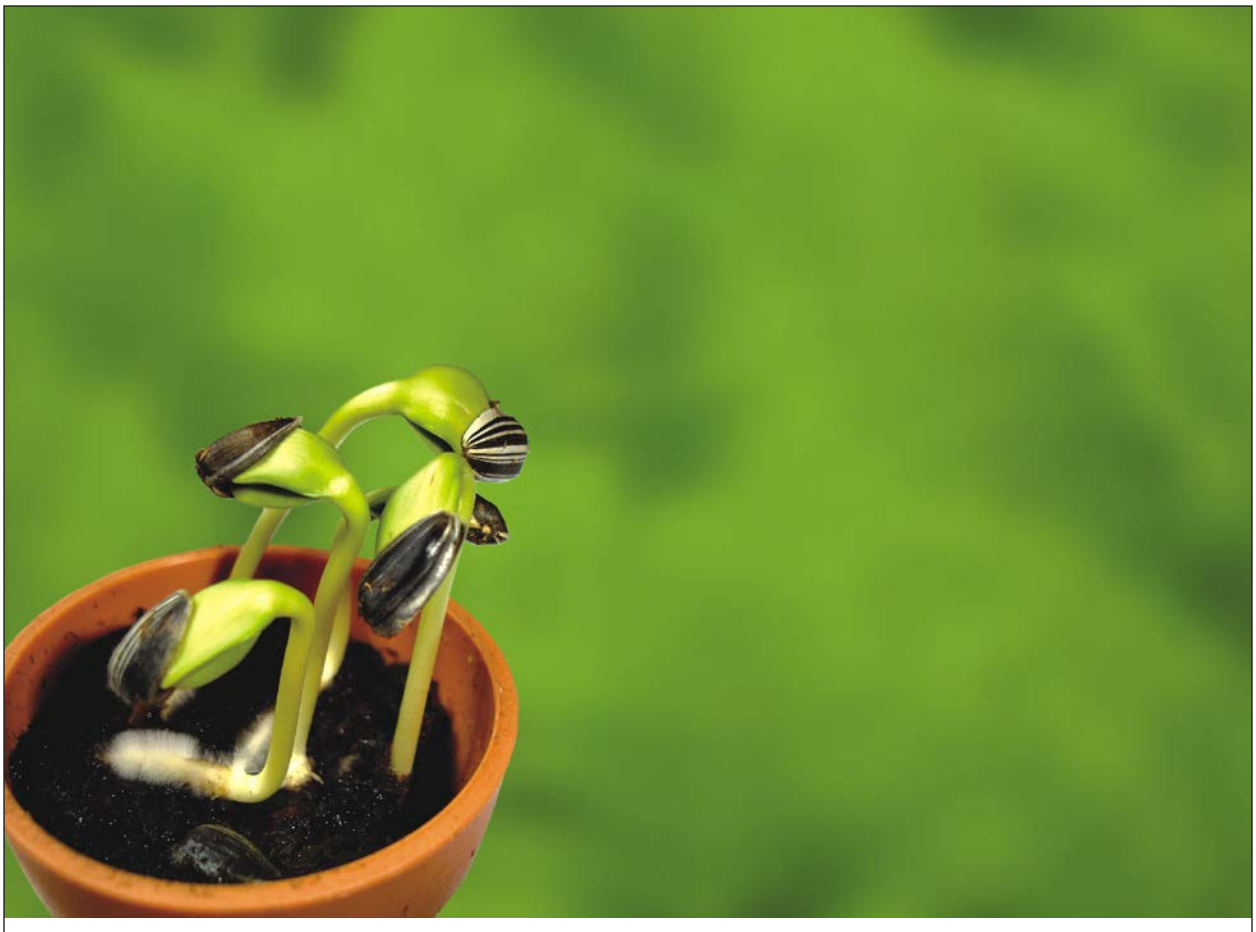

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Induction of Mutations in *Celosia argentea* using Dimethyl Sulphate and Identification of Genetic Variation by ISSR Markers

[1]Gehan G. Mostafa, [2]Asmaa M. Alfrmawy and [3]Hoda E. El-Mokadem
[1]Department of Horticulture (Ornamental Plants), Faculty of Agriculture, South Valley University, Qena, Egypt
[2]Department of Nucleic Acid Research, Mubarak City for Scientific Research and Technology Application, New Borg El-Arab, Egypt
[3]Department of Floriculture, Ornamental Horticulture and Garden Design, Faculty of Agriculture, Alex. University, Alexandria, Egypt

Corresponding Author: Gehan G. Mostafa, Department of Horticulture (Ornamental Plants), Faculty of Agriculture, South Valley University, Qena, Egypt

