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MORPHOLOGICAL AND GENETIC DIVERSITY ANALYSIS IN CALENDULA (CALENDULA OFFICINALIS L.) INFLUENCED BY MUTAGENIC EFFECT OF COLCHICINE

Ahmed A.M. Yassein[1]*, *Abd Allah H.A. Hassen*[2], *Ebtsam M. Abdel-Alah*[2], *Shoukry M. Selim*[2]

Address(es): Ahmed A.M. Yassein,
[1] Fayoum University, Faculty of Agriculture, Genetics Department, 63514, Fayoum, Egypt.
[2] Fayoum University, Faculty of Agriculture, Horticulture Department, 63514, Fayoum, Egypt.

*Corresponding author: aay00@fayoum.edu.eg

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ABSTRACT

Calendula officinalis L. (pot marigold) is one of the main aromatic and medicinal plants with many uses in food and medicines. This study was carried out to demonstrate the efficiency of six colchicine concentrations (0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 per cent, w/v) for Calendula improvement and induction of genetic variation. Colchicine treatments had a positive effect on the number of branches/plant, number of inflorescences, fresh and dry weight of inflorescences, inflorescence diameter, total soluble carbohydrates and β-carotene except for plant height, while seed germination and plant height were reduced. Estimation of heritability, genetic advance, genetic variability and selection of superior genotypes will be an important object in crop breeding and genetic improvement programs, and selection of genotypes with higher desirable characters. Heritability was high and ranged from 48.64 to 90.81, respectively (inflorescence diameter and plant height, respectively). Molecular markers based on a RAPD-PCR study elucidate the classification of induced Calendula mutants into two clusters. The coefficient of genetic diversity was estimated at 30%. A combination of morphological and physiological responses with molecular data contained in the various colchicine treatments illustrated the utility of RAPD-PCR as a method for identifying useful mutants and could be used to detect the colchicine effect significantly. Findings recommend the 0.05 per cent colchicine for efficient breeding calendula mutation and genetic improvement.

Keywords: Calendula, Colchicine, RAPD-PCR, Genetic diversity, Heritability

INTRODUCTION

Calendula officinalis L.(Asteraceae) is known as pot marigold, and it is an annual herb with yellow to orange inflorescences, originally to the Mediterranean region (**Ramos *et al.*, 1988**). *C. officinalis* is used in drugs, medicines, decoration and food (**Ramos *et al.* 1988**; **Della-loggia *et al.*, 1994**). Mutation breeding is one of plant breeding methods successfully conducted to enhance genetic diversity by means of crop improvement (***Kharkwal and Shu 2009***). Colchicine is poisonous alkaloid and is known as chemical mutagenic. More than studies suggested the effect of colchicine on plant as a mutagen, which prevents microtubules form forming and contributes to mutagenic effects (**Pickens *et al.*, 2006**), and mutant plants typically grow shorter stems and high inflorescences (**Pickens et al., 2006**). The application of various colchicine concentrations had a significant effect on content of β-carotene in plants, e.g. in *Sesamum indicum* (**Nura *et al.*, 2013**) and *Echinacea purpurea* **(Abdoli *et al.*, 2013**). Broad sense heritability and genetic advance were important for plant breeding programme (**Herbert *et al.* 1955**).
In addition to the phenotypic traits, the mutagenic effects can be accurately assessed using DNA molecular marker techniques used to identify and evaluate genetic diversity between plant species, genotypes and cultivars **(El-Nashar and Ammar 2016**; **Soubra *et al.*, 2018**). The goal of the present study was to assess the efficiency of different concentrations of colchicine in induction of new agronomic, chemical and yield components of Calendula mutant plants. Estimate heritability, genetic advance, genotypic coefficient of variability (GCV) and the phenotypic coefficient of variability (PCV). Genetic diversity evaluation of induced mutants controlled RAPD outcomes.

MATERIAL AND METHODS

Experimental layout

Local of pot marigold (*C. officinalis)* accession seeds, kindly provided by Aromatic and Medicinal Plant farmer, Beni Suef Governate, Egypt. During three successive seasons (2015/2016, 2016/2017 and 2017/2018), field experiments were carried out at a private farm, Beni Suef Governorate. Seeds were sown with peat-moss in plastic trays and incubated in kindergartens. After 45 days the grown seedlings were individually transplanted in plastic bags filled with claying soil.

