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Induced Mutations in *Gaillardia pulchella* Foug Plants by Chemical Mutagen and Detection Variation by (SRAP) Sequence-Related Amplified Polymorphism Markers

Eid, R. A. [1], Mahfouze, H. A. [3], El-Khateeb M.A.[2], Abd El-Aziz, N. G. [1], Ashour H.A.[2], and Radwan R.M.S[1*]

1- Ornamental Plants and Woody Trees Department, Agricultural and Biological Research Institute, National Research Centre, Dokki, Giza, Egypt.

2- Department of Ornamental Hort., Faculty of Agric., Cairo University, Egypt.

3- Genetics and Cytology Department, Biotechnology Research Institute, National Research Centre, Dokki, Giza, 12622, Egypt.

Abstract

Diethyl sulphate (DES) was used to induce genetic variability in *Gaillardia pulchella* plant to improving morphological characters. Besides, changes in the genomic DNA between mutant plants and the control were determined. The seeds were soaked in four different concentrations of DES (1000, 2000, 3000, and 4000 ppm) for 8 h. The results indicated that untreated plants gave the highest values of seed germination in M_1 and M_2, whilst 4000 ppm recorded the lowest value. The concentrations of 1000 and 2000 ppm increased the plant height, giving values of (125.47 and 118.96 cm in M_1) and (120.10 and 121.45 cm in M_2) compared to (110.07 cm in M_1 and 114.12 cm in M_2) for the control. The highest numbers of branches were (15.23 and 16.87 in M_1) and (17.10 and 18.17 in M_2) compared to (12.27 in M_1 and 14.20 in M_2) for the control. 3000 and 4000 ppm delayed flowering compared to the control. In contrast, 2000 ppm induced early flowering and increased the flowers number, compared to the control. Many mutants in leaves and inflorescences morphology were observed; the largest number of these mutants was obtained from 4000 ppm. SRAP mars were used to confirm the existence of genetic variability at the genomic DNA level among populations treated with DES and control, depending on the concentration of DES. Therefore, SRAP markers are considered an important tool in detecting the mutagenic effects of DES. Also, it will help to discriminate between populations showing mutations in morphological and floral characteristics.

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*Corresponding authors: **Radwan R.M.S**

E-mail: ragabradwan.54@gmail.com

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INTRODUCTION:

Gaillardia pulchella is a flowering plant called “blanket flower" or "American blankets" belong to *Asteraceae* family is a genus of annual, biennial, perennial herbs and subshrubs consist of 30 species, native to the Americas **(Ferner, 1981).** With single or double flower heads that have bands of colors, maroon, red or orange; it flowers from April to September **(Kale, 2002)**. The leaves are alternate and hairy; with smooth to tooth or lobed edges. Fruit has a pappus of scales. The plants are excellent for borders or mixed beds, house gardens, containers, streets, and tolerate dry conditions **(Agale *et al.*, 2017).** Inflorescences extract contains flavonoids, polysaccharides, amino acids, and tannins, which have important pharmaceutical uses **(Adzhiakhmetova *et al.*, 2020).**

Most cultivated flowering ornamental plants showed a narrow genetic base. So, induced mutations are considered one of the best methods for the improvement of ornamental herbs. Mutation breeding in ornamental plants by chemical mutagens is aimed to induce changes in one or many characters of an otherwise outstanding variety without altering the unique part of the genotype, such as flower characters (color, size, quality, morphology, and fragrance), leaf characters (form, size, and pigmentation), growth habit (compact, climbing, branching, and shorter growing period), and physiological traits, *i.e.*, changes in photoperiodic response, early flowering, and tolerance to biotic and abiotic stresses **(Kayalvizhi *et al.*, 2020).** A mutation is a sudden genetic alteration that takes place in an organism, it may occur naturally or be artificially induced, and the mutant that results will have altered chromosomes or genes **(De and Bhattacharjee, 2011).** The nuclear DNA is broken as part of the mutation induction mechanism, and as the DNA is repaired, new mutations may appear at random and are heritable. It is a quick, easy, and affordable method for getting the desired genotypes in crops. One of the most popular methods for using chemical mutagens to add more desirable character variation is induced mutation. Alkylating chemical substances like diethyl sulfate (DES), ethyl methane sulphonate (EMS), methyl methane sulphate (MMS), dimethyl methane sulphate (DMS), hydrazine and sodium azide, can be used to chemically cause mutations. When chemical mutagens from the alkyl group interact with DNA, the nucleotide sequence may change, and a point mutation may result. These can alkylate the phosphate groups in the phosphodiester backbone, as well as the different imino- or carbonyl groups on the purine or pyrimidine bases, and therefore react with DNA **(Spencer-Lopes *et al.*, 2018).** Chemical mutagenesis is means to cause mutations in plants in order to improve their agronomic traits. DES is a chemical mutagen and has been one of the most powerful mutagens in ornamental plants, being a strong mutagen in plants; it affects the different parts of the plants and their growth developmental phenomena by disturbing the metabolic **(Owias *et al.*, 1983).**

Molecular markers such as sequence-related amplified polymorphism (SRAP), can selectively amplify DNA coding regions, are widely used and have been reported to be highly stable, efficient, and suitable for direct use in different plants **(Li and Quiros, 2001).** Chemical mutagens have been

used in many studies to induce genetic variability in ornamental plants, **(Mangaiyarkarasi *et al.*, 2014)** on *Catharanthus roseus*, **(Mostafa *et al.*, 2014) on *Celosia argentea*, (El-Nashar and Asrar, 2016) on *Calendula officinalis*, (Chen *et al.* 2020) on *Chrysanthemum indicum*, (Elmenbawy *et al.*, 2020) on *Calendula officinalis*, (Habib *et al.*, 2021) on sunflower, (El-Khateeb *et al.*, 2022) on *Borgo officinalis*, and (El-Gazzar *et al.*, 2023)** on *Hibiscus rosa-sinensis*. The aim of this study was to investigate the effect of different concentrations of diethyl sulfate (DES) on inducing mutations to improve vegetative growth, flowering, and detection of DNA polymorphism among obtained mutants of *G. pulchella* by SRAP markers.

