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MUTATION TYPES AND FREQUENCY IN *NIGELLA DAMASCENA* L. IN THE M_2 AND M_3 GENERATION, USING ETHYL METHANESULFONATE, NITROSOMETHYLUREA AND A NEW DERIVATIVE OF DIMETHYLSULFATE, DG-2

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Aim. To identify mutations and evaluate the mutation frequency in *Nigella damascena* L. cultivars (cvs) Bereginya and Charivnytsya (M_2 and M_3 generation), following treatment of their seeds with ethyl methanesulfonate (EMS), nitrosomethylurea (NMU) and a new derivative of dimethyl sulfate, DG-2. **Methods**. Treated *Nigella* seeds of two cvs with the mutagens for 6 and 16 h and in concentrations of 0.01 and 0.5 % for EMS and NMU and 0.05 and 0.5 % for DG-2. **Results.** A wide range of mutations (59 types) was obtained, that was divided into six groups: five groups with changes in the morphological type and one group with changes in the physiological type. Among the detected mutations, there were both previously known mutations and those obtained in this culture for the first time. The highest mutation frequency (30 %) affecting synthesis of chlorophyll and structure of stem, shoots and leaves and 20 % for physiological features, was registered for NMU at 16 h and 0.05 % in cv. Bereginya. However, this NMU concentration appeared to be lethal for cv. Charivnytsya. **Conclusions.** The new mutagen DG-2 proved to be most effective for inducing mutations in the corolla petal color of nigella, namely 4.0 at a 0.5 % concentration of the mutagen and 16h exposure for cv. Bereginya and 4.0 % at the same concentration and exposure for cv. Charivnytsya. DG-2 caused a substantial number of mutations in all six mutation groups affecting morphological and physiological traits. The classic mutagen EMS was also effective across the spectrum of mutation groups in our study; however, it caused mutations at a lower frequency. The maximum mutation frequency under influence of EMC at a concentration of 0.05 % and an exposure of 16 h in cv. Bereginya was 11.0 %, and in cv. Charivnytsya 8.0 %. For all three mutagens used, an increase in the concentration of the active substance and of exposure time led to an increase in the mutation frequency in *N. damascena* plants. We will select mutants with economically valuable traits, such as tall, lodging-resistant plants and early maturing ones, for further work on the development of new cultivars of *N. damascena* for industrial cultivation.

Key words: mutagenesis, chemical mutagen, mutation, flower morphs.

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INTRODUCTION

The genus *Nigella* is widely distributed in the Mediterranean Region, Europe and SW Asia, and some of its species, including *N. damascena* are also cultivated for ornamentation. The species of this genus occur mostly in deciduous scrub steppe zones, from sea level to 1500 m (Dönmez et al, 2021). *Nigella sativa* L. and *Nigella damascena* L. found the greatest practical application (Prokhorov, 2021). They were already cultivated by the ancient Greeks and Romans and liked for the sweet-burning taste of their oily seeds. From a medical point of view, nigella seeds are an important source of provitamins of groups A, B, P, they contain about 40 % fatty oil, 0.5–1.5 % essential oil, 0.3 % damascenine alkaloid, vitamin E, lipase enzyme, as well as acetylcholines, catechins, cytokinins, calcium, iron, copper, zinc, phosphorus (Isakova et al 2015; Khazdair et al, 2021) The seeds of *N. damascena* in particular contain fatty acids (linoleic, oleic, palmitic acid) (Telci et al, 2014), proteins (e.g. albumin, gluten, globulin), and other biologically active compounds, such as β-elemene and its precursor, germacrene A (Kraker et

al, 1998), which is valued for anti- microbial activity. Furthermore, damascenine, an alkaloid known for antipyretic, analgesic, and anti-edematous properties. In addition to the medicinal value, seeds of *N. damascena* are also used as a food preservative and spice, due to their strawberry-like scent that is determined by caproic and butyric esters (Wais et al, 2009). Presently, plants are widely used in industry for the production of different kinds of primary and secondary metabolites, which are used as dyes, fragrances, food additives, insecticides, or drugs (Hussain et al, 2012; Klimek-Chodacka et al, 2020). It was demonstrated that *N. sativa* has some anticancer properties due to thymoquinone, the main component of its ether oil. The second sesquiterpene, β-element, which, in its turn, is intensely biosynthesized in *N. damascena*, also has therapeutic potential (Helvacıoğlu et al, 2021). *N. damascena* seeds are still used for example in Sicilian folk medicine as a galactagogue (Geraci et al, 2018). Other uses are as emmenagogue, vermifuge, and disinfectant (Heiss and Oeggl, 2005). This plant was also used as an anthelmintic and to treat hematuria, and skin diseases in the Serbian medieval medicine (Jarić et al, 2014). The former use has also been reported in Epirus, northwestern Greece (Vokou et al, 1993). Traditionally, *N. damascena* is used for treating trachoma in Tunisia and Italy (Leporatti and Ghedira, 2009). Apart from its use as herbal remedy, *N. damascena* is used as a condiment in several regions (Heiss and Oeggl, 2005), including in Morocco (Khabbach et al, 2011; Salehi et al, 2021). A rich source of β-elemene is the essential oil of *N. damascena*, in which β-elemene accounts for 47 %. Antimicrobial activity of this essential oil and β-elemene (against *Mycobacterium tuberculosis* strain H37Ra) was established by Sieniawska et al (2019).

Due to its many beneficial properties, the area of *N. damascena* cultivation has extended and exceeds at present the area of its natural occurrence considerably. Since *Nigella damascena* was previously used more often as an ornamental plant, and now it is gaining increasing interest as an oilseed and essential oil crop, breeding of new cultivars is necessary. To enlarge the sowing areas of nigella, it is necessary to create new highly performing cultivars with valuable traits, meeting the requirements for production by the processing industry, namely, increased performance, standing ability, early ripeness and high content of useful compounds. In order to obtain such cultivars mutagenesis, in particular induced mutagenesis, could be a solution (Lomtatidze et al, 2009).

