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INDUCED CHEMICAL MUTATION IN *LIATRIS LIGULISTYLIS* AND *LIATRIS PYCNOSTACHYA*

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ABSTRACT

The objective of the presented study was to induced mutation in two liatris species *L. ligulistylis* and *L. pycnostachya* using ethyl methane-sulfonate (EMS) at a concentration of 2.0 and 5.0 mM and characterization of the obtained mutants. The resulting phenotypic changes observed in liatris were mainly related to anthocyanin discoloration on shoots and inflorescence petioles, changes in the anatomy of inflorescence petioles and leaves, as well as the uneven rising of inflorescence buds. The EMS mutagen concentrations used in the experiment revealed an inhibitory effect on features such as the height and width of plants, the circumference and weight of bulbs and the number of buds on the bulb. It was found that, the frequency of all observed phenotypic changes in *L. ligulistylis* and *L. pycnostachya* was higher after the application of 2.0 mM EMS solution (11 and 3%, respectively).

For the assessment of genotypic changes at the DNA level the inter simple sequence repeat (ISSR) markers were adopted. Based on the dendrogram produced from a study analysis of the similarity coefficients using UPGMA it was found that the mutants under study showed a 43.5–75.7% similarity to each other.

Key words: mutants, DNA, EMS, ISSR analyssis, liatria.

INTRODUCTION

Liatris (*Liatris*) is characterized as ornamental plant with high aesthetic values. However, due to the only marginal interest among breeders, it is a little-known species. The genus *Liatris* belongs to the *Asteraceae* family and contains about 30 species.At least 13 species and several hybrids are grown as garden plants [7]. In the literature, there are few reports on the induction of mutations in this species. Chemical mutagenesis constitutes an approach allowing for an expansion of the range of variability, as well as for the provision of basic material for the breeding of new variants [10]. According to several authors [5, 18, 20], most mutational changes can be achieved by physical agents such as X-ray, gamma and neutron radiation. Of the great number of chemical compounds, the differences between which have proved to be mutagenic, only very few are used in applied mutagenesis. The most important ones include: EMS, DES (diethyl sulphate), sodium azide and other related agents. EMS has been widely used in plants because it case a high frequency of gene mutations and a low frequency of chromosome aberrations [5].

The proper evaluation of reactions to mutagenic agent response and the choice of ornamental plant mutants obtained in this manner depend on the availability of reliable diagnostic methods [12]. However, as each molecular marker has its own advantages and disadvantages, the sensitivity of mutant detection is quite different. ISSR markers are one of the screening techniques frequently used in genotyping [8].

The presented studies aimed to determine the phenotypic and genotypic changes in *Liatris ligulistylis* and *Liatris pycnostachya* using ethyl methane-sulfonate (EMS) at a concentration of 2.0 and 5.0 mM.

MATERIAL AND METHODS

The plant material consisted of seeds of twoliatris species sourced from the Jelitto seed company.

Mutagenic treatment

EMS solution in two concentrations of 2.0 and 5.0 mM, respectively, and prepared in phosphate buffer solution at pH 4 was used to induce mutations. Seeds of*L. pycnostachya* and *L. ligulistylis* (150 seeds per each concentration) were soaked in EMS mutagen solutions. The experimental control sample consisted of seeds soaked in sterile water. After 1h, the seeds were three times rinsed with sterile water and disinfected with 10% solution of sodium hypochlorite (NaOCl) for 15 minutes. Then, they were washed three times with sterile water and seeded on a multiple tray (with 2.5 × 2.5 cm openings) filled with deacidified peat. Multiple trays were placed in a growth room at a temperature of 22 ± 1ºC and 16 h lighting (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 10 days, the percentage of germinating seeds were performed. Nine-week-seedlings were transferred to soil and left for further growth. During the vegetative growth cycle, plants were fertilized with ammonium nitrate (2.0 $g \cdot m^{-2}$).