ABSTRACT

Plants of *Celosia argentea* var. *spicata* were treated with 0, 1000, 2000, 3000 and 4000 ppm. Dimethyl Sulphate (DMS) solution as soil drench to study the effect of DMS on the plant growth and phytochemical composition. Also, to produce new patterns of vegetative and flowering growth and identify them using Inter Simple Sequence Repeat (ISSR) markers. The concentration of 3000 ppm increased significantly the number of leaves, concentration of anthocyanin in the leaves and inflorescences in both generation and the concentration of phenols in the M_2-generation. The treatments of 1000, 2000 and 3000 ppm increased significantly the concentration of alkaloids in the M_2 generation. All treatments decreased significantly stem diameter, length of the inflorescence, delayed flowering in both generations and decreased plant height in the M_1-generation and chlorophyll content in the M_2 generation. Changes of leaf and inflorescence shape were found in the M_2 generation. The treatment of 3000 ppm produced one plant with bigger growth in the M_2 generations. Dwarfed plants were also obtained after treatments of 2000, 3000 and 4000 ppm. Plants with green vegetative growth, yellow nods and lacks of anthocyanin pigments were produced in the M_1 generation after the treatment of 3000 ppm and inherited in the next generation. The percentage of polymorphism of the amplified products was 41.8%. UPGMA dendrogram program classified the samples into two large clusters. The control sample was grouped in cluster I and the five mutants were grouped in cluster II.

Key words: *Celosia argentea*, phenols, mutants, ISSR marker, UPGMA dendrogram

INTRODUCTION

The genus *Celosia* consists of about 60 species belongs to the family Amaranthaceae. It is a native plant in subtropical and temperate zones of Africa, South America and South East Asia. *Celosia argentea* is a cultivated annual plant that is mainly used for planting flowerbeds in different types of gardens. Celosia grown under full light and warm conditions, reach heights up to 71 cm, tolerate a wide range of soil conditions. *C. argentea* is a well known medicinal plant for treating dysentery, diarrhea, acute abdominal pain, inflamed stomach, skin eruptions and exhibited antibacterial activity against, *Bacillus subtilis*, *Salmonella typhi*, *Escherichia coli*, *Agrobacterium tumefaciens* and *Mycobacterium tuberculosis*. *Celosia* is also one of the

main sources of natural pigments used in several industries and the seeds were used in bird-feed for poultry production (Eid *et al.*, 2006).

The curative properties of *C. argentea* are due to the presence of various complex chemical substances of different composition which occur as secondary metabolites. They are grouped as alkaloids, glycosides, flavonoids, saponins, tannins, carbohydrate (Patel *et al.*, 2010).

Alkaloids play some metabolic role and control development in living system (Edeoga and Eriata, 2001). They are also involved in protective function in animals and are used as medicine especially the steroidal alkaloids (Stevens *et al.*, 1992). Alkaloids are known to exhibit marked physiological activity when administered to animals (Okwu, 2004). Pure isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for analgesic antispasmodic and bactericidal effects (Stray, 1998). Phenolic compounds may be the reason for the theraputic, antiseptic, antifungal or bactericidal properties of the plants (Osuagwu *et al.*, 2007).

Chemical mutagenesis is a simple approach to create mutation in plants for their improvement of potential agronomic traits. Mutation methodology has been used to produce many cultivars with improved economic value and to study the genetics and plant developmental phenomena (Aruna *et al.*, 2010).

Dimethyl Sulphate (DMS) is a chemical compound with the formula $(CH_3O)_2SO_2$ which is monofunctional alkylating agents that have been shown to induce mutations, chromosomal aberrations and other genetic alterations in a diversity of organisms. As an alkylating agent DMS is a typical SN_2 agent attacking predominantly nitrogen sites in nucleic acids (Hoffman, 1980).

Inter Simple Sequence Repeat (ISSR) markers have been used with success to identify the mutants and study the genetic diversity of different medicinal plant species and crops (Shafie *et al.*, 2009; Farajpour *et al.*, 2011).

This investigation was carried out to study the effect of dimethyl sulphate on the growth and some phytochemical compositions of *Celosia argentea*, to produce a new pattern of vegetative and flowering growth and identify them using ISSR marker.

MATERIALS AND METHODS

The study was carried out at the Nursery of Floricultural and Ornamental Plants, Faculty of Agriculture, Alexandria University, Alexandria, Egypt during 2 successive generations of 2011 and 2012.