Effect of colchicine on seed germination

Calendula seeds were germinated in petri dishes with filter paper for 2 weeks. NaOCL 5% was used to surface sterilized with for 5 minutes prior to planting to avoid fungal invasion and then washed immediately with distilled water. For control and each treatment four replications of twenty five seeds were placed in 12-cm Petri dishes and then incubated with 25 °C for 2 weeks. Seeds were checked after 2 days for germination (radical length of greater than 5 mm was considered germination and were counted) and filter paper was remoistened as needed as described by **(Eberle *et al.* 2014**; **Kouchebagh *et al.* 2015**).

Colchicine treatments

The transplants were treated with colchicine concentrations at (0.0, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 %, w/v). Samples of M_1-plants from each treatment were obtained. Additionally, M_1-generation (first season) seeds were replanted for the next year's M_2-generation. Similarly, seeds obtained from the M_2-generatin (second season) have been replanted for generation M_3.

Vegetative growth characters

The three M generations were assessed for plant height (cm), number of branches/plant, number of inflorescences/plant, inflorescence diameter (cm), fresh and dry weight of inflorescences/plant (g).

Total soluble carbohydrates and β-carotene content

Both total soluble carbohydrates (%) **(Dubois *et al*., 1956**) and **β**-carotene mg^{-1} DW (**A.O.A.C. 1970**) were determined with the Last collection dried inflorescence.

DNA extraction and quality confirmation

At the 3-4 leaves stages leaf tissues were collected from the plants **(Hyam 1998**). Freeze-drying lyophilized the leaves, and then frozen in -80°C freezer until use. The tissue samples were ground by applying liquid nitrogen to a powder. Genomic DNA extraction was carried out using 0.1 g bulked tissue collected from individual plants equivalent weights of freeze-dried leaf samples. The bulked tissues were put into tubes of 2 ml eppendorf. Acetyltrimethyl ammonium bromide (CTAB) method (**Dellaporta *et al*. 1983**) has been used in isolation of DNA. In the TE buffer, the extracted DNA was re-suspended. Samples of 5 µl of isolated DNA used a 0.8 % agarose gel in TAE buffer, as defined by the sample, to assess the quality of DNA (**Sambrook *et al*., 2006**).

RAPD-PCR reaction

Reaction using extracted DNA, oligonucleotide primers (Table 1) was used for amplification to standardize the PCR conditions. In a DNA Thermo cycler the reactions were done. Each 25 *µl* reaction volume contained 12.5 *µl* Master Mix (one step PCR™), 2 *µl* of primer, 3 *µl* of genomic DNA (about 50 *ng*/µl) and 7.5 *µl* of sterile deionized water. In this study, RAPD primers that have been synthesized by Invitrogen, Biotechnology Co. Ltd. (USA) were used (Table 1). PCR reactions carried out with following conditions: initial denaturation step at 94°C for 5 min, then followed by 35 cycles of amplification at 94°C for 1 min, 36°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min were performed using thermal cycler 2720 (Applied Biosystems, USA). The PCR products were separated into a 1.2% agarose gel prepared with incorporated ethidium bromide by the 1X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3).

Table 1 List of primers were used and their nucleotide sequences.

No.	Primer code	Primers sequence (5'-3')
1	OPA-1	-5' CAGGCCCTTC 3'-
2	OPA-2	-5' TGCCGAGCTG 3'-
3	OPA-3	-5' AGTCAGCCAC 3'-
4	OPA-4	-5' AATCGGGCTG 3'-
5	OPA-5	-5' AGGGGTCTTG 3'-
6	OPA-6	-5' GCTCCCTGAC 3'-
7	OPA-7	-5' GAAACGGGTG 3'-
8	OPA-8	-5' GTGACGTAGG 3'-
9	OPA-9	-5' GGGTAACGCC 3'-
10	OPA-10	-5' GTGATCGCAG 3'-
11	OPC-1	-5' TTCGAGCCAG 3'-
12	OPC-2	-5' GTAAGGCGTC 3'-
13	OPC-3	-5' GGGGGTCTTT 3'-
14	OPC-6	-5' GAACGGACTC 3'-
15	OPC-7	-5' GTCCCGACGA 3'-
16	OPC-8	-5' TGGACCGCTG 3'-