MATERIALS AND METHODS:

Plant materials:

The seeds of *G. pulchella* (the local variety) were obtained from a bred strain in The Ornamental Horticulture Department of Agriculture Cairo University Egypt. A field experiment was conducted in this location during the two successive seasons of 2019/20 and 2020/21 for two generations (M_1 and M_2).

Treatment of seeds:

Seeds were pre-soaked in distilled water for 1 hour, 500 seeds were treatment with different concentrations of DES (0.0, 1000, 2000, 3000, and 4000 ppm), 300 seeds were measure the % seed germination in the lab. (Fig. 2), 200 seeds were sown in plastic trays filled with a mixture of peat moss, loam, and sand (1:1:1 by volume) on 5, October 2019, and 5, October 2020 for (M_1 and M_2 respectively) (Fig. 1) to produce seedling. After 10 days of sowing seeds began germination, and after 45 days of sowing, uniform Gaillardia seedlings (8-10 cm in height). The seedling of each treatments were transplanted into the open field (clay loam soil), in three rows at 60 cm apart and 50 cm between the hills within each row (two plants/hill), as every plot (3.5 x 1.8 m) contained 21 hills /plot.

Control **1000 ppm** **2000 ppm** **3000 ppm** **4000 ppm**

Fig. 1. Showing the seedlings of *Gaillardia pulchella* in plastic trays as affected by different concentrations of diethyl sulphate.

Control **1000 ppm DES** **2000 ppm DES** **3000 ppm DES** **4000 ppm DES**

Fig. 2. Showing the seed germination (%) of *G. pulchella* in Petri dishes as affected by different concentrations of diethyl sulphate (DES).

The first and second mutative generations 2019/20 (M_1) and 2020/21 (M_2)

The mass selection of seeds in M_1 plants was done from June to July 2020, where plants that survived in each treatment were evaluated, selected, and selfed in order to obtain the second mutative generation (M_2) seeds, according to **(Sinhamahapatra and Rakshit 1990).** Observations were taken during the vegetative growth and flowering periods. The seeds of all the M_1 viable plants that survived were harvested separately for each treatment when they reached maturity (June to July). In order to prevent cross-pollination between plants and some of them, whether by wind or insects, we used a bag of paper for the flower buds before opening in order to preserve the selected characters and to grow M_2 generation (seedlings) plants. In both generations all the recommended cultural practices namely, irrigation and fertilizer, were carried out during the plant's growth and flowering period. The fertilizers were supplied for each plot as recommended, using Kristalon mineral fertilizer (N:P:K) (19:19:19). The plants were fertilized monthly after a month of transplanting (1 g/hill). Irrigation was done with tap water according to the needed amount of water, and weeding was carried out as the soil needed.

Soil analysis:

Soil analysis indicated that, particle size distribution (%) was: sand: 26.6, silt: 26.3 and clay: 37.5 (texture: clay loam), pH: 7.4, EC ds.m-1: 0.94.

Genomic DNA isolation and SRAP analyses

DNA was extracted from fresh young leaves of *G. pulchella* plants mutated with DES and the control (M_2) by the DNA purification kit (Bio Basic Inc., Markham, Canada). Six SRAP primers (Table 1) were selected from **(Li and Quiros, 2001)** and were used to detected variation from *G. pulchella* original and mutant plants. PCR reaction contained 25 µl, 10X PCR buffer, 2 mM MgCl2, 0.2 mM dNTPs mixed, 10 pmol primers, 1.25 U Taq polymerase, and about 150 ng genomic DNA. And PCR conditions, initial denaturing step was performed at 94°C for 5 min, followed by 5 cycles at 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min, subsequently followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min with a final extension step at 72°C for 7 min. Amplification products were separated on 1.5% agarose gel containing 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) and 0.5 µg/ml ethidium bromide at 90 V.

Table (1): Sequence of primers used in this the study. The selective nucleotide sequences for each primer are underlined.

Primers name	Sequence	
	Forward	Reverse
SRAP-1	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTGTC
SRAP-2	TGAGTCCAAACCGGTAG	GACTGCGTACGAATTCGA
SRAP-3	TGAGTCCAAACCGGTCC	GACTGCGTACGAATTCAG
SRAP-4	TGAGTCCAAACCGGTCA	GACTGCGTACGAATTCTG
SRAP-5	TGAGTCCAAACCGGTCA	GACTGCGTACGAATTAAT
SRAP-6	TGAGTCCAAACCGGTGC	GACTGCGTACGAATTGTC

Data analysis:

A matrix for SRAP was generated by scoring reproducible bands as 1 for their presence and as 0 for their absence across the genotype. Genetic similarity coefficients were computed according to (Nei and Li, 1979). A dendrogram based on Jaccard similarity coefficients was constructed by using the un weighted pair group method of arithmetic averages (UPGMA) **(Sneath and Sokal, 1973)** employing sequential, agglomerative hierarchic and non-overlapping clustering (SAHN). All the computations were carried out using the PAST software **(Ryan *et al.*, 1995).** Correlation coefficients were calculated using similarity coefficients obtained from SRAP analysis.