Seeds of *Celosia argentea* var. *spicata* were sown on April 20th 2011 in 20 cm clay pots containing a mixture of clay: sand (1:1 v/v). After two months the seedlings were transplanted into clay pots containing the soil mixture of clay and sand (3:1 v/v). Then plants were treated with dimethyl sulphate solutions (0, 1000, 2000, 3000 and 4000 ppm) as a soil drench (10 mL for each pot). Seeds for the M_2-generation were sown on April 9th, 2012. The procedure of sowing and transplanting were made likewise the first generation.

All plants of the different treatments were examined daily to search for variation in the vegetative and flowering growth.

The experimental layout was a randomized complete block design containing three replications (Steel and Torrie, 1980). Each replication contained five treatments and every treatment consisted of five plants.

Recorded data

- Plant height (cm)
- No. of leaves plant^{-1}
- Stem diameter (cm) at the stem base and above the soil surface

- **Leaf area (cm^2):** The weight in g of one cm^2 of leaves was calculated as an average from two leaves taken from the 7th node of two plants, one leaf was taken from the main stem of each plant as a sample. The leaf area was then expressed as the average mean weight of a leaf divided by the mean weight of one cm^2
- **Flowering date:** Flowering date was calculated as days from seed sowing date to showing colour of inflorescences
- **Length of the inflorescence (cm)**
- **Chlorophyll content:** Total chlorophyll (SPAD units) was determined in the leaves at the flowering stage with SPAD apparatus as described by Yadava (1986)
- **Anthocyanin determination in the leaves:** The procedure of Fuleki and Francis (1968) was used to determine the anthocyanin content in the leaves
- **Anthocyanin determination in the inflorescence:** The determination was done as mentioned by the leaves
- **Alkaloids determination in the leaves:** The quantity of alkaloids in the sample was determined using Osuagwu *et al.* (2007) method. Five gram of the powdered sample was extracted with 10 mL of petroleum ether. The petroleum ether was removed using aspirator. One gram of the extract was suspended in 10 mL of double distilled water and the pH was adjusted to 7.6. After shaking for 1 h, the suspension was centrifuged. One milliliter of the supernatant was diluted to 50 mL with phosphate buffer. The absorbance was measured spectrophotometrically at 580 nm wave length
- **Phenols determination in the leaves:** The quantity of the phenols was determined as the procedure stated by Mostafa and Alhamd (2011)
- **Induction of variations:** All changes in the vegetative and flowering growth were recorded

ISSR analysis was carried out to identify the mutants. The identification and fingerprinting of the mutants was carried out at the Department of Nucleic Acid Research, Mubarak City for Scientific Research and Technology Application, New Borg El-Arab, Egypt.

DNA isolation and ISSR analysis: Genomic DNA was extracted from 1 g of leaf tissue using Biospin Plant Genomic DNA Extraction Kit (BioFlux, China).

Ten anchored ISSR primers were used (Table 1). PCR was performed in reaction volume of 25 μL using 12 ng of DNA of each sample, 25 pmol of each primer, 5X Taq DNA polymerase buffer

Table 1: ISSR primers, sequence, size of amplified fragment (bp), used to analyze genetic relationships among the control and treated plants of *Celosia argentea* var. *spicata*

Primer	Sequence (5'→3')	Size range (bp)
ISSR-1	(GA)8C	300-700
ISSR-2	(CA)8G	200-700
ISSR-3	(CAC)3GC	200-1000
ISSR-4	(GT)6CG	-
ISSR-5	(GT)6CC	100-800
ISSR-6	(GA)6CC	100-1000
ISSR-7	(CA)6GG	100-900
ISSR-8	(CT)8AC	200-700
ISSR-9	(TG)7G	200-900
ISSR-10	(CTC)5TGC	100-1000

(promega) including $MgCl_2$, 0.2 mM dNTPs and 0.5 U μL^{-1} Taq DNA polymerase (promega). ISSR amplification was carried out using Gen Amp PCR system 9700 Thermal cycling programmed with 5 min at 95° C for initial denaturation, followed by 40 cycle of 1 min at 95°C , 1 min at 45° C, 1 min at 72°C and a final extension at 10 min at 72°C. The amplified DNA fragments were separated on 2% agarose gel, stained with ethidium bromide, visualized on a UV Transilluminator and photographed by Gel Documentation system.

ISSR bands were scored as present (1) or absent (0) to form a binary matrix. Cluster analysis was conducted based on genetic similarity estimates using the unweighted pair-group method arithmetic average (UPGMA) procedure in NTSYSpc version 2.1 software package (Rohlf, 2000) in order to deduce genetic relationships among the control and the mutants.