It is important to find mutagens that will have the least effect on the survival of treated plants. Along with the application of classic chemical mutagens, there are studies on new compounds capable of inducing mutations, since there is a need to induce further specific changes and preferably with higher frequency. In addition, the need to protect the environment determined the search for compounds with high mutagenic effects, but with lower toxicity. Since mutagens are toxic to humans, because they are teratogenic, it is necessary to adhere to safety rules when working with these substances, see for example EU Chemical Abstract Service (CAS) documentation EINECS No: 201-058-1 (https://echa.europa.eu/documents/10162/8fcaf1f0-ea6d-4ec8-a129-f0405def7c33).

Mutations are a main cause of hereditary variation of all living organisms. Changes in the genetic material may be caused by physical (ultraviolet radiation, shortwave radiation, etc.), biological (viruses, bacteria), and chemical mutagens with much higher frequency than spontaneous mutations (Holme et al, 2019). Induced mutagenesis greatly facilitates obtaining valuable breeding material with hereditary properties and characteristics (Chaudhary et al, 2019). The creation of mutant cultivars is based on a high frequency of beneficial mutations, mobilizing the traits unattainable for other breeding methods (Ke et al, 2019). Mutagenesis is a relevant instrument of improving resistance, yield and quality characters. Mutagenesis is an important tool in crop improvement and is free of the regulatory restrictions imposed on genetically modified organisms (Kolakar et al, 2018). A direct genetic approach allows for the identification of improved or new phenotypes which can be used in traditional breeding programs. Powerful reverse genetic strategies that allow the detection of induced point mutations in individuals of the mutagenized populations can address the major challenge of linking sequence information to the biological function of genes and can also identify novel variation for plant breeding (Parry et al, 2009).

N-Nitroso-N-methylurea (NMU) and N-nitrosoethyl urea (NEU) supermutagens are used in selection to get enhanced induced mutation. In small concentrations these substances are used as growth stimulators. The above-mentioned super mutagens have an ability to enter the cell and cause such reconstruction of the genetic material when gene mutations are generated primarily and chromosome aberrations are insignificant. They frequently cause systemic mutations which serve as a basis for taxonomic differentiation. It is believed that the high genetic ability of super mutagens is caused

by the sum action of such compounds on the DNA molecules. Interaction of NMU and NEU with DNA molecules, followed by regrouping of pairs of nitrogenous bases, may occur in both ways: transition and transversion. These super mutagens induce more useful mutations in agricultural plants than the known earlier mutagen agents (Lomtatidze et al, 2009).

Ethyl methanesulfonate is a chemical mutagen, which is currently being used in plant breeding, to increase genetic variability in genes of agronomic interest, of species useful in agriculture. It primarily causes single base point mutations by inducing guanine alkylation, resulting in GC to AT transitions. Its effect is different between clones of a genotype and between genotypes of the same species. (Joya-Dávila and Gutiérrez-Miceli, 2020).

New mutagens of the DG series were first used in studies on flax by Tigova and Soroka (2018a and b; 2019). Mutagens of the DG series (DG-2, DG-6, DG-7, and DG-9) are new chemical compounds, derivatives of dimethyl sulfate, synthesized at the Institute of Bioorganic Chemistry and Petrochemistry of the National Academy of Sciences of the Ukraine and given to us by Ph.D. Dulnev. The essence of the mutagenic action of DMS is alkylation of the DNA molecule by the incorporation of ethyl or methyl groups. It is also known from the scientific literature that DMS often causes chromosomal ruptures and that most of the reunions are intrachromosomal, which leads to the formation of a large number of chromosomal inversions. The mutagen DG-2, which we chose for our study, was effective in inducing flower and seed color mutations in a study on flax. Moreover, mutagen DG-2 was effective for mutations to physiological attributes of growth and development and it was most effective in changing biochemical indexes of oil in seeds (Tigova et al, 2022).

In our earlier study on *N. damascena* plants of the M_1 generation, it was shown that the classical mutagen nitrosomethylurea strongly affects the survival of nigella plants, while the new mutagen DG-2 had the least effect on the survival of treated plants. (Gubanova and Soroka, 2019)

Aim: The study was aimed at detecting mutations and evaluating the mutation frequency in *Nigella damascena* L. of M_2 generation under the impact of the chemical mutagens EMS, NMU, and a new chemical mutagen DG-2, (a complex of 3-N,N dimethylaminosulfolane with dimethyl sulfate, mutagen DG-2 differs from the original compound of DMC by an additional group of sulfolane with dimethylamine, see Tigova et al, 2022) Our study intended to demonstrate the efficiency of the mutagens studied for *N. damascena*, and when so them in our further research and selection.

MATERIALS AND METHODS

Air-dried seeds of *Nigella damascena*, cvs Bereginya and Charivnytsya, were treated with chemical mutagens – ethyl methanesulfonate (EMS), nitrosomethylurea (NMU), and a new chemical mutagen, DG-2 (a complex of 3-N,N-dimethyl aminosulfolane with dimethylsulfate) (Tigova et al, 2022). In each variant, 350 seeds were treated at one time. The seeds were placed in cotton bags and soaked in 0.01 and 0.05 % aqueous solutions of mutagens EMS and NMU, and 0.05 and 0.5 % aqueous solutions of DG-2. The use of the 10-fold higher concentration of DG-2 (which was used only once before this study) was based on our team's experience with the compound (Tigova et al, 2022). Seeds of the corresponding cultivar, soaked in distilled water, were used as the control. The exposure lasted 6 and 16 h. After the treatment, the seeds of each variant were washed for one hour in the running water and sown in rows of 2.5 m with an interrow spacing of 20 cm and a 50 cm distance between plots on the same day in an open field. Prior to blossoming, the nigella plants were isolated with separate micro-perforated polypropylene bags for pollination. The plants in the experimental and control groups were observed on the experimental plots of the Institute of Oil Crops, the NAAS, in 2019–2021. The scheme of nigella sowing in the second mutant generation was as follows: row length 1.5 m, interrow distance 0.3 m, and the distance plots 0.5 m. The scheme of sowing in the third mutant generation: the row length 1 m, interrow distance 0.4 m and distance between plots 0.5 m.