Data recorded:

The following data were collected on *G. pulchella* plants that were grown until 50 % of the flowers were opened, that is, about a month after the flowers start to appear for each treatment: (**a)** Seed germination (%), the germination percentage of seeds was measured using the following equation;

$$\text{Germination (\%)} = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds sown for germination}} \times 100$$

(b) Vegetative parameters [plant height (cm) and No. of main branches/plant]; **(c)** Flowering parameters [No. of days from planting to flowering (DPF) and No. of flowers (inflorescences)/plant]; **(d)** Plants abnormalities [leaves and inflorescences abnormalities]; **(e)** Molecular marker analyses using SRAP markers of plants mutant with DES.

Statistical analysis:

Statistical analysis was conducted using COSTAT software; a randomized complete block design was used, with three replicates for each treatment and 10 plants in each replicate. The results of the four trials were statistically analysed using **(Snedecor and Cochran's, 1980),** and the means were separated using (Duncan, 1980) multiple range tests and compared using the L.S.D test at 0.05 probability.

RESULTS AND DISCUSSION:

a. Seed germination (%)

Data in (Fig. 2) and (Table 2) showed that all concentrations treated by (DES) decreased in germination percentage $M_1$and M_2, treatments 1000 and 2000 ppm achieved the maximum germination percentages (81.33 and 81.66%), then (87.66 and 89.00%), respectively, and the highest concentration of 4000 ppm, which recorded the minimum germination percentages by (62.33 and 64.66%) in $M_1$and M_2, respectively. Compared the control that recorded the highest value in M_1 and M_2 (84.0 and 89.33%), respectively. In this regard, (Kulkarni, 2011) stated that the reduction in seed germination by using DES may be owing to, one of the physiological effects of DES mutagen; **(Deepika *et al.*, 2016)** reported that the reduction in germination percentage may be related to disruptions in the synthesis of enzymes involved in the process of germination or the action of DES mutagens on the meristematic tissues of the radical/plumule could cause a reduction in seed germination. These results are similar to those found by **(Mangaiyarkarasi *et al.*, 2014)** on *Catharanthus roseus*, used EMS at 30, 40, 50, 60, and 70 mM, and found that the seed germination increased when EMS concentrations decreased; **(Chen *et al.*, 2020)** mutated *Chrysanthemum indicum* plants by four different concentrations of EMS (0, 0.1, 0.2 and 0.5%) for 8

h, and indicated that with increasing EMS concentrations, the germination rate decreased

Table (2): Effect of diethyl sulphate on seed germination (%), plant height (cm), number of main branches/plant, number of days to flowering (DPF) and number of flowers /plant of *G. pulchella* plant, during the $M_1$and M_2 generations (2019/2020) and(2020/2021).

Characters			Vegetative Parameters		Flowering Parameters	
Treatments		Seed germination (%)	Plant height (cm)	Number of main branches/plant	Number of days to flowering (DPF)	Number of flowers /plant
Control	M_1	84.00 a	110.07 c	12.27 c	171.00 b	95.61 c
	M_2	89.33 a	114.12 b	14.20 b	160.00 c	105.26 c
1000 ppm DES	M_1	81.33 a	125.47 a	15.23 ab	160.23 d	115.60 b
	M_2	87.66 a	120.10 a	17.10 a	152.16 d	126.43 b
2000 ppm DES	M_1	81.66 a	118.96 b	16.87 a	164.20 c	129.12 a
	M_2	89.00 a	121.45 a	18.17 a	149.31 e	133.40 a
3000 ppm DES	M_1	75.66 b	115.40 b	13.77 bc	163.00 c	97.02 c
	M_2	80.00 b	119.20 a	12.63 bc	165.63 b	106.19 c
4000 ppm DES	M_1	62.33 c	105.44 c	9.86 d	185.06 a	82.70 d
	M_2	64.66 c	103.03 c	10.60 c	177.91 a	88.09 d

b. Vegetative parameters

1. Plant height (cm)

The results in (Table 2), showed that increase of plant height in 1000, 2000 and 3000 ppm in M_1 and M_2, respectively, (125.47, 118.96 and 115.40 cm) and (120.10, 121.45 and 119.20 cm), comber 4000 ppm that recorded the lowest value in M_1 and M_2 (105.44 and 103.03 cm), compared to the control (110.07 and 114.12 cm). **(Joshi *et al.*, 2011)** suggested that the increase in plant height using low concentrations of DES may be attributed to an increase in the rate of cell division or cell elongation. For the decrease in the plant height using the high concentration of DES may be due to hindering cell development and growth, as reported by **(Neagu, 1984)** on *Helianthus annuus*; **(Badr *et al.*, 2000)** on *Tagetes erecta*, **(El-Nashar, 2006)** on *Amaranthus*. These results are similar to those observed by (Krupa-Makiewicz *et al*., 2010) on the petunia plant, they soaked the seeds in DES at 0.5 and 1.0 mM, EMS at 0.5 and 1.5 mM, and MMS at 1.5 and 2.0 mM (for 60 min), and found that the low levels of all mutagens increased the plant height as compared to the control; (Kapadiya *et al*., 2014) treated the chrysanthemum plants with three concentrations of EMS and DES (0.02, 0.03, and 0.04%) for six h, and recorded that the highest concentration of both mutagens reduced plant height; **(Kayalvizhi *et al.*, 2017)** investigated the effects of DES on tuberose, and indicated that the plant height was greater with low levels of (DES); **(Sedaghathoor *et al.*, 2017)** tested the influence of DES on tulip plants, and stated that the low concentrations of DES enhanced the plant height; **(Ghosh *et al.*, 2020)** on *Jasminum grandiflorum* used EMS at

25, 30, 35, and 40 mM. They mentioned that with increasing EMS concentrations, plant height was shortened.

