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New mutations of flower shape in *Nigella damascena* L., its pleiotropic effects and patterns of inheritance

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All relevant data are within the paper and its Supporting Information files.

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Abstract: Two mutants with short sepals were identified after ethyl methanesulfonate treatment of *Nigella damascena* seeds. In one of them ("*shs1*" gene = short sepal 1), isolated from the line with double flowers, the sepals, in addition to reduced size, were divided into several rounded lobes, which granted the flower an original rose-like appearance of ornamental value. Another mutant with reduced sepals ("*shs2*" gene = short sepal 2) was isolated from the line with simple flowers. The allelism test showed that these two genes were non-allelic. Both mutants as pollen parents were crossed with the same line with single flowers. In a dihybrid cross, simple flower, non-reduced sepals (wild type) × double flower, reduced sepals ("*shs1*" gene) F_1 hybrids demonstrated a wild phenotype. F_2 progeny, in addition to two parental classes, showed two recombinant classes in a 9:3:3:1 ratio, indicating that flower shape and sepal size were inherited monogenously and independently, and the plant with rose-like flowers was a double recessive homozygote. Reduced sepals ("*shs2*" gene) in crosses with the single flower line of wild type were inherited as a monogenic recessive trait, showing a 3:1 segregation ratio in F_2. Both mutant genes had a number of similar pleiotropic effects, which, however, were different in strength. Thus, both mutant genes shortened leaf segments, divided the cotyledon leaves into several lobes, and caused disturbances in the female generative sphere, leading to a lack of seed setting. At the same time, the identification of mutants as early as at the cotyledon stage, due to the pleiotropic effect, makes it possible to select and maintain them, especially with regard to the mutant with rose-like flowers, which is highly decorative.

1. Introduction

Nigella damascena L. is an annual herbaceous plant of the Buttercup family (*Ranunculaceae*). This is a crop of wide application, the products of which are used in medicine, food industry, and perfumery. Its seeds contain about 50% fats, which consist mainly of unsaturated fatty acids, up to

20% protein, 2-3% essential oil, enzymes, more than 20 macro- and microelements, including essential ones (Riaz *et al.*, 1996).

Nigella is best known for the fact that nigedase is obtained from its seeds, a lipolytic enzyme preparation that is widely used in medical practice. The absence of animal protein and bile components in its composition permits to prescribe this enzyme preparation for allergies, as well as in cases where the presence of bile acids is highly undesirable. The literature contains information on the pharmacological activity of other biologically active compounds isolated from this plant, in particular, fatty oils and a number of essential oil components (Helvacıoğlu *et al.*, 2021; Salehi *et al.*, 2021; Benazzouz-Smail, 2023). The variety of applications of *Nigella* has initiated research to develop various *in vitro* biotechnologies for this crop from callus culture to protoplast culture (Klimek-Chodacka *et al.*, 2020).

In addition to the above, *Nigella damascena* has long been among the highly ornamental annual plants. Its high decorativeness is granted by rather large petal-like sepals of white, different shades of blue, purple, pink and even red colors. In floriculture, the shape of the flower is no less valuable than the color of the flower for giving the appearance to an ornamental plant. The presence of floral dimorphism in *Nigella damascena*, which ensures the shape of single or double flowers, and a variety of sepal colors allowed breeders to create a series of wonderful varieties.

It has long been shown that the floral dimorphism is monogenically controlled, with the ‘single’ morph being dominant and the ‘double’ morph being recessive (Toxopeus, 1927). In recent years, flower dimorphism and different types of petal modifications in *Nigella damascena* have served as the basis for using this plant as a model for elucidating the molecular control of floral dimorphism and identifying genes expressed during petal development (Jabbour *et al.*, 2015; Zhang *et al.*, 2020, Galipot *et al.*, 2021).

As a result of studies on induced mutagenesis in *Nigella*, we have identified two mutations affecting the size of the sepals, which ultimately alters the shape of the flower. These mutations, as well as their pleiotropic effects, are described in this article, which also presents the inheritance patterns for the mutant traits.

2. Materials and Methods

In our studies on chemical mutagenesis, two mutations with a similar phenotypic appearance, expressed in the deformation of the sepals, were identified in *Nigella damascena*. In one case the malformation was manifested in shortening the sepal and rounding its edge, so that the sepal instead of a pointed shape had an oval shape. It was by the presence of a shorter sepal and its rounded edge that this mutation was originally isolated. Another mutation was only designated by shortened sepals. Both mutations were identified in M_3 generation. The first mutant was isolated from a variety with double flowers after seed treatment with ethyl methanesulfonate at the concentration of 0.01% and exposure for 16 hours, the other was found from a variety with single flowers as a result of seed treatment with the same mutagen at the same concentration for 6 hours.

In order to check whether these two mutations are allelic, an allelism test was performed.

To study the inheritance of the mutant traits, both mutants, using them as pollen parents, were crossed with the same line with single flowers. F_1 hybrids were self-pollinated and in F_2 families the segregation ratios were analyzed. In the cross combination “single flower, non-reduced sepals × double flower, reduced sepals”, four classes were considered, and in the combination “single flower, non-reduced sepals × single flower, reduced sepals”, the F_2 population was divided into two classes.