The experiment was randomized and performed in triplicate. Each time numbering of seed samples before each new sowing was changed, genotypes were swapped and order of sowing changed. Only the control plot remained unchanged. The M1, M2 and M3 generation was planted in three consecutive years. The number of families in the samples corresponded to the rules of classical mutagenesis and was quite large, see **Tables 1 to 4**). The generations M_2 and M_3 were sown in field conditions by families: family in M_2 – generation of one plant from generation M_1 (all the capsules, each capsule 5 fused true seed pods, from one isolator from one plant after self-pollination); family in M_3 – generation of one family from M_2 (after self-pollination in the isolators). As for generation M_2, 100 families of each treatment variant were sown, except the ones which

had been treated with nitrosomethylurea, because it affected vitality too much. The data about the number of families in the variants of the second mutant generation are indicated in Tables 1–4. The families of M_2, in which a percentage of mutations were lethal, were collected completely to detect again lethality in generation M_3. During the vegetation period, there were phenological observations, the plants with changed morphologi-

Table 1. The number of families with mutations in *Nigella damascena* L. in generations M_2 of cv. Bereginya in per cent (exposure of 6 h)

Mutagen	EMS		NMU		DG-2		Control
Conc. (%)	0.01	0.05	0.01	0.05	0.05	0.5	0
Families (number)	100	100	67	17	100	100	100
group I	0	2.0 ± 1.40	3.0 ± 2.08	0	1.0 ± 0.99	0	0
group II	4.0 ± 1.96	5.0 ± 2.18	0	5.88* ± 5.70	3.0 ± 1.71	6,0 ± 2.37	0
group III	0	1.0 ± 0.99	0	0	2.0 ± 1.40	3.0 ± 1.71	0
group IV	3.0 ± 1.71	7.0 ± 2.55	0	0	3.0 ± 1.71	5.0 ± 2.18	0
group V	0	1.0 ± 0.99	0	0	0	0	0
Group VI	0	4.0 ± 1.96	3.0 ± 2.08	5.88* ± 5.70	2.0 ± 1.40	0	0

Note. Conc. – mutagen concentration; group I – VI – groups of mutations in the M_2 generation; * – statistically significant differences from control, at significance level $p < 0.05$.

Table 2. The number of families with mutations in *Nigella damascena* L. in M_2 generations of cv. Bereginya in per cent (exposure 16 h)

Mutagen	EMS		NMU		DG-2		Control
Conc. (%)	0.01	0.05	0.01	0.05	0.05	0.5	0
Families	100	100	13	10	100	100	100
group I	1.0 ± 0.99	4.0 ± 1.96	7.7* ± 7.39	30.0* ± 14.49	2.0 ± 1.40	5.0 ± 2.18	0
group II	0	11.0 ± 3.13	15.4* ± 10.00	30.0* ± 14.49	5.0 ± 2,18	8.0 ± 2.71	0
group III	0	0	0	0	0	1.0 ± 0.99	0
group IV	3.0 ± 1.71	w.0 ± 1.71	7.7* ± 7.39	10.0* ± 9.49	4.0 ± 1,96	10.0 ± 3.00	0
group V	2.0 ± 1.40		0	0	3.0 ± 1.71	4.0 ± 1.96	0
Group VI	1.0 ± 0.99		7.7* ± 7.39	20.0* ± 12.65	0	5.0 ± 2.18	0

Note. Conc. – mutagen concentration; I – VI group – groups of mutations in the M_2 generation; * – statistically significant differences from control, at significance level of $p < 0.01$.

Table 3. The number of families with mutations in *Nigella damascena* L. in M_2 generations of cv. Charivnytsya in per cent (exposure 6 h)

Mutagen	EMS		NMU		DG-2		Control
Conc. (%)	0.01	0.05	0.01	0.05	0,05	0,5	0
Families	100	100	64	40	100	100	100
group I	2.0 ± 1.40	3.0 ± 1.71	1.6 ± 1.55	7.5 ± 4.16	5.0 ± 2.18	7.0 ± 2.55	0
group II	1.0 ± 0.99	3.0 ± 1.71	0	2.5 ± 2.47	7.0 ± 2.55	7.0 ± 2.55	0
group III	0	0	0	0	1.0 ± 0.99	2.0 ± 1.40	0
group IV	3.0 ± 1.71	3.0 ± 1.71	6.3 ± 3.03	7.5 ± 4.16	2.0 ± 1.40	1.0 ± 0.99	0
group V	0	0	0	0	0	0	0
Group VI	1.0 ± 0.99	2.0 ± 1.40	1.6 ± 1.55	2.5 ± 2.47	0	0	0

Note. Conc. – mutagen concentration; I – VI group – groups of mutations in M_2 generation.

Table 4. The number of families with mutations in *Nigella damascena* L. in M_2 generations of cv. Charivnytsya in per cent (exposure 16 h)

Mutagen	EMS		NMU		DG-2		Control
Conc. (%)	0.01	0.05	0.01	0.05	0,05	0,5	0
Families	100	100	25	0	100	100	100
group I	7.0 ± 2.55	8.0 ± 2.71	4.0* ± 3.92	–	4.0 ± 1.96	10.0 ± 3.00	0
group II	2.0 ± 1.40	6.0 ± 2.37	16.0* ± 7.33	–	7.0 ± 2.55	13,0 ± 3.36	0
group III	2.0 ± 1.40	2.0 ± 1.40	0	–	4.0 ± 1.96	6.0 ± 2.37	0
group IV	2.0 ± 1.40	4.0 ± 1.96	4.0* ± 3.92	–	5.0 ± 2.18	10.0 ± 3.00	0
group V	0	0	0	–	2.0 ± 1.40	4.0 ± 1.96	0
Group VI	4.0 ± 1.96	6.0 ± 2.37	16.0* ± 7.33	–**	2.0 ± 1.40	4.0 ± 1.96	0

Note. Conc. – mutagen concentration; I – VI group – groups of mutations in M_2 generation; * – statistically significant differences from control at a significance level of the sum of mutations $p < 0.05$; ** – this concentration proved to be lethal for the seeds, treated for 16 h

cal and physiological traits were noted, the following generation was checked for the inheritance of the isolated changes. All kinds of mutations were registered at each stage of plant growth and development. Only the changes in the traits of plants, which were inherited in subsequent generations, were considered to be mutations. The mutations were isolated based on the absence of these changes in the control group plants of the corresponding cultivars in all the observed generations of the untreated plants.