2. Number of main branches /plant

Data in (Table 2) indicated that using DES at 1000 and 2000 ppm in M_1 and M_2, respectively, induced a significant increase in the number of branches, giving values (15.23 and 16.87 branches) and (17.10 and 18.17 branches) by increment % (24.12 and 37.48%) and (20.42 and 27.95%) compared to the untreated plants (12.27 and 14.20 branches). On contrast, the highest concentration of DES 4000 ppm in M_1 and M_2, respectively, had a negative effect on the formation of branches, gave the lowest number of branches (9.86 and 10.60), by decrement % (19.64% and 25.35%) compared to the control. These results agreement with **(Kapadiya *et al.*, 2014)** they found that the high concentration (0.04%) of DES reduced the branches number of chrysanthemum plants; **(Mangaiyarkarasi *et al.*, 2014)** used EMS at 30, 40, 50, 60 and 70 mM on *Catharanthus roseus*, they concluded that as the concentrations decreased, the branches number increased; (El-Nashar and Asrar, 2016) applied DES on *Calendula officinalis*, They indicated that lowering mutagen concentrations (1000 and 2000 ppm) of DES had an enhanced on number of branches; (Sedaghathoor *et al*., 2017) reported that the low concentration (0.1%) of DES enhanced the growth of tulip plants; **(El-Gazzar *et al.*, 2023)** used (EMS) and (DMS) at (0.1, 0.2 and 0.3%) to treat the cuttings of *Hibiscus rosa-sinensis* plant. They observed that the plant height, number of the leaves and branches, were decreased with increasing the concentrations of (EMS) and (DMS).

c. Flowering parameters

1. Number of days from planting to flowering (DPF)

The results in (Table 2) revealed that treating the plants by DES at (1000, 2000, and 3000 ppm) in M_1 induced early flowering, and shortened the vegetative growth phase by (10.77, 6.80 and 8.00 days), compared to the control. Whereas, 4000 ppm DES increased the number of days elapsed to flowering by 14.06 days over the control. In the M_2, it was found that concentrations of 1000 and 2000 ppm DES led to an early in flowering phase by (7.84 and 10.69 days) compared to the control. On the other hand, treating the plants with 3000 and 4000 ppm DES, prolonged the vegetative growth phase by (5.63 and 17.91 days) compared to the control. **(Neagu, 1984)** on *Helianthus annuus*, reported that the high levels of chemical mutagens, hindered cell development, decreased growth rate and delayed flowering phase; (Badr *et al*., 2000) on *Tagetes erecta*, and **(El-Nashar, 2006)** on *Amaranthus*, they stated that the physiological damage caused by increasing chemical mutagen levels may be the cause of flowering inhibition. Similar results were reported by **(Kapadiya, *et al.*, 2014)** on chrysanthemum plants, used DES with different concentrations of (0.02, 0.03, and 0.04%) for 6 h, and showed that the flowering was delayed by up to 7 days with high levels. (Patel *et al*., 2018) on gladiolus, who found that the low concentrations (0.15 and 0.2%) of DES induced early flowering, and **(Ghosh *et al.*, 2020)** on *Jasminum grandiflorum*, used 25, 30, 35, and 40 mM of EMS, and indicated that the early flowering was related to small EMS concentrations.

2. Number of flowers (inflorescences)/plant

The results in (Table 2) showed that the concentrations of 1000 and 2000 ppm DES,

increased the formation of inflorescences in M_1 and M_2, respectively, giving (115.60 and 129.12 inflorescences) and (126.43 and 133.40 inflorescences), by an increment of (20.90 and 35.04%) and (20.11 and 26.73%), compared to the control (95.61 and 105.26 inflorescences). In contrast, the concentration of 4000 ppm, decreased the number of inflorescences in M_1 and M_2, respectively, (82.70 and 88.09) by decreasing % (13.50 and 16.31%), compared to the control. The results obtained are in consistent with **(El-Nashar and Asrar, 2016)** on *Calendula officinalis*, they observed that the low concentration of DES (1000 ppm) increased inflorescences number; **(Kayalvizhi *et al.*, 2017)** on tuberose plant, reported that the low level of DES (15 mM) gave the greatest number of flowers; **(Sedaghathoor *et al.*, 2017)** on tulip plant, stated that the low levels of DES improved the production of flowers; **(Elmenbawy *et al.*, 2020)** treated *Calendula officinalis* seeds with three different concentrations of EMS (1000, 3000, and 10000 ppm), and observed that inflorescences number decreased with raising EMS levels; **(Ghormade, *et al.*, 2020)** found that the low levels of EMS (0.01, 0.05, 0.1, 0.5, 1.0, and 1.5%) on chrysanthemum, increased the formation of flowers, and **(El-Gazzar *et al.*, 2023)** who used (EMS) and (DMS) on *Hibiscus rosa-sinensis* plant, and found that the low concentration (0.1%) of both mutagens increased the number of flower, but the concentrations (0.3%) decreased it.