RESULTS AND DISCUSSION

All concentrations of dimethyl sulphate decreased significantly the plant height in the M_1-generation as shown in Table 2, while the concentration of 2000 ppm increased significantly the plant height in the M_2 generation compared to the control (62.2 and 50.3, respectively). These results are in agreement with the finding of Aliyu and Adamu (2007) and Roychowdhury and Tah (2011).

Plants treated with high concentrations of dimethyl sulphate 3000 and 4000 ppm gave the highest number of leaves in both generations compared to the control (201.7, 122.1 and 104.6 for M_1-generation and 200.3, 121.5 and 104.0 for M_2 generation, respectively).

The concentration of 4000 ppm DMS produced the largest leaf area in both generations (20.08 and 19.24 cm^2 for $M_1$and M_2, respectively) compared to control (17.7 and 17.17 cm^2).

The stimulative effect of dimethyl sulphate might be attributed to cell division rates as well as an activation of growth hormones, e.g., auxin (Zaka *et al.*, 2004; Joshi *et al.*, 2011).

All concentrations of dimethyl sulphate decreased significantly stem diameter and length of the inflorescences in both generations as shown in Table 2, while insignificant differences were found in plants treated with 1000 ppm with respect to inflorescences length in the M_1 generation compared to control (9.0 and 10.8, respectively). Low concentration of DMS induced some stimulation effect on plant growth, while the higher concentrations resulted in an inhibiting effect as found by further studies (Mostafa, 2009; Mostafa and Alhamd, 2011; Roychowdhury and Tah, 2011). This inhibition effect can be due to physiological damage produced cumulatively by

Table 2: Effect of dimethyl sulphate concentrations on plant height (cm), number of leaves, leaf area (cm^2), stem diameter (cm), flowering date (days) and length of the inflorescences (cm)

	Plant height (cm)		No. of leaves		Leaf area (cm^2)		Stem diameter (cm)		Flowering date (days)		Length of the inflorescences (cm)	
Treatments	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
Control	49.3^{a}	50.3^{b}	104.6^{c}	104.0^{bc}	17.7^{b}	17.17^{ab}	0.90^{a}	1.10^{a}	118.7^{d}	104.7^{c}	10.8^{a}	16.0^{a}
1000 ppm	43.8^{b}	52.1^{ab}	93.0^{d}	89.5^{bc}	12.8^{c}	12.54^{bc}	0.60^{b}	0.77^{b}	121.0^{b}	122.2^{a}	9.0^{ab}	11.6^{b}
2000 ppm	41.7^{b}	62.2^{a}	72.8^{e}	71.6^{c}	17.6^{b}	17.91^{a}	0.61^{b}	0.81^{b}	120.5^{bc}	115.4^{b}	8.0^{bc}	10.9^{b}
3000 ppm	37.4^{c}	41.0^{b}	201.7^{a}	200.3^{a}	12.6^{c}	12.99^{c}	0.63^{b}	0.86^{b}	119.7^{cd}	117.2^{ab}	5.1^{d}	7.8^{c}
4000 ppm	43.4^{b}	45.0^{b}	122.1^{b}	121.5^{b}	20.0^{a}	19.24^{a}	0.61^{b}	0.88^{b}	122.8^{a}	104.8^{c}	6.5^{cd}	12.3^{b}
$LSD_{0.05}$	4.3**	11.6*	3.1**	38.1**	1.4**	4.3*	0.17*	0.18*	1.1**	5.9**	2.0**	2.3**

Values in the same column not followed by the same letter are significantly different at the 5% level of probability
*, **Significant at p = 0.05 and 0.01, respectively

Table 3: Effect of dimethyl sulphate concentrations on chlorophyll content, concentration of the anthocyanin in the leaves and inflorescences, concentration of the alkaloids and phenols

	Chlorophyll content (SPAD unit) leaves		Concentration of the anthocyanin in the leaves (mg mL^{-1})		Concentration of the anthocyanin in the inflorescences (mg mL^{-1})		Concentration of the alkaloids (mg mL^{-1})		Concentration of the phenols (mg mL^{-1})	
Treatments	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2	M_1	M_2
Control	37.7^{a}	45.6^{a}	25.7^{c}	34.9^{c}	166.0^{d}	168.8^{e}	0.324^{a}	0.216^{c}	1.164^{a}	1.492^{b}
1000 ppm	27.9^{b}	44.2^{ab}	36.8^{b}	37.1^{b}	232.7^{b}	242.7^{b}	0.292^{a}	2.758^{a}	1.154^{a}	1.515^{b}
2000 ppm	36.0^{a}	39.5^{bc}	22.7^{d}	41.9^{a}	168.6^{d}	179.6^{d}	0.355^{a}	1.478^{b}	1.103^{a}	1.458^{b}
3000 ppm	43.0^{a}	34.8^{c}	38.9^{a}	41.4^{a}	250.0^{a}	265.0^{a}	0.308^{a}	1.494^{b}	1.215^{a}	1.621^{a}
4000 ppm	43.2^{a}	36.5^{c}	37.3^{b}	33.8^{c}	208.2^{c}	219.1^{c}	0.199^{b}	0.233^{c}	1.296^{a}	1.458^{b}
$LSD_{0.05}$	7.6**	5.5**	1.6**	2.1**	16.6**	8.3**	0.090*	0.949**	NS	0.102*