Before the end of the vegetative growth cycle, biometric measurements establishing the height and width of the plants, the circumference and weight of the bulbs, and the number of the buds on the bulb were evaluated. During the experiment, phenotypic assessment was evaluated successively. Among liatris mutants of different phenotypes than those of the control, leaves were collected for experiments at the DNA level.

ISSR analyssis

The genetic differences between control and mutants of liatris were determined using the ISSR technique. DNA was prepared from 100 mg of fresh leaves using liquid nitrogen and Genomic DNA Prep Plus kit (A&A Biotechnology). To determine the quantity and purity of each DNA samples, a GeneQuant RNA/DNA Calculator (Pharmacia LKB) spectrophotometer was used. PCR mixtures (25 µl) contained: 1.5 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl, pH 8.3, 0.1% Triton X-100, 0.2 µM of each dNTPs, 1.0 unit of*Taq* DNA polymerase (Thermo Scientific) and 50 ng template genomic DNA. 27 ISSR primers were used (UBC Canada). Amplifications were performed according to [26] protocol. The PCR products were electrophoresed in 2% agarose gel in presence of ethidium bromide (5.0 $mg \cdot dm^{-3}$) then UV visualized and photographed using a Minibus Pro DNR camera (Bio-Imaging System Ltd Israel). O'RangeRuler 200bp DNA Ladder (Thermo Scientific) was used as a size markers (3000 – 200 bp). The relative mobility position of all bands for each analyzed samples was calculated and transformed in a data matrix in which the character "1" mean the presence of a specific band and "0" represent its absence.

Data analysis

Gel Quant (Bio-Imaging System Ltd Israel) and Diversity One 1.3 (Pharmacia LKB) softwares were used to construct dendrogram by the UPGMA (unweighted pair group with arithmetic mean) method. The strength of the internal branches from the resulting tree were tested by TREECON bootstrap analysis application using 2000 replicates [24].

RESULTS AND DISCUSSION

Chemical and ionizing radiation mutagenesis have been routinely used to generate genetic variability for practical breeding and genetic studies, especially in ornamental crops [6, 11, 17]. Induced mutation are highly effective to enhance natural genetic resources and have successfully assisted in developing improved and new cultivars among both seed and vegetatively propagated crops [6]. Mutants induced by gamma radiation are often generated by deletion of large DNA fragments, whereas chemical mutagens EMS alkylates DNA and causes base mispairing (mutation) during polynucleotide synthesis. As compared with physical mutagens, chemicals may give rise to relatively more gene mutations rather than chromosomal changes [1, 19]. The enhancement of mutation frequency and alteration of mutation spectrum in a predictable manner are two important goals of mutation research [25].

Phenotype changes

In the present study, for each combination of the experiment, 150 *L. ligulistylis* and *L. pycnostachya* seeds were counted out. A comparable sample size was adopted by [2] while analyzing between 105 and 225 banana (*Musa spp.,* AAA Group) apical meristems, [3] – 250 vining morning glory (*Ipomea purpurea*) seeds and [10] – 150 petunia (*Petunia* × *atkinsiana* D.Don) seeds. According to [15], even with a relatively small number of plants, mutational changes can be observed after the application of EMS.

The concentrations of EMS mutagen used in the experiment exhibited a negative effect on the seed germination capacity of both *L. pycnostachya* and *L. ligulistylis* (Tab. 1). The lowest germination capacity (47%) was observed for *L. ligulistylis* seeds soaked in 2.0 mM EMS solution, the highest (84%) in 5.0 mM EMS solution. In terms of*L. pycnostachya* seeds, an inverse relationship was observed. Higher germination (92%) was found after treatment with the solution of 2.0 mM EMS as compared to seeds treated with 5.0 mM of mutagen solution (59%).The results of analysis presented here are in concordance with results obtained by [23] and [10]. Similar results were presented for genus *Petunia.*

Tab. 1. The seed germination capacity [%] of *L. ligulistylis* and *L. pycnostachya*

Species	Germination ability [%]		
	control	2.0 mM EMS	5.0 mM EMS
L. ligulistylis	100	47	92
L. pycnostachya	100	84	59

mean	100	65.5	75.5

In terms of the concentrations of chemical mutagen used to induce mutation in *L. ligulistylis* and *L. pycnostachya* most of the phenotype changes were observed on shoots and inflorescence petioles.