d. The leaves and inflorescences abnormalities during the period of experiment

1. Leaves abnormalities

The leaf abnormalities in (Fig. 3) illustrated that all concentrations of DES induced many variations of the leaves compared to the control, such as deformed leaves, simple undulate, deeply loped with large size, curvature and undulate, bending and lobed leaves, corrugated, crenate, wrapped, variegated, doubly serrate, doubly serrate with colored midrib, serrated deeply lobed, biforked. It was found that, the largest number of these variations was observed with the concentration (4000 ppm) of DES. These mutations in the leaves may be attributed to the result of chromosomal disruptions, also may be due to the result of layer rearrangement caused by chemical mutagens, according to **(Abd El-Maksoud, 1988); (Mato, *et al.*, 2000)** stated that variegated leaves may be caused by genetic changes in chloroplast DNA and gene and /or plastid changes, (El-Nashar and Asrar, 2016) on calendula plant, reported that the mutations occur as a result of chromosomal, chromatid, or subchromatid abnormalities, alterations in chromosome number, inhibition of cell division, or mitotic activity induction. In this concern, **(Srivastava *et al.*, 2018)** on orchid (*Aerides crispa*) applied EMS at (0.025, 0.05, 0.1, 0.2, and 0.3%) for 6 h, and recorded the following leaf shapes; lanceolate leaf, straita leaves, maculate leaf, oblong, waxy, viridis leaf, short, and broader leaves; **(Chen *et al.*, 2020)** on *Chrysanthemum indicum*, found that using EMS different concentrations of 0, 0.1, 0.2, and 0.5% for 8 h, induced many abnormalities in shape of leaves; **(El-Khateeb *et al.*, 2022)** treated *Borgo officinalis* seeds with different concentrations of DES (0.1, 0.2, 0.3 and 0.4%) for 6 h, and obtained many leaf morphological changes in leaf size, shape, margin, and petioles.

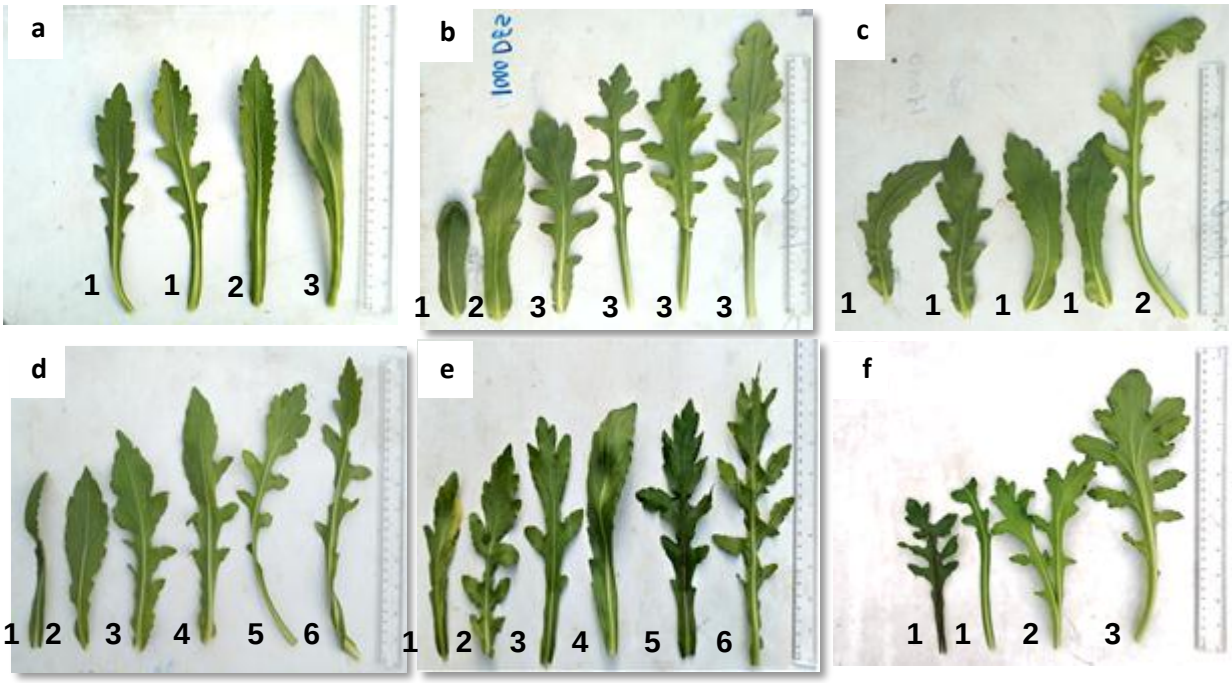

Fig. 3. Showing the plant abnormalities of *G. pulchella* as affected by different concentrations of DES on leaves shape in M_1 and M_2 generations.

(a), Control original leaves plants (1, Loped; 2, Dentate and 3, Serrulate); **(b)** 1000 ppm DES (1, Deformed; 2, Simple undulate and 3, Deeply loped with large size) in M_1 and M_2; **(c)** 2000 ppm DES (1, Curvature and undulate and 2, Bending and lobed leaves M_1 and M_2; **(d)** 3000 ppm DES (1, Corrugated; 2, Crenate; 3, Doubly serrate; 4, Lobed; 5, Curvature and deeply lobed and 6, Wraped and lobed leaf in M_1 and M_2; **(e)** 4000 ppm DES (1, Variegated; 2, Doubly serrate; 3, Lobed; 4, Corrugated; 5, Doubly serrate with colored midrib and 6, Serrated deeply lobed in M_1 and M_2; **(f)** 4000 ppm DES (1, Deformed; 2, Biforked and 3, Doubly serrate in M_2.