Values in the same column not followed by the same letter are significantly different at the 5% level of probability
NS: Not significant, *, **Significant at p = 0.05 and 0.01, respectively

increased chemical mutagen concentrations. Reduced growth due to higher doses was explained differently by different workers. It may be attributed to one or more of the following reasons: The increase in destruction on growth inhibitors, drop in the auxin level or inhibition of auxin synthesis and decline of assimilation mechanism as reported by Roychowdhury and Tah (2011).

Concerning to flowering date, significant differences were obtained among the treatments in both generations. In general all treatments delayed flowering in both generations as shown in Table 2. This result agrees with the results of Mostafa and Alhamd (2011) and Gad (2012).

The data shown in Table 3 indicated that, chlorophyll content was decreased gradually with increasing the concentration of dimethyl sulphate in the M_2-generation. The concentration of 1000 ppm DMS decreased significantly chlorophyll content in the M_1-generation compared with the control (27.9 and 37.7, respectively). But insignificant differences were found between the other treatments compared to the control.

These slightly decrease in chlorophyll content with high concentrations of dimethyl sulphate was supported by the results of Pandey *et al.* (2012) where they found chlorophyll damage caused by various physical and chemical mutagens.

The concentrations of 3000, 4000 and 1000 ppm. DMS increased significantly the concentration of anthocyanin in the leaves in the M_1 generation compared to control (38.9,37.3,36.8 and 25.7 mg mL^{-1}) as shown in Table 3. In the M_2 generation, the concentrations of 2000, 3000 and 1000 ppm. DMS increased significantly the concentration of anthocyanin in the leaves compared to control (41.9, 41.4, 37.1 and 34.9 mg mL^{-1}).

All treatments increased significantly the concentration of anthocyanin in the inflorescences in both generations except that of the 2000 ppm. DMS (168.6 mg mL^{-1}) compared to control (166.0 mg mL^{-1}) in the M_1- generation.

The results of the alkaloids concentration for both generations are presented in Table 3. The results showed that the concentrations of 1000, 2000 and 3000 ppm did not differ significantly from the control in the M_1-generation, while the concentration of 4000 ppm gave the lowest value (0.199) and differed significantly from the control (0.324). For M_2 generation, the concentrations of 1000, 2000 and 3000 ppm DMS increased significantly the alkaloids concentration compared to the control (2.758, 1.478, 1.494 and 0.216, respectively).

An increase in the alkaloids concentration was found in the second generation with comparable to first generation with using 1000, 2000 and 3000 ppm dimethyl sulphate, this may be due to a recessive mutant which was not indicated in the M_1 generation. Mutations are mostly recessive and they cannot be selected until the second generation as reported by Toker *et al.* (2007). Similar results were reported by Gad (2012).

No significant differences were obtained among the treatments in the M_1-generation with respect to the phenols concentration. In the M_2 generation, the concentration of 3000 ppm DMS increased significantly the phenols concentration compared to the control (1.621 and 1.492 mg mL^{-1}, respectively).

The treatments of 2000, 3000 and 4000 in the first generation and 1000, 2000 and 3000 ppm in the second generation produced changes in the leaf form as shown in Fig. 1 and 2. These changes of leaf form or shape may be due to chromosomal disturbances. Also these changes could be referred to the layer rearrangement as a result of the chemical mutagens effect (El-Nashar, 2006; Mostafa, 2009).

