bangladesh J. Bot.* 17: 211-214.

Castro CM, Oliveira AC and Calvaho FIF (2003). Changes in allele frequencies in colchicine treated ryegrass population assessed with APD marker. *Agrociencia* 9: 107-112.

Chopra VL (2005(. Mutagenesis: Investigating the process and processing the outcome for crop improvement. *Cur. Sci*. 89)2(: 353-359.

de Oliveira AC, Richter T and Bennetzen JL (1996). Regional and racial specificities in sorghum germplasm assessed with DNA markers. *Genome* 39: 579-587. http://dx.doi.org/10.1139/g96-073

Deikman J and Ulrich M (1995). A novel cytokinin-resistant mutant of *Arabidopsis* with abbreviated shoot development. *Planta* 195: 440-449. http://dx.doi.org/10.1007/BF00202603

Hoelzel AR (1998). Molecular genetic analysis of populations. 2nd edn. Oxford University Press, New York.

Hu FR, Liu HH, Wang F, Bao RL, et al. (2015). Root tip chromosome karyotype analysis of hyacinth cultivars. *Genet. Mol. Res.* 14: 10863-10876. http://dx.doi.org/10.4238/2015.September.9.24

Jaccard P (1908). Nouvelles recherches sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat.* 44: 223-270.

Kharkwal MC and Shu QY (2009). The role of induced mutations in world food security. In: Induced plant mutations in the genomics era (Shu QY, ed.). Food and Agriculture Organization of the United Nations, Rome, 33-38.

Lewis R, Gaffin D, Hoefnagels M and Parker B (2002). Life. McGraw Hill, New York.

Li GY and Quiros CF (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica. Theor. Appl. Genet.* 103: 455-461. http://dx.doi.org/10.1007/s001220100570

Lockhart PJ, Steel MA and Larkum AW (1996). Gene duplication and the evolution of photosynthetic reaction center proteins. *FEBS Lett.* 385: 193-196. http://dx.doi.org/10.1016/0014-5793(96)00383-3

Mahna SK, Garg R and Parvateesam M (1989). Mutagenic effects of sodium azide in black gram. *Curr. Sci.* 58: 582-584.

Maluszynski M, Szarejko I, Barriga P and Balcerzyk A (2001). Heterosis in crop mutant crosses and production of high yielding lines using doubled, haploid system. *Euphytica* 120: 387-398. http://dx.doi.org/10.1023/A:1017569617715

Micke A (1988). Genetic improvement of grain legumes using induced mutations: an overview. In: Improvement of grain legume production using induced mutations (Micke A, ed.). IAEA, Vienna, 1-51.

Nassar NM, Graciano-Ribeiro D, Fernandes SD and Araujo PC (2008). Anatomical alterations due to polyploidy in cassava, *Manihot esculenta* Crantz. *Genet. Mol. Res.* 7: 276-283. http://dx.doi.org/10.4238/vol7-2gmr399

Novak FJ and Brunner H (1992). Plant breeding: Induced mutation technology for crop improvement. *IAEA Bull.* 4: 25-32.

Nura S, Adamu AK, Mu' Azu S and Dangora DB (2011). Chemical mutagenesis for improved quality traits in jute (*Corchorus olitorius* L.). *Cont. J. Biol. Sci* 4: 22-27.

Nura S, Adamu AK, Mu' Azu S, Dangora DB, et al. (2013). Morphological characterization of colchicine-induced mutants in sesame (*Sesamum indicum* L.). *J. Biol. Sci.* 13: 277-282. http://dx.doi.org/10.3923/jbs.2013.277.282

Panwar P, Nath M, Yadav VK and Kumar A (2010). Comparative evaluation of genetic diversity using RAPD, SSR and cytochrome P450 gene based markers with respect to calcium content in finger millet (*Eleusine coracana* L. Gaertn.). *J. Genet.* 89: 121-133. http://dx.doi.org/10.1007/s12041-010-0052-8

Pickens KA, Cheng ZM and Kania SA (2006). Effect of colchicine and oryzalin on callus and adventitious shoot formation of *Euphorbia pulchurrima* "Winter Rose". *HortScience* 41: 1651-1655.

Richardson AD, Duigan SP and Berlyn GP (2002). An evaluation of noninvasive methods to estimate foliar chlorophyll content. *New Phytol.* 153: 185-194. http://dx.doi.org/10.1046/j.0028-646X.2001.00289.x

Rohlf FJ (2005). NTSYS-pc: Numerical taxonomy and multivariate analysis system, ver. 2.2. Exeter Publishing Ltd, New York.

Roychowdhury R and Tah J (2011). Chemical mutagenic action on seed germination and related agro-metrical traits in M1 *Dianthus* generation. *Curr. Bot* 2: 19-23.

Shehata SM, Ammar MH, Abdelkalik AF and Zayed BA (2009). Morphological, molecular and biochemical evaluation of Egyptian jasmine rice variety and its M_5 derived mutants. *Afr. J. Biotechnol.* 8: 6110-6116.

Singh G, Sareen P and Saharan R (1997). Mutation studies in mungbean. *J. Nucl. Agric. Biol* 26: 227-231.

Souza VF, Pagliarini MS, Valle CB, Bione NC, et al. (2015). Meiotic behavior of *Brachiaria decumbens* hybrids. *Genet. Mol. Res.* 14: 12855-12865. http://dx.doi.org/10.4238/2015.October.21.5

Steel RGD and Torrie JH (1980). Principles and procedures of statistics: A biometrical approach. 2nd ed. McGraw-Hill, New York.

Uno G, Storey R and Moore R (2001). Principles of botany. McGraw-Hill, New York.

Wang Y, Reighard GL, Layne DR, Abbot AG, et al. (2005). Inheritance of AFLP markers and their use for genetic diversity analysis in wild and domesticated pawpaw. *J. Am. Soc. Hortic. Sci.* 130: 561-568.

Wooten JA and Tolley-Jordan LR (2009). Validation of phylogenetic signals in amplified fragment length data: testing the utility and reliability in closely related taxa. *BMC Res. Notes* 2: 26. http://dx.doi.org/10.1186/1756-0500-2-26

Wu FH, Yu XD, Zhuang NS, Liu GD, et al. (2015). Induction and identification of *Stylosanthes guianensis* tetraploids. *Genet. Mol. Res.* 14: 12692-12698. http://dx.doi.org/10.4238/2015.October.19.13