2. Inflorescences abnormalities

The inflorescence abnormalities in (Figs 4 and 5) illustrated that using DES had an evident effect on inducing many abnormalities in the color and formation of the inflorescences such as, dark burgundy radial, red double inflorescence, burgundy semi-double inflorescence, red semi-double inflorescence, radial pink flowers tinged with light yellow, semi-double inflorescence with radial dark red flowers tinged with yellow tops, single double inflorescence with radial pink flowers tinged with light yellow, yellow radial flowers tinged with red at the bottom, inflorescence with two disc flowers, deformed inflorescence, fasciation flower, orange inflorescence, radial orange flowers tinged with light yellow, red radial flowers and one petal appear in the disc flowers, and slender ray flowers,. It was found that the highest concentration of DES (4000 ppm) gave the largest number of inflorescence variations. The inflorescences abnormalities by DES mutagen may be attributed to a deficiency or delay in the development of flowers, as well as a proliferation of inflorescence-like structures in their place, according to **(Coen and Carpenter, 1993),** (Nakatsuka *et al.*, 2005) stated that the abnormalities may be attributed to the result of a gene mutation that caused the floral meristem to be replaced with meristems that contain some or all of the flowers characters. In this regard, **(Kolar *et al.*, 2015)** used EMS on *Delphinium malabaricum* plant, obtained many changes in morphological of flowers; **(Samatadze *et al.*, 2019)** treated *Calendula officinalis* seeds with 0.04 and 0.08% DES, and recorded many mutants

in vegetative and floral parameters; (**Chen *et al.*, 2020)** on *Chrysanthemum indicum*, found that using EMS different concentrations of 0, 0.1, 0.2, and 0.5% for 8 h, induced many abnormalities in shape of leaves and flowers; **(Elmenbawy *et al.*, 2020)** on *Calendula officinalis*, subjected the seeds to EMS at 1000, 3000, and 10000 ppm. All applications of EMS induced variations in color and shape of flowers.

Fig. 4. Showing the color variations of inflorescences abnormalities of G. pulchella as affected by different concentrations of DES on inflorescence color in M1 and M2 generations.

(a) Control original color, the outer half yellow and the rest of the ray red. **(b)** 1000 ppm DES, Dark burgundy radial in M_1 and M_2. **(c)** 1000 ppm DES, Red double inflorescence in M_1 and M_2. **(d)** 2000 ppm DES, Burgundy semi-double inflorescence in M_1 and M_2. **(e)** 2000 ppm DES, Red semi-double inflorescence in M_1 and M_2. **(f)** 3000 ppm DES, Radial pink flowers tinged with light yellow in the tips in M2. **(g)** 4000 ppm DES, Red double inflorescence in M_1 and M_2. **(h)** 4000 ppm DES, Semi-double inflorescence with radial dark red flowers tinged with yellow tops in M_1 and M_2. **(i)** 4000 ppm DES, Orange single inflorescence in M_1 and M_2. **(j)** 4000 ppm DES, Single double inflorescence with radial pink flowers tinged with light yellow in the ends of petals in M_2.

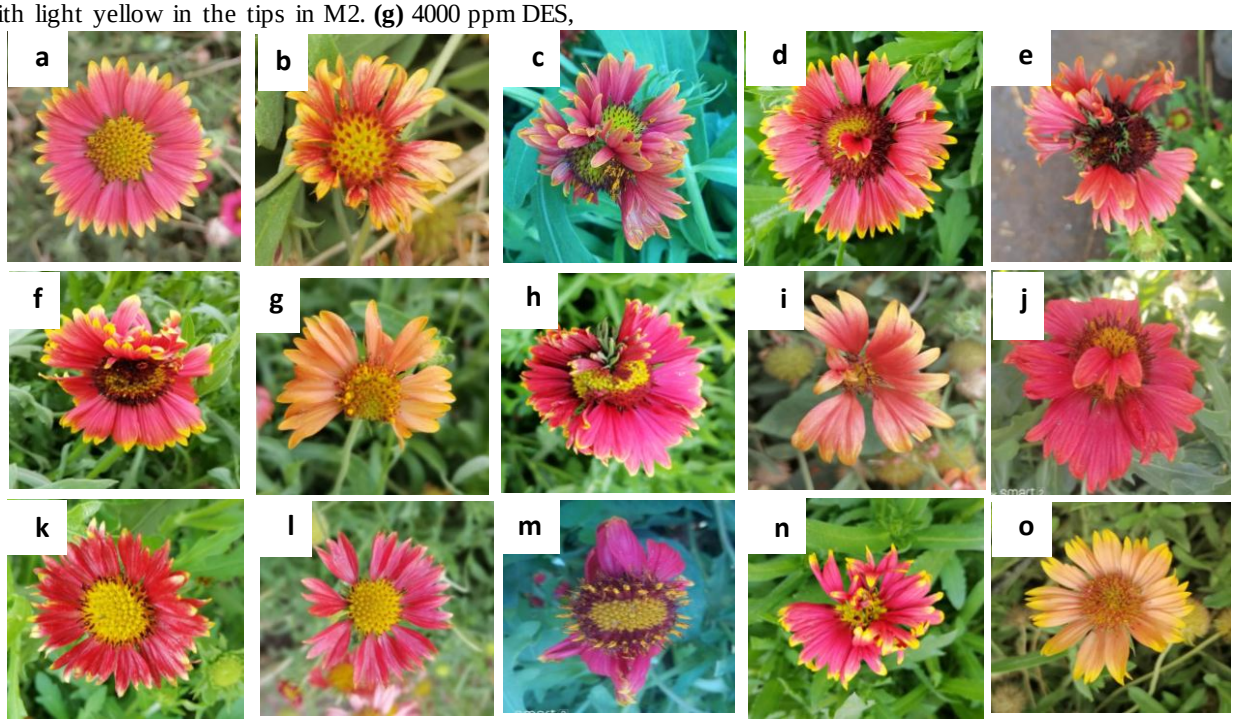

Fig. 5. Showing the deformation of inflorescences abnormalities of G. pulchella as affected by different concentrations of DES on inflorescence in M1 and M2 generations.