The noted mutations included 1) mutations ones related to the disrupted chlorophyll synthesis, 2) mutations in the structure of stem, shoots, and leaves, the mutations in the flower (changes in the corolla petal color, the form of petals, and buds), 3) mutations in the structure of seed capsules and 4) mutations related to the physiological traits of growth and development. Therefore, each variant considered all the types of mutational variation of nigella and the number of plants of each type. Each plant with the mutant trait in M_2 was taken into account once.

The frequency of mutant changes was determined in per cent as the ratio between the number of mutant families and their total number in generation M_2. The final conclusion about the presence of mutations in M_2 was made after their confirmation in generation M_3.

The results of the observations were calculated using standard mathematical and statistical methods (Wasserman, 2004). The main statistical characteristics of the quantitative change in the investigated indices were dispersion (s2), standard deviation (s), standard error of the mean (sx), and criterion χ^2 (Wasserman, 2004).

The application of the χ^2 criterion allowed us to obtain a good approximation of the binomial criterion for all values of the manifestation of p and its absence *q* for tables of the 2 × 2 type, where the sample size *n* exceeded 50. In the case of a two-digit population, the test statistic χ^2 was defined as follows (1.1)

$$\chi^2 = \Sigma^2_{i=1} [(|P_i - Q_i| - 0.5)2/Q_i],$$

where P_i is the actual (empirical) frequency in the cell of the conjugation table; Q_i are expected, theoretically calculated frequencies in the same cell.

The cv. Bereginya is a morph with single simple blue flowers, cv. Charivnytsya a morph with double blue flowers

RESULTS

After the nigella seeds were treated with chemical mutagens EMS and NMU in the concentrations of 0.01 and 0.05 % and mutagen DG-2 in the concentrations of 0.05 and 0.5 %, we obtained a wide spectrum of mutations in M_2 generation which was represented by 59 types of changes, divided into six groups – five groups with morphological and one group with physiological changes (Tables 1–4).

To classify the mutants in our study, we used the classification, developed by Morgun and Logvinenko 1995), amended by Tigova and Soroka (2019). We amended the classification of Tigova and Soroka as follows: from the classification, developed by Tigova and Soroka, we used the following of their mutation groups: a) disrupted chlorophyll synthesis, b) structure of stem, shoots, and leaves, and c) physiological traits of growth and development. We divided Tigova and Soroka's group of flower mutations into

Fig. 1. *N. damascena* cv. Bereginya plants treated with 0.05 % NMU with an exposure of 16 h, M_3 generation, 2 years and 2 months after treatment, with so-called light green margin mutation

two groups: 1) mutations in the flower structure and 2) those in the corolla petal color. We also found a group of mutations not described by Tigova and Soroka (2019), namely mutations in the structure of the seed capsule.

Mostly we took the terms for the names in the classification of chlorophyll mutations from the classification of Morgun and Logvinenko (1995), Holm G. (1954). Therefore, the types of mutations with similar phenotypical manifestations have Latin names in our study. Some mutations were first revealed by us, so we amended the original classification. For instance, we were the first to detect the so-called light green margin mutation (**Fig. 1**) – these are the plants with first white leaves and actual leaves with a light green margin at the edge. The plants were fertile and progeny was obtained from the mutants. We also obtained for the first time mutants with a phenotype showing white parts randomly distributed on the stems (**Fig. 2**). The mutants with this trait are fertile. This trait is passed on to the offspring of mutants, but splits. The trait is also inherited by some plants from the mutant family that did not have the trait in the phenotype.

In our experiment, we discovered, as far as we know, for the first time, hereditary changes such as witches' broom-like leaf bushes (**Fig. 3**), widely spaced internodes (**Fig. 4**, ***a***).

Fig. 2. *N. damascena* cv. Charivnytsya plants treated with 0.05 % DG-2, with an exposure of 6 h, M_3 generation, 2 years, 2 months and 2 weeks after treatment, with the mutation of white plant parts