These changes were manifested as an anthocyanin coloration of inflorescence shoots (Fig. 1a) which differed from that of the controls (Fig. 1d), crooked inflorescence petioles and down-directed leaves (Fig. 1b), and irregular growth of the inflorescence buds (Fig. 1c). In addition, both liatris species grown from seeds treated with EMS solution blossomed seven days later than the control. The mutagen used in this experiment significantly decreased *L. pycnostachya* plant resistance to low temperatures, in relation to the control. This resulted in the appearance of anthocyanin discolorations on the whole plant and a clearly weaker growth speed. The frequency of all observed phenotypic changes in *L. ligulistylis* and *L. pycnostachya* was higher after the application of 2.0 mM EMS solution for the induction of mutation, and was estimated at 11 and 3%, respectively. Among liatris grown from seeds treated with 5.0 mM of mutagen concentrate, plants of phenotypes different from that of the control were described only for *L. ligulistylis*. The frequency of these changes was 8%. Similar results obtained in the present work have been described by [22, 23] and [10] in petunia, [17] and [13] in chrysanthemum, [9] in tillandsia and [11] in kalanchoe.

Fig. 1. *L. ligulistylis* with phenotype variations a) anthocyanin coloration of inflorescence shoots; b) crooked inflorescence petioles and down-directed leaves; c) irregular growth of the inflorescence buds; d) control

Our results showed that the mutagen concentrations used in the experiment had an inhibitory effect on features such as the height and width of plants, the circumference and weight of bulbs, and the number of buds on the bulb. Plants of both liatris species were lower than were seen in the control. *L. ligulistylis* was lower in comparison to the control by 17 and 32% (plants derived from seeds treated with 5.0 and 2.0 mM EMS solution, respectively) and *L. pycnostachya* by 39% (Tab. 2). Plant width (measured at the widest point) for *L. ligulistylis* remained at the same level as for the controls (25.4 cm); however, *L. pycnostachya* was narrower than the control by 29 and 38% (plants derived from seeds treated with 2.0 and 5.0 mM EMS solution, respectively). Moreover, the plants obtained from seeds treated with EMS solution developed smaller bulbs in comparison to the control (Tab. 2). Only in case of *L. ligulistylis*, the circumference of bulbs was similar to that for the control after the application of 5.0 mM mutagen concentrate (98% of control). The circumference of bulbs in the remaining mutants was reduced by 23–42% in comparison to the control. A similar association was observed for the weight of knotted bulbs. *L. pycnostachya* mutants obtained from seeds treated with 2.0 mM EMS solution (27% of control) produced the smallest bulbs,

the largest were produced by *L. ligulistylis* obtained from seeds treated with 5.0 mM EMS solution (98% of the control). No significant differences in terms of the number of knotted buds on bulbs were observed between the obtained mutants and the control (Tab. 2).

Tab. 2. Mean values of morphological traits of *L. ligulistilis* and *L. pycnostachya* after mutagenic treatment

Species		Morphological traits									
		Plants height [cm]		Width of plants [cm]		Circumference of bulbs [cm]		Weight of bulbs [g]		Number of buds on the bulb	
Liatris ligulistilis	Control	18.0	a*	25.4	a	12.9	a	19.7	a	4.3	a
	2.0 mM EMS	12.3	c	23.7	c	9.9	c	12.4	c	3.5	bc
	5.0 mM EMS	15.0	b	24.9	ab	12.6	a	19.3	a	3.3	c
	Mean	15.1	b	24.7	b	11.8	b	17.1	b	3.7	b
	LSD0.05	**0.36**		**0.47**		**0.23**		**0.34**		**0.29**	
Liatris pycnostachya	Control	41.9	a	46.0	a	19.4	a	73.1	a	5.0	a
	2.0 mM EMS	26.0	c	32.8	c	11.3	c	19.8	d	4.9	a
	5.0 mM EMS	25.4	c	28.6	d	11.9	c	25.6	c	4.5	a
	Mean	31.1	b	35.8	b	14.2	b	39.5	b	4.8	a
	LSD0.05	**0.87**		**0.51**		**0.82**		**0.61**		**0.74**	