(a) Control original color, the outer half yellow and the rest of the ray red; **(b)** 1000 ppm DES, Yellow radial flowers tinged with red at the bottom in M_2; **(c)** 1000 ppm DES, Inflorescence with two disc flowers in M_2; **(d)** 1000 ppm DES, Red radial flowers and a yellow color at the end and the appearance of one petal

in the disc flowers in M_2; **(e)** 1000 ppm DES, Deformed inflorescence in M_2; **(f)** 2000 ppm DES, Fasciation flower in M_1 and M_2; **(g)** 2000 ppm DES, Orange inflorescence in M_1 and M_2; **(h)** 3000 ppm DES, Fasciation flower in M_2; **(i)** 3000 ppm DES, Radial orange flowers tinged with light yellow in M_1 and M_2; **(j)** 4000 ppm DES, Red radial flowers and one petal appear in the disc flowers in M_1; **(k)** 4000 ppm DES, Slender ray flowers in M_2; **(l)** 4000 ppm DES, Slender ray flowers in M_1 and M_2; **(m)** 4000 ppm DES, Fasciation flower in M_1 and M_2; **(n)** 4000 ppm DES, Deformed inflorescence in M_2; **(o)** 4000 ppm DES, Orange radial flowers tinged with yellow in M_1 and G_2.

e. Molecular marker analyses using SRAP markers of plants mutant with DES

Changes in genomic DNA induced by DES led to genetic variations were indicated using SRAP markers, which were carried out by six SRAP primers. Six SRAP primers were used for identifying DNA polymorphism among *G. pulchella* plants mutated by DES and the control.

A total of 32 amplified fragments, ranging from 100 to 930 bp, were recorded. Thirteen amplicons out of 32 loci were polymorphic (40.63%), while 19 fragments were monomorphic (59.38%). The highest number of bands was produced by primers SRAP-2 and SRAP-5 (seven amplicons), followed by primer SRAP-3 (six bands), while the lowest number of amplicons was generated by primers SRAP-1, SRAP-4 and SRAP-6 (four bands). On the other hand, primer SRAP-3 displayed 66.67% polymorphism, followed by primers SRAP-1 and SRAP-6, which gave 50% polymorphism. In contrast, SRAP-5 scored the lowest polymorphism (28.57%). However, no polymorphic bands were scored in primer SRAP-4, it gave only monomorphic bands.The results of SRAP markers indicated that the treatment of *G. pulchella* plants by DES induced the appearance or disappearance of loci compared with the untreated ones (Fig. 6) and (Table 3).

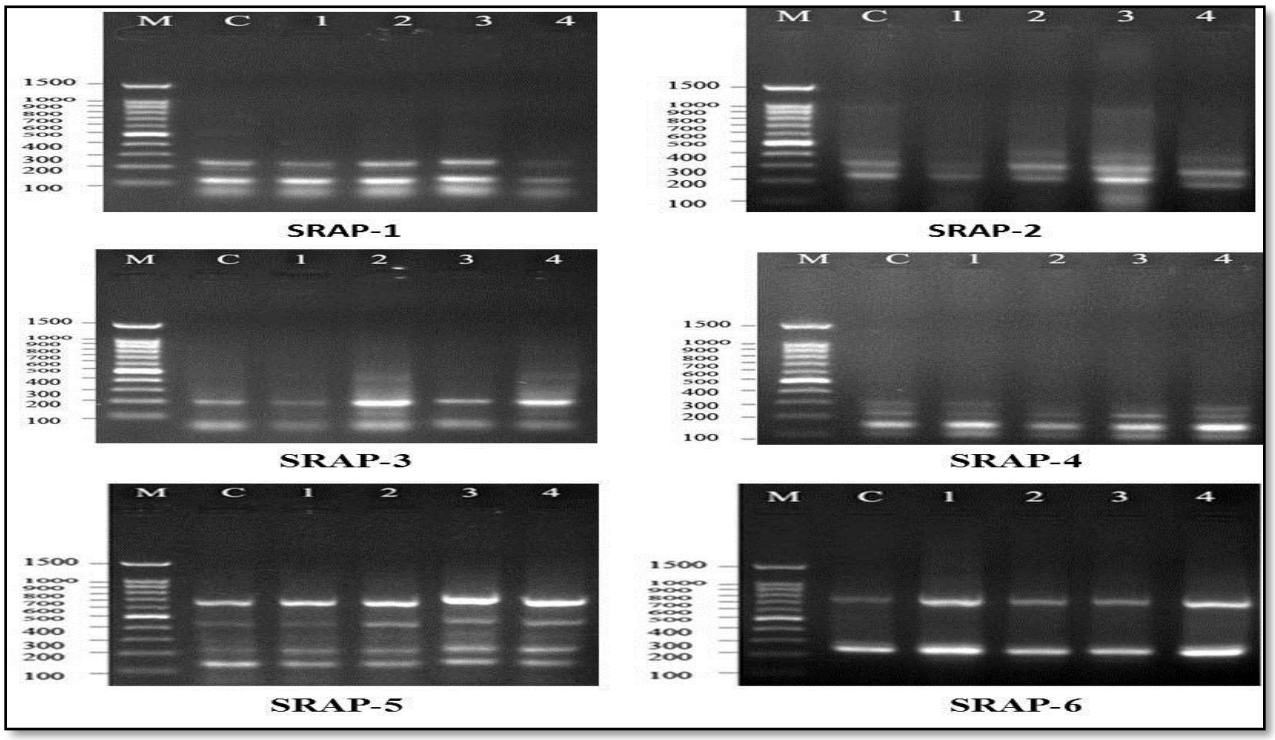

Fig. 6. SRAP-PCR analysis of *G. pulchella* plants mutated with four different concentrations of DES, using primers SRAP-1, SRAP-2, SRAP-3, SRAP-5, and SRAP-6. Lane M: 100 bp DNA ladder; lane C: The control plant; lane 1: 1000 ppm DES; lane 2: 2000 ppm DES; lane 3: 3000 ppm DES and lane 4: 4000 ppm DES.