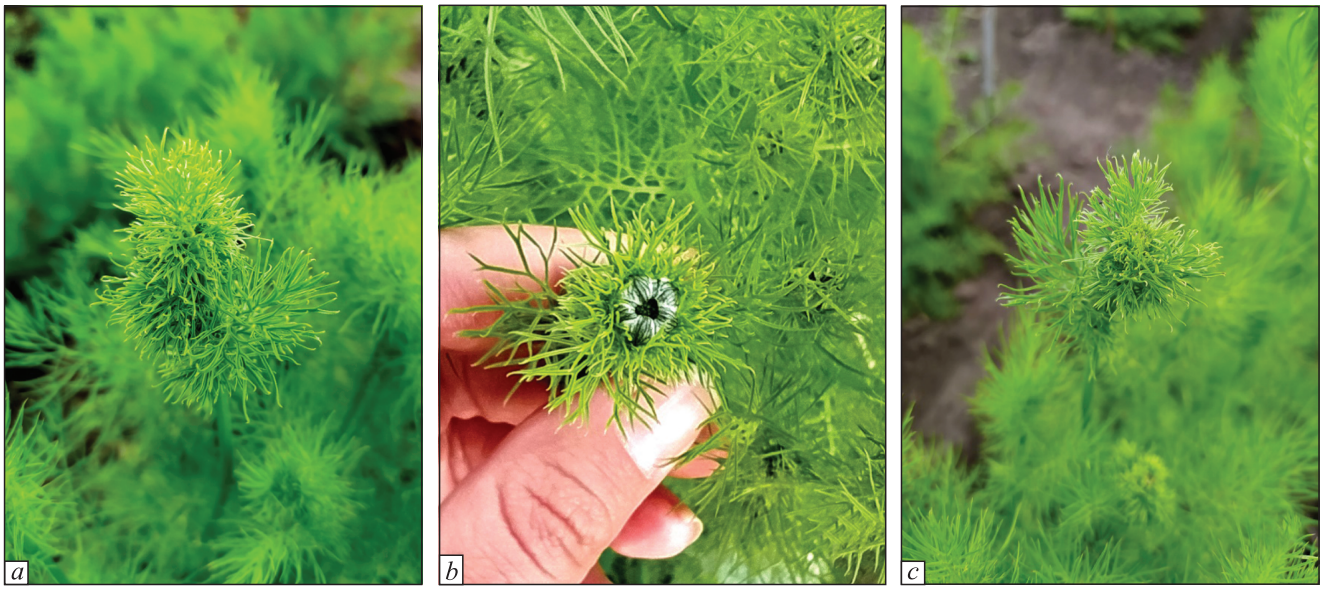

Fig. 3. *N. damascena* cv. Charivnytsya plants treated with 0.01 % EMS, with an exposure of 6 h, M_3 generation, 2 years, 2 months and 2 weeks after treatment, with a mutation giving witches 'broom-like leaf bushes: *a*, *b*, *c* – plants with the trait

We discovered, to our knowledge, for the first time the hereditary change in the color of apetalous morph (so-called double flower) of Nigella damascena – a mutation of a flower with a blue brim (Fig. 4, *b* and **Fig. 5**).

In the control plants of the cv. Charivnytsya (a cultivar with apetalous morph -so-called double flower) the flower, after blooming, fills with color as the gynoecium and androecium develop. At first, the flowers are light, gradually they turn blue and by the time the petal-like sepals fall off, they turn dark blue. In mutants with the trait flower with a blue margin, the light ring in the color of the flower is retained at the

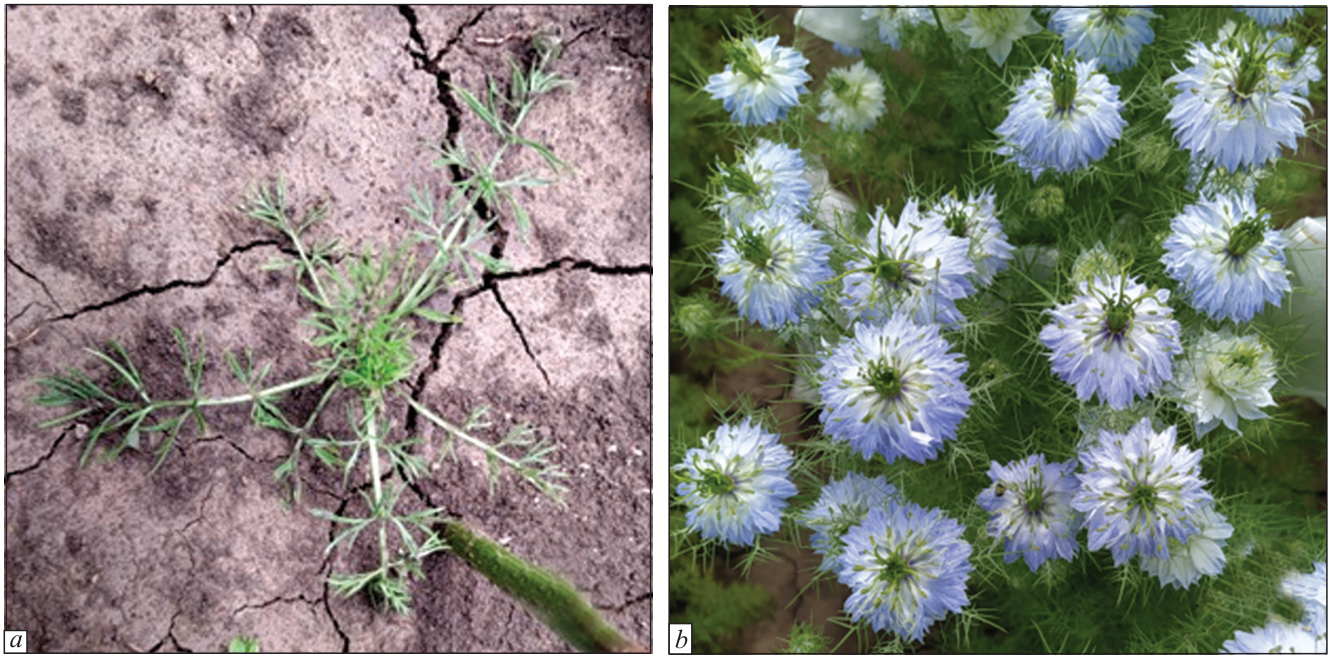

Fig. 4. *N. damascena* cv. Charivnytsya plants treated with 0.01 % NMU, with an exposure of 6 hours, M_3 generation, 2 years, 2 months and 2 weeks after treatment, with (*a*) the mutation of widely spaced internodes, and (*b*) cv. Charivnytsya plants treated with 0.5 % DG-2, with an exposure of 16 hours, M_3 generation, 2 years and 3 months after treatment the apetalous morph of *N. damascena*, so called double flower, mutation of a white flower with a blue brim (*b*)

Fig. 5. *N. damascena* cv. Charivnytsya plants treated with 0.5 % DG-2, with an exposure of 16 h, M_3 generation, 2 years and 3 months after treatment the apetalous morph of *N. damascena*, so called double flower, mutation of a white flower with a blue brim