RAPD-PCR Analysis

RAPD-PCR Analysis was used to evaluate the DNA samples' genetic diversity. Hence, visually scored as present (1) or absent (0) for the reproducible, polymorphic and monomorphic bands. Also faint reproducible RAPD bands were scored as in the program of (NTSYSpc, ver. 2.1). For each primer, the total number of bands per line was recorded and the percentages of polymorphic band were determined.

Statistical analysis

The experimental design used was randomized complete blocks with three replications and data analysis was performed by (SPSS 25 software). Duncan's multiple range tests were outline to assess the significance between treatments $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of colchicine on seed germination

With colchicine use, the percentage of seed germination has been reduced. The highest rates were 98% and 88% respectively, from control and low concentration of colchicine, (0.025%). Low germination rate was observed at high concentrations of 0.4 and 0.8 per cent colchicine (Table 2). These findings are in agreement with various reports in ornamental plants which confirm the reduction of germination with colchicine application (Ramos *et al*., 1988, **Dellaloggia *et al*., 1994**, **Abdoli *et al*., 2013**).

Table 2 Effect of colchicine on seed germination (%).

Colchicine	Seed germination (%)
Control	98.00^{A}
0.03%	88.00^{B}
0.05%	68.00^{C}
0.10%	36.00^{D}
0.20%	32.00^{D}
0.40%	12.00^{E}
0.80%	12.00^{E}

Legend: Means denoted by a different letter indicate significant difference between treatments p≤0.05

Vegetative growth characters

Plant height

Results in Table (3) indicate that colchicine treatment at all concentrations decreased plant height in a negative way. It is found, however, that plant height is associated with rising concentration of colchicine. For plants treated with 0.8 per cent colchicine, the shortest height was 39.22 cm.

These results may be due to negative reflection on plant height of the treatment desires with colchicine effect on cells division, and enlargement cells in plant organs. Similar results were reported by (**El-Nashar and Ammar 2016**; **Estaji *et al*., 2017**; **Kushwah *et al*., 2018**; **Samatadze *et al*., 2019**). They found the maximum height of plants was observed in the controls. Plant height was reduced linearly by enhancing concentration of colchicine.

Table 3 Effect of colchicine on plant height (cm), No. branches/plant, No. inflorescences/plant, inflorescence diameter (cm), inflorescence fresh and dry weight (g), total soluble carbohydrates (%), β-carotene (mg^{-1} DW) of *C. officinalis* plant (mean of 3 seasons).

Colchicine	plant height (cm)	No. branches/plant	No. Inflorescences/ plant	inflorescence diameter (cm)	fresh weight (g)	dry weight (g)	total soluble carbohydrate (mg^{-1} DW)	β-carotene (%)
Control	58.97^{A}	5.89	45.53^{B}	6.95^{B}	6.15	1.03^{D}	12.72^{B}	0.41^{B}
0.03%	55.28^{AB}	6.61	49.03^{B}	6.83^{B}	6.23	1.06^{CD}	12.99^{B}	0.45^{A}
0.05%	51.44^{BC}	8.88	58.19^{A}	7.78^{A}	7.89^{A}	1.32^{A}	14.12^{A}	0.45^{A}
0.10%	46.67^{CD}	8.03	55.33^{A}	6.70^{B}	7.22^{B}	1.22^{B}	13.62^{AB}	0.44^{AB}
0.20%	46.81^{CD}	7.78	53.78^{A}	6.77^{B}	6.43^{C}	1.15^{B}	13.36^{AB}	0.44^{AB}
0.40%	42.25^{D}	7.16	49.83^{B}	6.93^{B}	6.11	1.05^{CD}	12.99^{B}	0.43^{AB}
0.80%	39.22^{D}	6.56	47.42^{B}	6.56^{B}	5.47^{C}	1.02^{D}	12.89^{B}	0.42^{B}

Legend: Means denoted by a different letter indicate significant difference between treatments p≤0.05.