*Means in the same column followed by the same letter are not significantly different ($p < 0.05$; Least Significant Differences test LSD)

Other observations in comparison to those from our study were described by [10, 11] for petunia and kalanchoe. The results from these latter studies revealed that the mutagens used in the study had a stimulating effect on features such as height, number of flowers and the length of roots for both petunia and kalanchoe. Mutants examined by the author outweighed the control in terms of the analyzed features by 5 to 90% for petunia and by 20 to 80% for kalanchoe.

Genotype changes

The use of molecular markers such as RAPD (Random Amplified Polymorphic DNA) or ISSR to detect genetic variability induced by mutagenic treatment has been widely demonstrated [4, 10, 14, 16, 23]. Molecular techniques provide different approaches to identify variability among individuals. The choice of technique for any given application depends upon the material used and the nature of the question being addressed [21].

In our study, the ISSR technique was used for the assessment of genetic similarity between the obtained mutants and the control. 7 plants (4 – *L. ligulistylis* and 3 – *L. pycnostachya*) were selected for the analysis. Among the 27 ISSR primers, eight generated visible products (Tab. 3). In total, for the liatris genotypes under study, 55 ISSR products were obtained, of which 23 (41.8%) were either polymorphic or monomorphic, and 9 (16.4%) were species-specific. The most polymorphic ISSR products (6) were obtained after the application of primer 840 for amplification. The use of five of the ISSR primers, i.e. 801, 810, 815, 820 and 823, generated genotype-specific products (Tab. 4). In reaction with one primer a mean of 7 ISSR loci were amplified from 500 to 3000 bp long. The longest product (3000 bp) was obtained when using primers 810, the shortest (500 bp) – primer 823. The highest number of polymorphic loci (6), from 1600 to 2700 bp long, were obtained with the use of primer 840. The least (1) polymorphic product visible on agarose gels were obtained when primers 810 and 815 were used for ISSR (respectively: 2700 and 2100 bp).

Tab. 3. A characteristic of ISSR-PCR products obtained for two analyzed liatria species

Primer no.	Primer sequences 5'–3'	Fragment size range (bp)	Number of amplified loci											
			Total	Mono-morphic	Poly-morphic	Accession-specific	*L.ligu-listylis*	*L.ligu-listylis*	*L.ligu-listylis*	*L.ligu-listylis*	*L.pycno-stachya*	*L.pycno-stachya*	*L.pycno-stachya*	Generated amplicons
801	(AT)8T	1000–2200	5	1	3	1	4	4	5	1	4	4	4	26
810	(GA)8T	1800–3000	5	3	1	1	4	3	5	3	3	4	4	26
815	(CT)8G	800–2800	8	4	1	3	5	4	4	4	4	7	6	34
820	(GA)8YC	1000–2700	9	3	3	3	5	4	4	8	5	3	3	32
823	(TC)8C	500–2400	6	3	2	1	4	4	5	5	4	5	4	31
840	(GA)8GT	1600–2700	6	0	6	0	2	2	3	2	4	4	4	21
847	(CA)8RC	800–2400	8	5	3	0	5	5	5	5	6	8	8	42
848	(CA)8RG	1000–2300	8	4	4	0	7	7	7	5	6	8	6	46
Total			55	23	23	9	36	33	38	33	36	43	39	258
Mean			7	3	3	1	5	4	5	4	5	5	5	33

Tab. 4. ISSR accession-specific products amplified for 7 accession of liatris

Accession [no. of accession]	Primers and generated products
L. ligulistylis [3]	801[1600], 810[2600]
L. ligulistylis [4]	820[2700, 2600]
L. pycnostachya [5]	820[1200]
L. pycnostachya [6]	815[1800, 1000], 823[1400]
L. pycnostachya [7]	815[800]