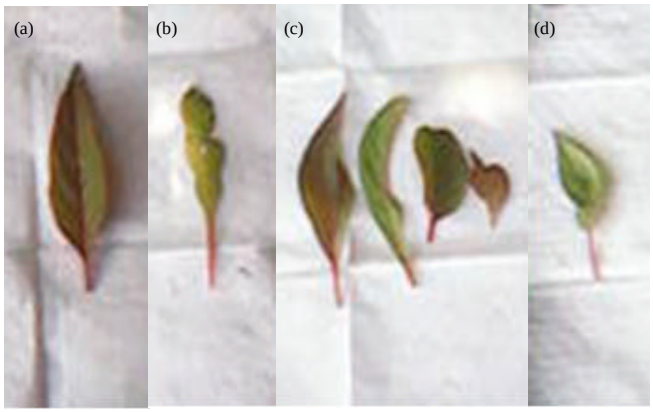

Fig. 1(a-d): Changes in the leaf form in the M_1-generation as a result of the treatment with dimethyl sulphate, (a) Control, (b) 2000, (c) 3000 and (d) 4000 ppm

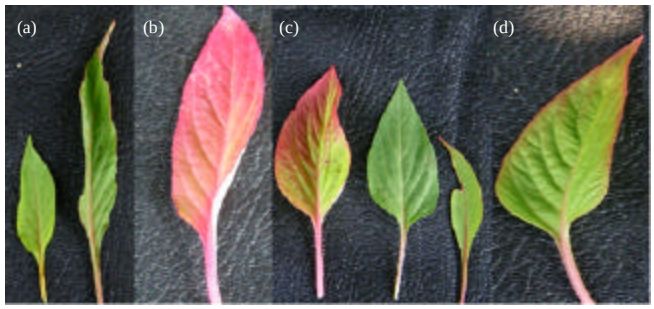

Fig. 2(a-d): Changes in the leaf form in the M_2-generation as a result of the treatments with dimethyl sulphate, (a) Control, (b) 1000, (c) 2000 and (d) 3000 ppm

The treatment of 3000 ppm produced one plant with greater growth (Taller and thicker branches) than the control as shown in Fig. 3. The stimulatory effect of the mutagen may be attributed to the increase in the rate of cell division or cell elongation as reported by Joshi *et al.* (2011).

Dwarfed plants were obtained from the plants treated with 2000, 3000 and 4000 ppm in both generations as shown in Fig. 4. This dwarfed growth may be due to physiological damage resulted in the alteration from normal to dwarf growth. Joshi *et al.* (2011) explained the dwarfed growth to auxin destruction, changes in ascorbic acid content and physiological and biochemical disturbances.

Fig. 3(a-b): Plant growth in M_2-generation as a result of the treatment with dimethyl sulphate at 3000 ppm, (a) Control and (b) plant with greater growth

Fig. 4(a-d): Dwarfed plants obtained in the M_2-generation as a result of the treatments with dimethyl sulphate (a) Control, (b) 2000, (c) 3000 and (d) 4000 ppm

Many changes of inflorescences shape were found after the treatments of 2000, 3000 and 4000 ppm in the M_2-generation as shown in Fig. 5 and 6. These abnormalities are inflated of inflorescence, splitting inflorescence apex and later splitting. Splitting the inflorescence may be due to a gene mutant resulted in floral meristem being replaced with meristems that have some or all of the characteristics of inflorescence. In this case there is a failure, or delay in the production of flowers and a proliferation of inflorescence-like structures in their place as reported by Coen and Carpenter (1993).

Plants with many inflorescences produced at the main stem directly from the lateral bud without slightly branches were found in the M_2-generation after the treatment of 3000 ppm as shown in Fig. 7.

Two plants having green vegetative growth (branches and leaves) with yellow nodes were found in the M_1-generation after the treatment of 3000 ppm as shown in Fig. 8. Lacks of anthocyanin pigments in the vegetative growth mutant was transmitted to the M_2- generation.

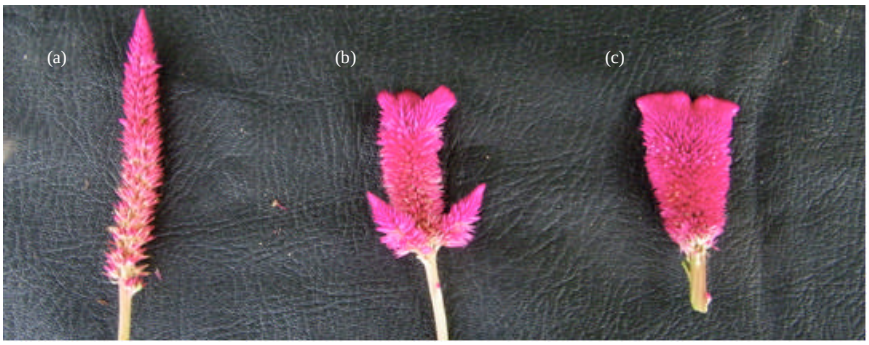

Fig. 5(a-c): Changes in the inflorescence form in the M_2-generation as a result of the treatments with dimethyl sulphate, (a) Control, (b) 2000 and (c) 3000 ppm