Number of branches/plant

Application of different concentrations of colchicine increased branches/plant numbers Table (3). Moderate concentration, as opposed to other treatments and control, 0.05 per cent had the most effect in terms of number of branches per plant. The maximum number of branches/plant recorded was 8.8 from plants received 0.05 per cent colchicine. The findings generally showed that the comparative analysis between treated and control plants showed significant differences in the number of branches. This result may be due to high vigour growth differentiated by Calendula plants especially branching ratio under moderate levels of colchicine but with increasing concentration of colchicine, the number of branches decreased. Such findings are compatible (**Amiri *et al*., 2010**) with *Datura stramonium*, (**Yassein and Aly 2014**) with *Brassica napus*, and (**El-Nashar and Ammar 2016**) with *C. officinalis*. They reported that when colchicine was applied the number of branches per plant increased. This may be due to the influence of colchicine concentrations on high apical meristems development for the auxiliary Calendula plant branches. The maximum number

of branches may be due to regular colchicine supply which increased vegetative growth.

Number of inflorescences/plant

Table (3) showed that with all the different concentrations of colchicine the number of inflorescences/plant was increased. The maximum number of inflorescences (58.19) was reported by 0.05 per cent colchicine. The moderate concentration of colchicine, 0.05 and 0.1 per cent observed high inflorescences number than low and high concentration rates. There appears to be correlations between number of branches and number of inflorescences, so with increasing number of branches due to consuming more colchicine causes an increasing number of inflorescences. This may be due to Calendula plants distinguishing high vigour growth especially branching ratio under moderate levels of colchicine but with increasing concentration of colchicine, the number of inflorescences decreased.
These findings are in line with (**Hannweg *et al*., 2013**) on *Crocosmia aurea* and (**El-Nashar and Ammar 2016**) on *C. officinalis*. They found that number of inflorescences/plant in most moderate treatments was increased by treating plants with colchicine relative to control plants.

Diameter of inflorescence

Results provided in Table (3) show that colchicine significantly increased the diameter of inflorescences using 0.05 per cent relative to control, and measured 7.78 cm. This result revealed that inflorescences diameter of Calendula plants increased as concentration rate of colchicine increased and reached its peak values at 0.05 per cent. Similar results were reported by various authors as (**Zhang *et al*., 2016**) on *Trollius chinensis* (**Wang *et al*., 2017**) on *Fagopyrum tataricum* and (**El-Nashar and Ammar 2016**) on *C. officinalis*. They found that colchicine treatments had a positive and significant effect on diameter of inflorescences compared to untreated plants.

Fresh and Dry weight of inflorescence

Results in Table (3) showed that the moderate concentrations of colchicine had a positive impact on fresh and dry inflorescence weight, especially 0.05 per cent, with the highest significant records on fresh and dry weight, respectively with 7.89 and 1.32 g. These findings comply with (**El-Nashar and Ammar 2016**) on *C. officinalis,* (**Wang *et al*., 2017**) on *F. tataricum,* and (**Kushwah *et al*., 2018**) on *C. carinatum.*

Total soluble carbohydrates

Results presented in Table (3) clarified that colchicine treated plants increased the total soluble carbohydrates content compared with untreated plants, especially at 0.05 per cent with 14.12%. Similar results (**Estaji *et al*., 2017**) reported on *S. leriifolia* and (**Abdoli *et al*., 2013**) *E. purpurea*.

β-carotene pigment content

Data tabled in Table (3) showed that the content of β-carotene increased slightly wi moderate levels of colchicine. The highest significant content of β-carotene was 0.45 mg DW derived from 0.05 per cent colchicine treated plants. Different results (**Nura *et a* 2013**), published on *S. indicum* and *E. purpurea* (**Abdoli *et al*., 2013**). They concluded th the amount of β-carotene inflorescence was increased as a result of an increased treatment colchicine relative to control plants.