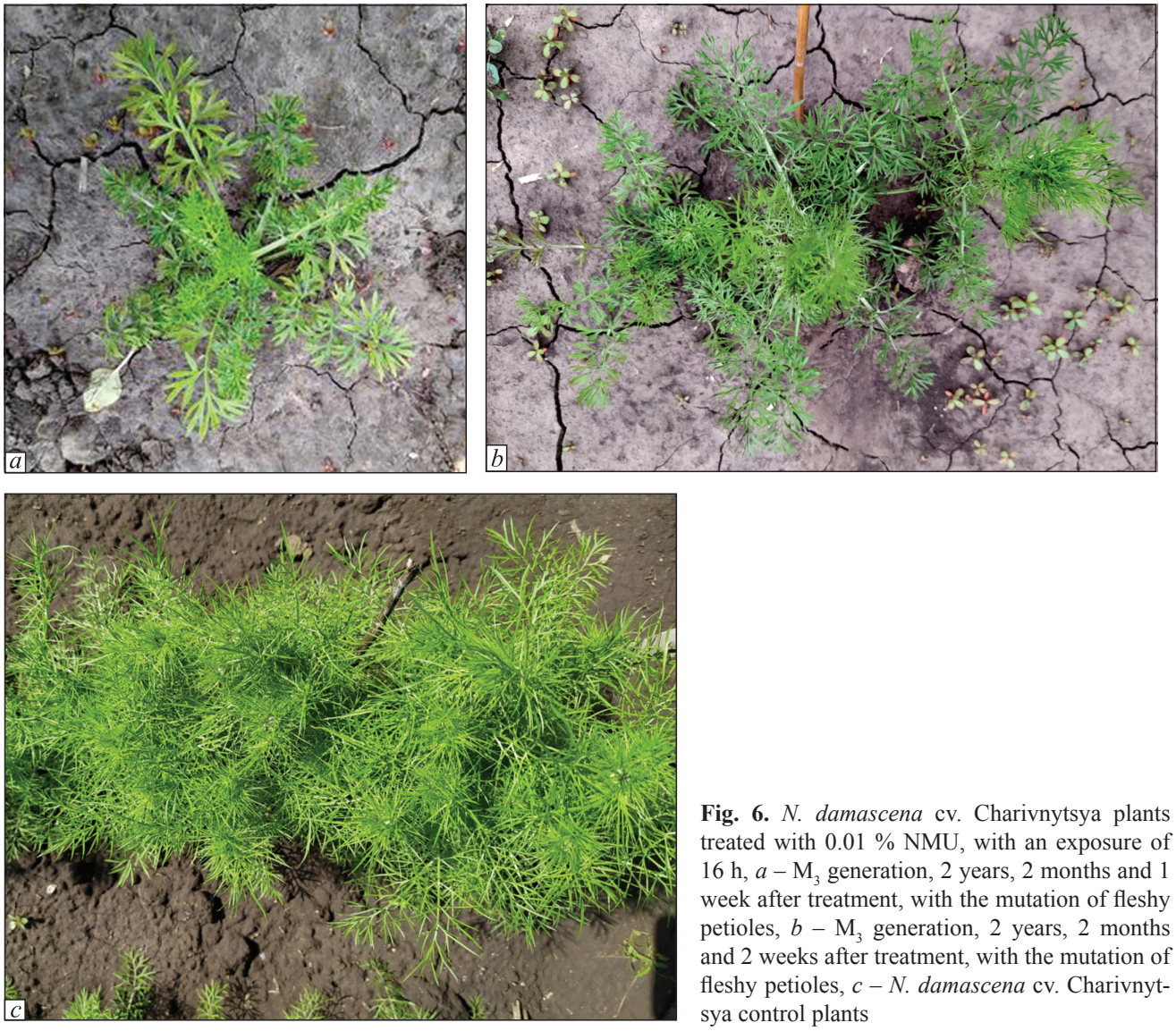

Fig. 6. *N. damascena* cv. Charivnytsya plants treated with 0.01 % NMU, with an exposure of 16 h, *a* – M_3 generation, 2 years, 2 months and 1 week after treatment, with the mutation of fleshy petioles, *b* – M_3 generation, 2 years, 2 months and 2 weeks after treatment, with the mutation of fleshy petioles, *c* – *N. damascena* cv. Charivnytsya control plants

Fig. 7. *N. damascena* cv. Bereginya plants treated with 0.5 % DG-2, with an exposure of 6 hours, M_3 generation, 2 years, 2 months and 3 weeks after treatment, with the mutation flower rotated 90 degrees

Fig. 8. *N. damascena* cv. Charivnytsya plants treated with 0.5 % of DG-2, with an exposure of 6 hours, M_3 generation, 2 years, 2 months and 2 weeks after treatment, with the mutation of convolute (crooked) flower spike and stem

later stages of flower development, and the flowers are two-colored. Plants with this trait are fertile, the trait is passed on to offspring and does not split.

In our experiment, we also discovered, as far as we know, for the first time fleshy petioles mutation (**Fig. 6, *a*, *b***). The flower of Nigella damascena is different from the flower of black cumin, but Datta, Biswas (1985) also noted flower deformations in the latter. The mutation which we called flower rotated 90 degrees was first discovered for this species in our study (**Fig. 7**).

The following groups of mutations were observed:

I. Mutations with disrupted chlorophyll synthesis (11 types): albina, virido-albina, xantha, chlorina, viridis, striata, corroded, light green margin, yellow lower leaves, dark green plants, white parts of the plant.

II. mutations in the structure of the stem, shoots, and leaves (14 types): cotyledon, divided into several parts, witches 'broom-like leaf bushes, deformed leaves, convolute or curly leaves, convolute (crooked) flower

Fig. 9. *N. damascena* with the mutation *a–b*: ribbed capsule, *c* – spherical round capsules, *d* – multisegmented capsule, *e* – several fused capsules, *f* – capsules with helicoidal horns, *g* – elongated capsule, *h* – seed capsule deformation, *i* – pentagonal

spike and stem (**Fig. 8**), abnormal branching, extended leaf blade, widely spaced internodes, fleshy petioles, absence of petioles on lower leaves, multiflorant (branched), high plants, low plants, dwarfs.