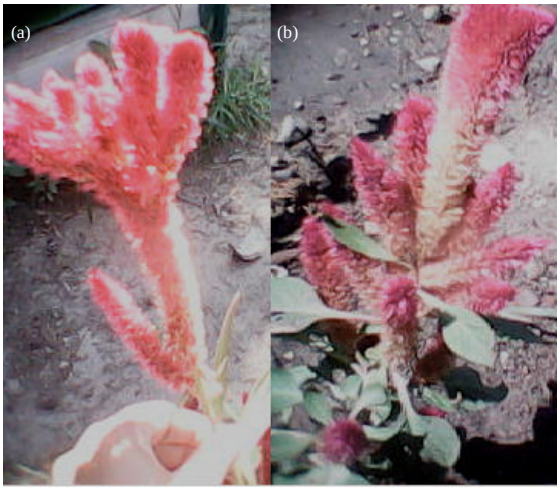

Fig. 6(a-b): Changes in the inflorescence form (a) Splitting apex and (b) Bases of the inflorescences in the M_2-generation as a result of the treatment with dimethyl sulphate at 4000 ppm

Fig. 7(a-b): Plants with many inflorescences at the main stem in the M_2-generation as a result of the treatment with dimethyl sulphate, (a) Control and (b) 3000 ppm

Fig. 8: Plants with green vegetative growth and with yellow nods in the M_2-generation as a result of the treatment with dimethyl sulphate at 3000 ppm

Five mutants were used to identify them from the control (Table 4) using Inter Simple Sequence Repeat (ISSR) markers.

The genetic relationship among control and mutants were evaluated using ISSR assay. Ten ISSR primers amplified a total of 74 bands, 31 of them were polymorphic. The percentage of polymorphism of the amplified products was 41.8% (Table 5). The size of amplified bands ranged from 100-1000 bp (Fig. 9). Primer ISSR4 did not yield clear bands and primer ISSR5 did not