Heritability and Genetic Variability

In trying to determine the variability in agronomic and yield components, which a responsible for yield variation between different cultivars, heritable components must b determined. Genetic advances and heritability estimates are important preliminary criteria any plant breeding program and the heritable variation is powerful for plant genet improvement. Table (4) describes the genotypic coefficient of variability (GCV), t phenotypic coefficient of variability (PCV), broad sense heritability and genetic advance percentage of mean for traits. The GCV values for plant height, No. branches, fresh and d weight were moderate (14.18, 13.85, 12.05 and 9.76, respectively). The remaining featur registered low GCV values. The heritability of broad sense for all traits has been calculate The estimated high variation in heritability between traits ranged from 48.64 to 90.81 p cent in plant height (Table 4). High heritability was observed for plant height along wi high genetic advance, estimation of genetic advance is effective as selection criterion wh viewed in combination with heritability estimates (**Herbert *et al*., 1955**). High estimates heritability for plant height, fresh weight, No. inflorescences, dry weight and No. branch suggest a strong selection response in these traits. Various authors reported similar resu (**Rahim *et al*., 2010**; **Eshghi *et al*., 2012**; **Yassein and Aly 2014**).

Table 4 Estimates of genetic parameters

Genetic parameters	Plant height	No. branches	No. Inflorescences	inflorescence diameter	inflorescence fresh weight	inflorescence dry weight	total soluble carbohydrate	β-carotene
h^2	90.806	80.871	85.52	48.643	87.077	84.701	65.317	51.628
Gs	27.839	25.158	16.53	7.0851	23.165	18.497	5.7007	4.539
GCV	14.182	13.58	8.68	4.9314	12.051	9.7563	3.4241	3.0665
PCV	14.882	15.101	9.38	7.0706	12.914	10.601	4.2368	4.2678

Legend: Heritability h^2, Genetic advance (Gs), Genetic coefficient variance (GCV), and phenotypic coefficient variance (PGV) for plant height, No. branches/plant, No. inflorescences, inflorescence diameter, fresh and dry weight of inflorescences, total soluble carbohydrates and β-carotene content.

RAPD Conditions for Amplification

In the beginning a total of 16 random primers were screened for Calendula consistent amplification. In the present study eleven random decamer primers have been successfully amplified and used. The RAPD profile obtained was produced in approximately 20 high bands between 200 up to 2000 bp (Fig. 1). The polymorphism was 60 per cent and the band average was 7.2 per primer. The greater number of fragments that one primer yielded was 8 bands. The Polymorphism Information Content (PIC) values varied between 0.0 and 0.35 (Table 5). Variants and variations in ornamental plants were detected using molecular markers (**Mohapatra and Rout 2005**). DNA markers such as RAPD are useful options for genetic diversity assessment. For plant breeding and genetics studies (**Mikhaĭlovskiĭ *et al*., 2007**), Genetic variations can be used to identify species, genotypes and cultivar and to estimate phylogenetic relationships. RAPD-PCR technique was used to characteri calendula genotypes as powerful tool for detecting of genetic differences, requiring simp DNA content, low amount of DNA, easy and quick detection of DNA polymorphism, an producing numerous polymorphic bands for comparative analysis (**Hassan and Yasse 2014**). Analysis of morphological and molecular traits among Calendula genotypes an concentrations of colchicine revealed diversity up to 30% (Fig. 2) indicating high genet variability between the genotypes due to colchicine treatments, which can be detected b RAPD-PCR technique. Similar observations in ornamental plants have been reported b different investigators, and genotypes could be distinguished using RAPD-PCR, an genotypes and cultivars can also be calculated for genetic variability (**Sano *et al*., 2016**).

Table 5 Characterization of selected RAPD primers with *C. officinalis*

Primer	Amplified products	Polymorphic product	% polymorphism	Polymorphism Information Content (PIC) values
OPA1	5	3	60	0.277
OPC2	4	0	0	0
OPC3	9	8	89	0.345
OPA4	9	8	89	0.336
OPA5	9	7	78	0.254
OPA6	6	2	33	0.109
OPA7	5	2	40	0.131
OPA8	11	6	55	0.23
OPA9	8	5	63	0.224
OPA10	9	8	89	0.318
OPC2	7	0	0	0