III. Mutations in the flower structure (7 types): flower rotated 90 degrees, altered sepals, half-grown corolla (reduced petals), large flowers, deformed flowers, 2–3 flowers together, and the upper flower dies back.

IV. Mutations in the seed capsule structure (11 types): (**Fig. 9**.) seed capsule deformation, a large capsule, multisegmented capsule, several fused capsules, two-segment capsules, spherical capsules, pentagonal capsule, ribbed capsule, egg-like capsule, elongated capsule, capsules with helicoidal horns

V. Mutations in the color of the corolla petals (4 types): a white flower (simple), a white flower (double), a

Fig. 10. *N. damascena* with the mutation a white flower (double) (left) a blue flower with white margin (right)

white flower with a blue brim (Fig. 5, *b* and 6), and a blue flower with white (**Fig. 10**).

VI. Mutations in the physiological traits of growth and development (4 types): early ripe plants, sterile plants, plants resistant to lodging, and plants lodging strongly (control plants of both cultivars have medium resistance to lodging; mutants either have strong resistance to lodging, or, conversely, are lodging strongly).

Similar to the classic mutagens EMS and NMU, the mutagen DG-2 induced hereditary morphological and physiological changes with different frequencies in different groups. The efficiency of each mutagen is in direct proportion to the frequency of the mutation induced by it. Yet the studied compounds were considerably different in the spectrum of the mutations. For instance, mutagen DG-2 was found to be most effective in obtaining the group of mutations in flower, including the change in the color of the corolla petals (group V) for both cv. Bereginya and cv. Charivnytsya after the longer 16h exposure (Table 2).

For the exposure of 16 h to the mutagens we obtained the following results (also see Table 2 and 4): Among all the investigated mutagens, DG-2 at 0.5 % initiated the maximum mutation frequency (causing change in the color of the corolla petals for both cultivars used at 16h exposure), namely 4 % (group V, Table 2). The frequency of the classic mutagen EMS at 0.05 % in this group was c. 2.0 % for Bereginya, and zero for the cv. Charivnytsya. It is noteworthy that EMS caused mutations in all the six groups isolated in our study at 0.05 %. In addition to flower mutations DG-2 also caused mutations in all other groups at 0.5 %. Mutations, affecting the change in the seed capsule structure (group IV) and disrupted chlorophyl synthesis (group I), were most frequent for DG-2 (10.0 % in both cultivars and 5 and 10 % in cv. Bereginya and cv. Charivnytsya respectively). The percentage mutations in group II (stems, shoots, leaves) were also high for DG-2, namely 8 and 13 % respectively.

Using NMU at 0.05 % and 16 h exposure, cv. Bereginya had a higher survival rate than cv. Charivnytsya. NMU had highest mutation frequency for several groups of traits, namely, for the disrupted chlorophyll synthesis (group I), 30.0 %, for the physiological traits of growth and development (group VI), 20.0 %. For group IV (capsule structure) NMU had a similar mutation frequency as DG-2 at 0.5 %, namely 10 % (Table 2).

EMS was also effective in all the groups of traits. The highest frequency of mutagens was induced by EMS at 0.05 % and 16 h exposure, variety in the structure of stem, shoots and leaves (group II 11.0 % in cv. Bereginya and 6 % in cv. Charivnytsya. In group I (disrupted chlorophyll synthesis) it was 4.0 and 8 % for the respective cultivars.

DISCUSSION

Based on the study of Datta and Biswas (Datta, Biswas, 1985) on black cumin, a close relative of *Nigella damascena*, we can conclude that some mutations found in our experiment, are similar to those discovered by Datta and Biswas. For instance, these were:

cotyledon, divided into several parts, deformed leaves, convolute (crooked) flower spike and stem (Fig. 3), abnormal branching, extended leaf blade, absence of petioles on the lower leaves, multiflorants (branched, high plants, low plants, dwarfs. These are mostly mutations in the structure of the stem, shoots, and leaves.

Ethyl methanesulfonate (EMS) is a classic mutagen that proved itself in the breeding practice to obtain breeding material with different characteristics. EMS was effectively used to obtain plants of the genus *Nigella* L. with changed morphological and physiological traits (Gilot et al, 1967; Phai, 1976; Biswas and Datta, 1983; Datta and Biswas, 1985; Asif and Ansari, 2019; Gubanova and Soroka, 2021). In our study, EMS was also found to be efficient evoking mutations, in all six groups of traits for the cv. Bereginya, but not in group V (flower mutations) in cv. Charivnytsya. In our hands EMS had a lower mutation frequency than NMU or DG-2, but it has been shown that EMS in combination with X-ray treatment (Datta and Biswas, 1985, using *N. damascena*) or gamma-radiation (Gosh and Datta, 2005, using *N. damascena*; Kaul and Bahn, 1977, using rice and Tamilzharasi et al, 2022, using black mung bean) can enhance mutation frequency (Datta and Biswas, 1985). This could be a direction of our future research. Amin et al (2019) using *N. sativa* found that the frequency of morphological variants increased when increasing the mutagenic dose. The maximum frequency was observed in a combination of EMS and gamma rays. In our study, lower concentrations of this substance were used without radiation, but, as in the study of Amin et al (2019), with increasing dose, the number of mutations also increased.

Nitrosomethylurea (NMU) gave the highest percentage of mutations, but had a strong negative effect on the survival of nigella plants, that was also observed in our earlier study using M1 plants (Gubanova and Soroka, 2019). Usatov et al (2019) using similar concentrations of NMU induced effectively plastid mutations in sunflower. Despite its high lethality NMU induced mutations with very high frequency, up to 30 % in a considerable part of the groups of detected traits. In the study by (Phai, 1976), nitrosomethylurea proved to be one of the most effective mutagens, second only to ethyleneimine. They also used a concentration of nitrosomethylurea of 0.01 % like us, the rest of their concentrations were lower than ours. The nitrosomethylurea concentration of 0.05 % seems to be too high as it greatly affects the survival of the treated Nigella plants. In the study of Phai (1976), nitrosomethylurea was also effective in inducing chlorophyll mutations.