The ISSR markers for the assessment of the genetic variability of the mutants and control of petunia was also investigated by [23]. In the ISSR reaction, 309 amplification products were generated, of which 83.4% were polymorphic products. Similarly, [10] applied 37 primers to assess the genetic similarity of 30 mutants and control plants of petunia of the M_1 generation, and 13 of the M_2 generation. The frequency of the occurrence of polymorphic products ranged from 96.7% (M_1) to 68.3% (M_2), and genotype-specific products, which were not observed in the control plants, ranged from 0.44% (M_1) to 1.4% (M_2).

Based on our results obtained with the use of ISSR primers, multiple genotype-specific loci were amplified differentiating mutants from the control plant genotype. Moreover, the presence of polymorphic products on electrophoregrams confirmed a large range of variation within the sequence under study. On the basis of genetic similarity coefficients and UPGMA cluster analysis, a genetic similarity tree was plotted, on which the plants of *L. ligulistylis* and *L. pycnostachya* constituted a separate group (Fig. 2). Liatris plants showed a mutual similarity in the range of 43.5 to 75.7%. The most similar to each other (75.7%) were *L. ligulistylis* mutants obtained from seeds treated with 2.0 mM EMS solution. However, the most distant were*L. ligulistylis* and *L. pycnostachya* plants obtained from seeds treated with 2.0 mM EMS solution (43.5%).

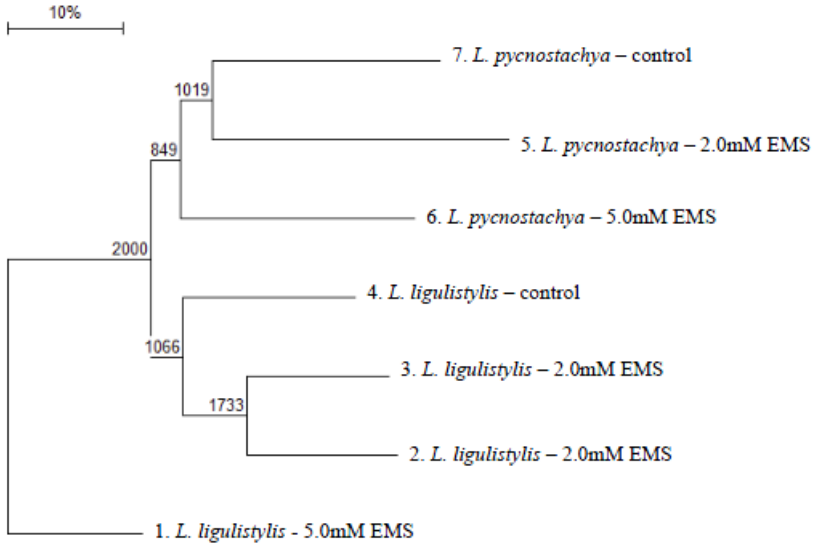

Fig. 2. Dendrogram of the liatria genotypes – variants and control. Numbers at the forks indicate 2000 bootstrap replicates

CONCLUSION

1. The highest number of phenotypic changes after mutagenic treatment in both liatris species were observed in shoots and inflorescence petioles.
2. Higher frequency of all observed phenotypic changes in *L. ligulistylis* and *L. pycnostachya* was found after treatment with 2.0 mM of the EMS solution (11 and 3%, respectively). However, the frequency of the obtained mutants among*L. ligulistylis* treated with 5.0 mM EMS, was 8%.
3. Chemical mutagen used to induced mutation had an inhibitory effect on the features of both liatris species; for example, the height and width of the plant, the circumference and weight of the bulbs and the number of buds on the bulb.
4. The results obtained in the present paper have revealed a high usefulness of ISSR markers for characterization of variability in liatris mutants. The obtained differences consisted in different number and weight of PCR products in all obtained plant population.

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