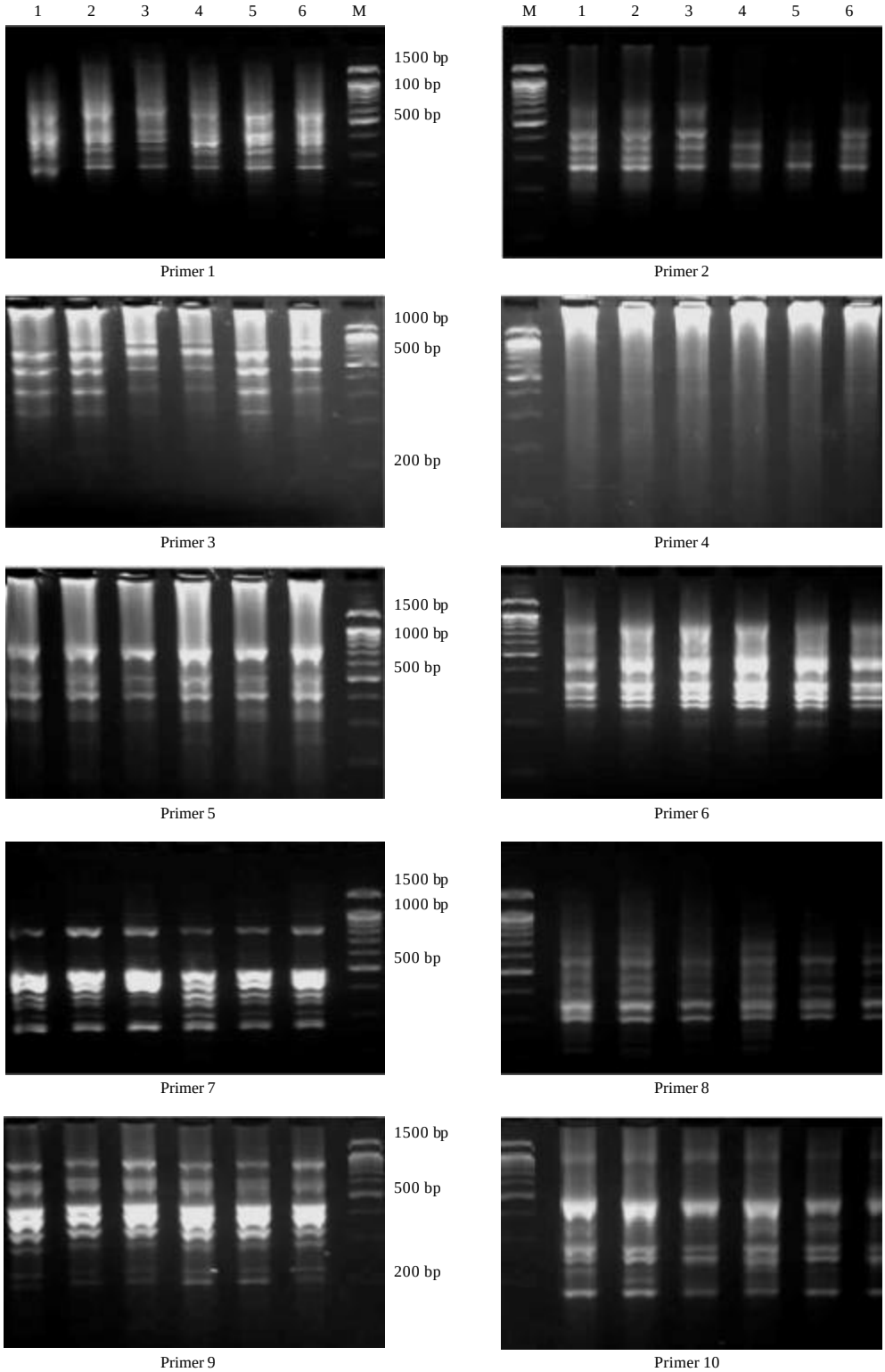

Fig. 9: ISSR patterns of *Celosia argentea* var. *spicata* generated by primer ISSR (1-10). Lane 1: Control, lanes 2-6: The mutants plants produced by dimethyl sulphate and M: 100 bp DNA ladder, respectively

Table 4: Description of the mutants

Sample No.	Variation describe
1	Normal plant as a control
2	Having green vegetative growth with yellow nodes (reddish color disappeared)
3	Plant with greater growth (Taller and thicker branches)
4	Dwarfed plant
5	Inflated of inflorescence and stem
6	Splitting inflorescence and leaf shape abnormalities

Table 5: Number of amplified and polymorphic bands and percentage of primer polymorphism used to analyze genetic relationships among the control and mutants of *Celosia argentea* var. *spicata* produced by ISSR marker using ten primers

Primer	No. of amplified bands	No.of polymorphic bands	Primer polymorphism (%)
ISSR-1	10	9	60.0
ISSR-2	8	3	37.5
ISSR-3	5	2	40.0
ISSR-4	-	-	-
ISSR-5	4	0	0.0
ISSR-6	9	2	22.2
ISSR-7	10	4	40.0
ISSR-8	10	5	50.0
ISSR-9	9	2	22.2
ISSR-10	9	4	44.4
Total	74	31	41.8

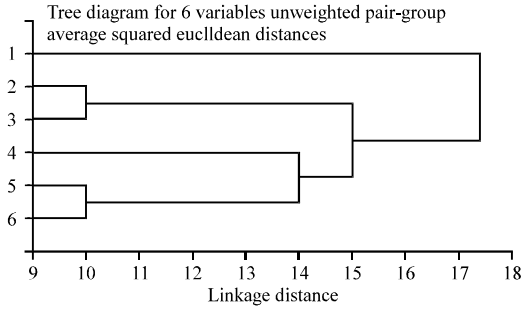

Fig. 10: Dendrograme constructed on the basis of ISSR profile for the control and 5 mutants of *Celosia argentea* var. *spicata* using ten ISSR primer

produced polymorphic band patterns. The largest number of polymorphic bands was nine bands with the primer ISSR1, while primers: ISSR3, ISSR6 and ISSR9 generated low number of polymorphic bands (two bands) as shown in Table 5. UPGMA dendrogram program which constructed with coefficient of similar matrix (Table 6) based on ISSR data classified the samples into two large clusters: Cluster I and II. The control sample was grouped in cluster I and the other five different morphological characters are were grouped in cluster II (Fig. 10). These results referred to that those morphological characters genetically different from the control. These variation came from mutagenic effect of dimethyl sulphate treatments as an alkylating agents which led to induce chromosome damage, chromosom

Table 6: Genetic similarity of DNA among five mutants and control plants of *Celosia argentea* produced by dimethyl sulphate treatments

	1	2	3	4	5	6
1	100.0					
2	74.6	100.0				
3	66.1	83.0	100.0			
4	74.6	71.8	80.0	100.0		
5	75.0	77.7	69.2	75.0	100.0	
6	79.6	49.4	76.2	79.6	83.0	100.0

aberrations and base pair substitution, resulting in amino acid change which change the function of proteins (Khan and Tyagi, 2009).

CONCLUSION

It is quite evident that, dimethyl sulphate could be suitable for inducing genetic variability in the natural gene pool of *Celosia*. It is also appropriate to induce valuable mutants. It can be concluded that also, ISSR marker can be used for the identification and fingerprinting the mutants of *Celosia argentea*.

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