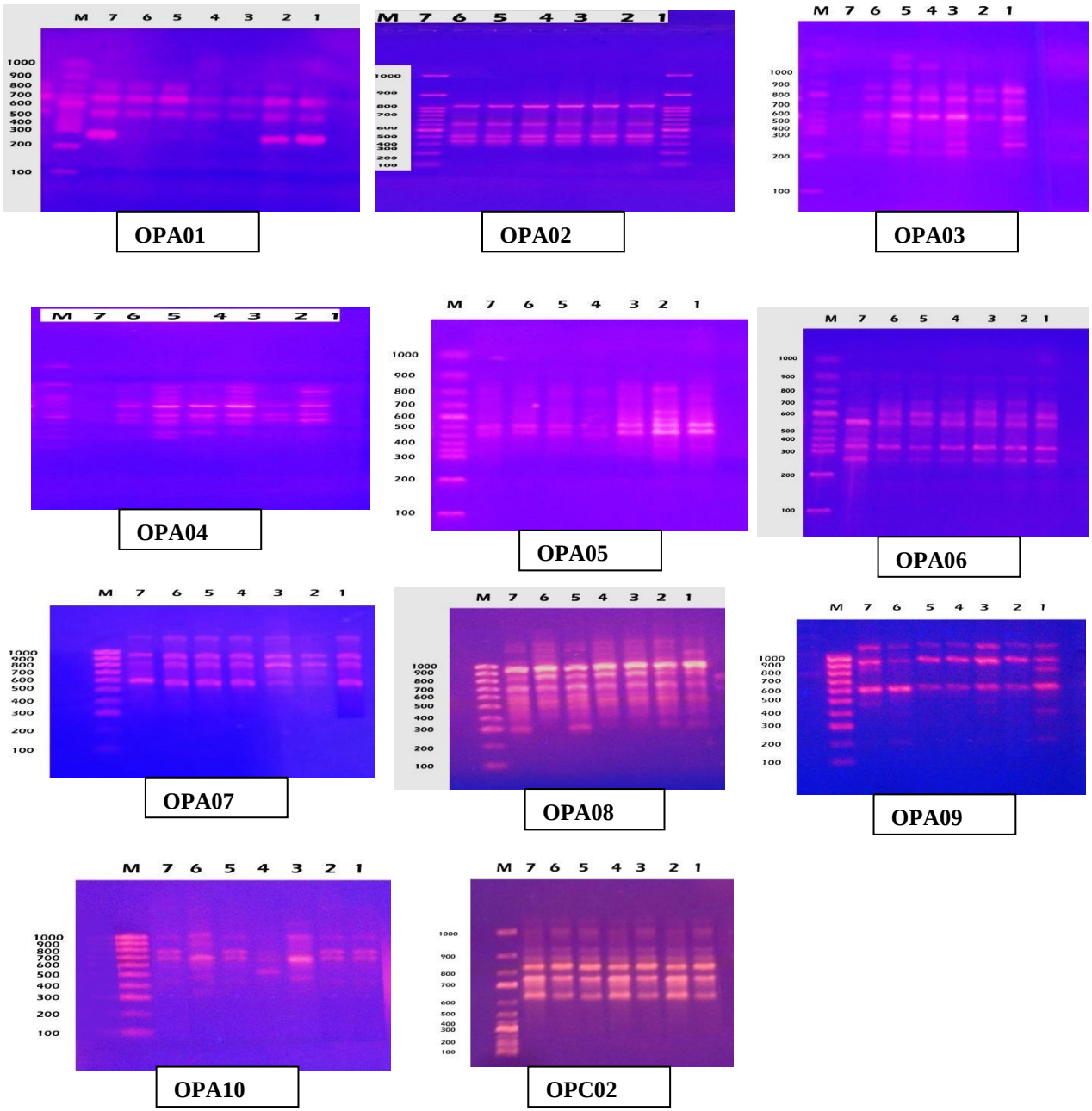

Figure 1 RAPD primer profiles amplified in *C.officinalis* genotypes.
Legend: M, DNA ladder, and lanes from 1 to 7 represent 0.8%, 0.4%, 0.2%, 0.1%, 0.05%, 0.025% and 0.0% colchicine respectively.