DG-2 was again the most optimal mutagen, inducing mutations of morphological and physiological nature in all the groups detected by us with low lethality and this was also established in our research on the M1 generation (Gubanova and Soroka, 2019). Similar to the study of Tigova, in which mutagen DG-2 was used for the first time (Tigova, Soroka, 2018a, mutagen DG-2 was found especially effective in inducing chlorophyll and flower mutations (color of corolla petals).

Ke et al (2019) showed that non-presoaked seeds are more sensitive to EMS treatment than presoaked seeds. This is consistent with the experience that presoaking of seeds can reduce injury caused by chemical mutagens. In our study, we did not use pre-soaking the seeds. We washed the seeds in running water after treatment with mutagens.

CONCLUSIONS

A wide spectrum of inheritable mutations in *Nigella damascena* cvs Bereginya and Charivnytsya of the M2 and M3 generation (59 types), (divided into six groups according to the phenological manifestations at different stages of development), were obtained using three different mutagens, namely EMS, NMU and GG-2. These mutations included both well-known types and a few new types which were not previously known for this species.

The highest mutation frequency in most groups was induced by NMU. However, this mutagen at 0.05 % and 16 h exposure was found to be lethal for cv. Charivnytsya.

EMS was also found effective in our study, inducing mutations with a frequency, lower than that for NMU and the spectrum fairly similar to that of DG-2, It did not impact the change in the color of corolla petals in cv. Charivnytsya.

Noteworthy was the wide spectrum of mutations invoked by the relatively new mutant DG-2, a derivative of dimethylsulfate. It proved to be most effective in inducing the mutations of the change in the color of the flower corolla petals (4.0 % at a concentration of 0.5 % and 16 h exposure). The overall mutation frequency for DG-2 was 1.0–30.0 % in cv. Bereginya and 1.0–16.0 % in cv. Charivnytsya. For all three mutagens it was established that an increase in their concentration and exposure time led to increase in mutation frequency.

The mutated *N. damascena* material obtained in the M2 and M3 will be used in our future research where we will investigate mutants with economically useful traits. We are going to select mutants with economically valuable traits, such as tall, lodging-resistant plants and early maturing ones, in order to develop new cultivars of *N. damascena* for industrial cultivation.

Adherence to ethical standards. This study was performed by the author in compliance with the ethics requirements and did not envisage any research involving the participation of animals or people.

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Мутації та їх частота у *Nigella damascena* L. у поколіннях M_2 і M_3 залежно від типу хімічного мутагену: етилметансульфонату, нітрозометилсечовини та нового похідного диметилсульфату – ДГ-2

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Мета. Виявити мутації та оцінити їх частоту у *Nigella damascena L.* покоління M_2 і M_3 сортів Берегиня та Чарівниця залежно від впливу хімічних мутагенів етилметансульфонату, нітрозометилсечовини та нового похідного диметилсульфату – ДГ-2. **Методи.** Обробили насіння чорнушки двох сортів мутагенами ЕМС, НМС у концентраціях 0,05 та 0,01 % та ДГ-2 у концентраціях 0,5 та 0,05 % з експозиціями 6 та 16 год. **Результати.** Отримано широкий спектр мутацій (59 типів), виявлених у поколінні M_2. Вони були поділені на 6 груп: 5 груп із змінами морфологічного типу та 1 група із змінами фізіологічного типу. Серед виявлених мутацій були як відомі раніше, так і отримані вперше на цій культурі типи. Найбільший вплив на частоту мутацій мала нітрозометилсечовина з експозицією 16 год. У сорту Берегиня при концентрації даної речовини 0,05 % була виявлена максимальна в нашому дослідженні частота – 30 %. В таких групах мутацій даного сорту чорнушки при максимальній експозиції та максимальній концентрації НМС було виявлено максимальну частоту, а саме: частота групи мутацій з порушенням синтезу хлорофілу – 30,0 %, частота групи мутацій структури стебла, пагонів і листків – 30,0 %, частота групи мутацій фізіологічних ознак зростання та розвитку – 20,0 %. Концентрація нітрозометилсечовини 0,05 % при експозиції 16 год виявилася летальною для рослин сорту Чарівниця. **Висновки**. Новий мутаген ДГ-2 виявився найбільш дієвим задля індукування мутацій забарвлення пелюсток віночку чорнушки. Він викликав мутації з частотою 4,0 % у рослин сорту Берегиня та з такою самою частотою у рослин сорту Чарівниця при концентрації 0,5 % та експозиції 16 год. Також ця речовина справила значний вплив на весь діапазон виявлених у нашому дослідженні мутацій, що впливають на морфологічні та фізіологічні ознаки. Класичний мутаген етилметансульфонат також був ефективним щодо всього спектру груп мутацій у нашому дослідженні; проте він викликав мутації із меншою частотою. Максимальна частота мутацій під впливом ЕМС з концентрацією 0,05 % та експозицією 16 год у сорту Берегиня становила 11,0 %, а у сорту Чарівниця – 8,0 %. Згідно з нашими даними, в досліді, збільшення концентрації діючої речовини та експозиції призводило до зростання частоти мутацій у рослин чорнушки дамаської. Доцільно використовувати ці мутанти в подальших дослідженнях і селекції. Будемо відбирати мутанти з господарсько цінними ознаками, такими як високорослі, стійкі до вилягання, ранньостиглі та ін. для подальшої роботи над створенням нових сортів *N. damascena* для промислового вирощування.

Ключові слова: чорнушка, мутагенез, хімічний мутаген, етилметансульфонат, нітрозометилсечовина, диметилсульфат, мутація.

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