Table (3): SRAP analysis of *G. pulchella* plants (M_2) mutated by DES.

Primer name	Size range of the scorablebands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	% Polymorphism	Unique markers	Molecular size of markers (bp)
SRAP-1	105-500	4	2	2	50	-2	-190; -500
SRAP-2	100-930	7	4	3	42.86	0	0
SRAP-3	113-600	6	2	4	66.67	0	0
SRAP-4	105-300	4	4	0	0	0	0
SRAP-5	112-610	7	5	2	28.57	+1	+960
SRAP-6	180-790	4	2	2	50	+1	+780
Total	**100-930**	**32**	**19**	**13**	**40.63%**	**4**	**12.5%**

f. Cluster analysis

The genetic identity values among *G. pulchella* plants mutated by DES and the control ranged from 0.67 to 0.89 (Table 4). The lowest genetic similarity was found between the control and 4000 ppm DES (0.67%), while the highest genetic identity was scored between (the control and 3000 ppm DES) and (2000 and 4000 ppm DES) (0.89%) (Table 4). A dendrogram indicated three different groups. The first group (I) involved the individuals treated with 3000 ppm DES and the control. The second group (II) included 1000 ppm DES mutants. The third group (III) contained individuals treated with 2000 and 4000 ppm DES (Fig. 7).

Table (4): Distance matrix depended on Jaccard similarity coefficients in *G. pulchella* plants mutated by DES.

DES Conc.	Control	1000 ppm DES	2000 ppm DES	3000 ppm DES	4000 ppm DES
Control	1.00				
1000 ppm DES	0.81	1.00			
2000 ppm DES	0.70	0.72	1.00		
3000 ppm DES	0.89	0.72	0.79	1.00	
4000 ppm DES	0.67	0.69	0.89	0.70	1.00

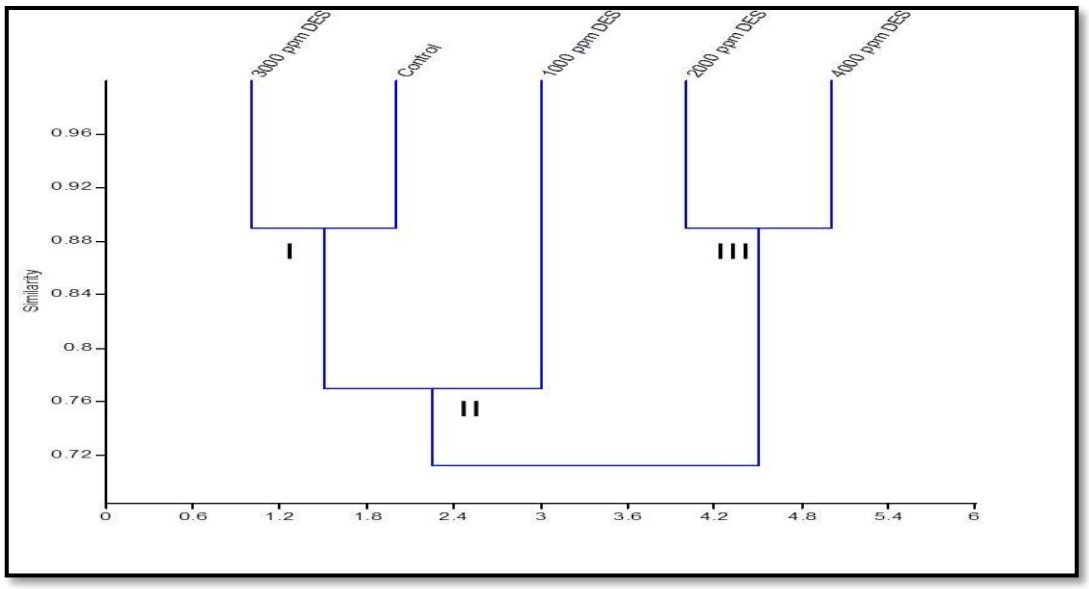

Fig. 7. Dendrogram of *G. pulchella* plants mutated by DES based on Jaccard's similarity coefficients, compared with the control.

CONCLUSION:

This study highlights the genetic enhancement *G. pulchella* plant by chemical mutagens of DES. The results indicated that the low concentrations of (1000 and 2000 ppm) had significant impacts on vegetative growth (plant height and No. of branches per plant), flowering growth (early flowering and an increase in flowers number) in M_1 and M_2 generations, compared to the control. In contrast, the high concentration of 4000 ppm decreased these characters, and prolonged vegetative growth. Also, all concentrations of DES induced many mutations in the shape and structure of leaves and induced color mutants and deformation of the inflorescences compared to the control. On the other hand, SRAP markers were used to confirm the existence of genetic diversity at the genomic DNA level among populations treated with DES and control, depending on the dose. Therefore, SRAP markers are considered an important tool in detecting the mutagenic effects of DES. Also, it will help to discriminate between populations showing mutations in morphological and floral characteristics.

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