Evaluation of genetic diversity based on RAPD-PCR

Results of RAPD-PCR were applied to search out the differences between the various concentrations of colchicine in seedling stage. Phylogenetic relationships between different concentrations of colchicine and existing genetic diversity were illustrated in tree diagram upon PCR results. The genetic diversity matrix was applied through NTSYS pc software for cluster analysis. Genetic diversity assessment could be of great importance for the classification of genotypes, species and treatments. Estimating of genetic relationship and selecting of superior genotypes would be of great goals for selecting genotypes with high desirable characters and improving plant breeding programs. Analysis of the dendrogram showed high genetic diversity among genotypes, around 30%. Phylogenetic tree represents two clusters; first cluster included only the control, while the second cluster divided into sub-clusters the higher doses in one and the reminder of genotypes in the other sub-cluster Figure (2). Data gathered from dendrogram illustrated the effect of treatment diversification. Various authors used the RAPD technique to detect the changes in DNA patterns and to calculate genetic similarity/diversity between genotypes/species. Genetic variation in *D. grandiflora* using RAPD-PCR (**Kumar *et al*., 2005**) was studied because of the high level of RAPD polymorphism used to identify cultivars and the genetic variability was used to study genetic distances among genotypes and treated plants. The RAPD analysis was used in *Dendranthema grandiflora* cv. Snow Ball to detect genetic polymorphism among the mutants variants by mutagenesis *in vitro* (**Kaul *et al*., 2011**). Genetic diversity was estimated using RAPD (**Zainudin *et al*., 2014**) in *Jatropha curcas* mutants and in *B. napus* (**Yassein and Aly 2014**) and the results showed that genetic diversity can be calculated on the base of RAPD within the mutants.

Mutation can be responsible for the appearance of new bands if they occur in a sufficie number of cells at the same locus (**Atienzar and Jha 2006**). The appearance a disappearance of bands could be associate with changes or mutations of colchicine induc fer plant DNA (**Atienzar *et al*., 1999**; **Atienzar and Jha 2006**). The high number disappeared bands was observed at 0.05 per cent concentration suggests that colchicine this concentration was able to induce DNA alterations that resulted in loss of band. T appearance of new PCR bands may reveal a change in some oligonucletide priming due t mutations and juxtaposition two sequences that matching the primers sequence **(Atienzar *al*., 1999**). RAPD has the potential to represent genetic differences in different ornament crops up to species and cultivar levels (**Kaul *et al*., 2011**). SRAP marker technique was us to verify the existence of genetic variability at molecular-level as a result of the colchici mutagen concentrations (**El-Nashar and Ammar 2016**). Diethyl sulphate (DES) a Diethyl sulphate (DMS) used in low concentrations and had a positive effects morphological and yielding traits and genetic polymorphism among Calendula cultiva mediated FISH-based visualization of 45S and 5S rDNA correlates with variability in t cultivar characteristics (**Samatadze *et al*., 2019**).

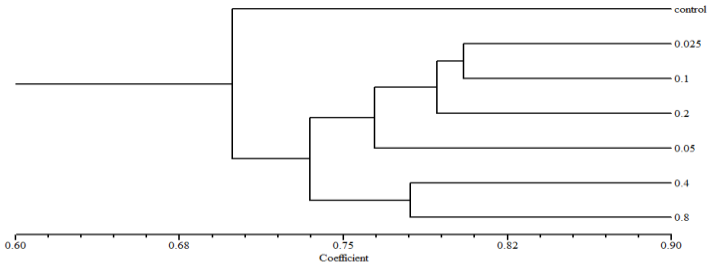

Figure 2 Dendrogram of *C.officinalis* genotypes induced by colchicine based on RAPD-PCR

CONCLUSION

Results highlight the usefulness of colchicine application in Calendula induction mutation and found high heritability in the majority of traits, which help in plant breeding programme selection criteria. RAPD-PCR as a tool for detecting the effects of colchicine on Calendula and could be used significantly in the detection of useful mutants. The latest could be used inbreeding programs to improve inflorescence yield and quality as well as in detecting similarities at the molecular level among the different mutants. Results recommend the 0.05 per cent colchicine for efficient breeding Calendula mutation.

Conflict of interest: The authors declare that they have no conflict of interest.

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