To test if the observed frequencies of plants in F_2 populations correspond the expected ones a Chi-square test was used (Griffiths *et al.,* 2004).

3. Results and Discussion

Flowers of two mutants with deformed sepals are shown in figure 1. The flower of the mutant isolated as a result of mutagenic treatment of seeds of the double-flowered *Nigella* plant is shown in Figures 1a and 2b. As can be seen from the figures, the mutant, in contrast to the usual double flower (2d), was characterized by shortened and more rounded sepals (“*shs1*” = shortened sepals with rounded edges). The incompletely opened flower of the mutant plant resembled the shape of a rose flower.

In a cross combination of “single flower, non-reduced sepals (wild type) × double flower, reduced sepals (mutant type)”, F_1 hybrids had a single flower and non-reduced elongated sepals like the wild type parent. That is, a single flower completely dominated

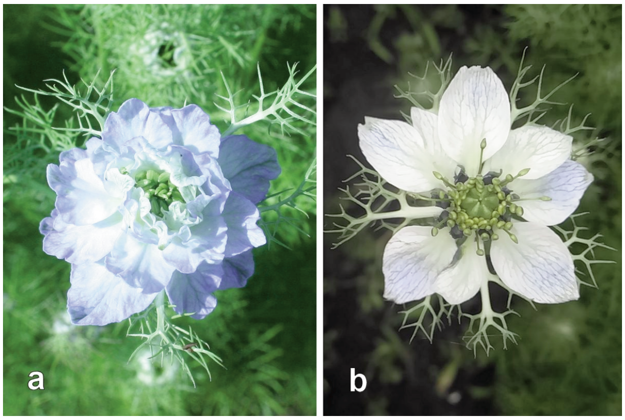

Fig. 1 - Flowers of two *Nigella damascena* mutants with reduced sepals: a) rose-like double flower at the beginning of opening (shs1 gene); b) single flower with reduced sepals (shs2 gene).

the double one, and non-reduced sepals over reduced ones ("*shs1*"). In this cross combination the parents differed by two genes and, if these genes are inherited independently, we have to obtain a typical dihybrid pattern with the four unique phenotypes in a 9:3:3:1 ratio in F_2. Two F_2 families showed a segregation ratio where, in addition to the parental classes of single flower, non-reduced sepals (2a) and double flower, reduced sepals (2b), two recombinant classes appeared - single flower, reduced sepals (2c) and double flower, non-reduced sepals (2d) in approximately equal proportions (Table 1, Fig. 2). In both F_2 families, there was a complete correspondence of the observed segregation ratios to the theoretically expected frequencies. The identified segregation model indicated an independent combination of flower morph and sepal shape traits and, consequently, the absence of linkage between the genes that determine those traits.

The flower of another mutant with deformed sepals, isolated after mutagenic treatment of seeds of a plant with simple flowers, in contrast to the first mutant, was characterized by a stronger shortening of the sepals and the absence of roundness at their ends ("*shs2*" = shortened sepals) (Fig. 1b). The reduction in the sepal length was accompanied by a significant deformation of the flower pistil, which was visually revealed in the strong shortening of the styloides. Some flowers of this mutant lacked them altogether.

Sepals reduced in length ("*shs2*") in crosses with the single flower line of wild type (with non-reduced sepals) were inherited in a monogenic recessive pat-

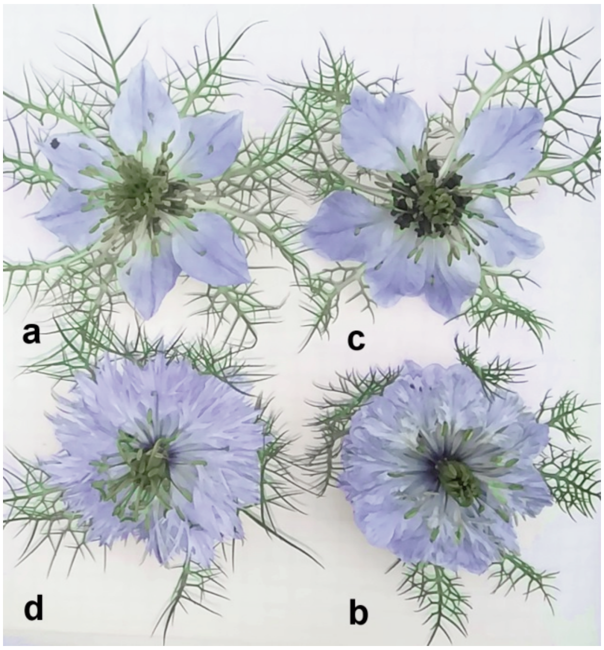

Fig. 2 - Phenotypic classes in F_2 *Nigella damascena* cross combinations single flower, non-reduced sepals (wild type) × double flower, reduced sepals (*shs1* mutant): a) single flower, non-reduced sepals; b) double flower, reduced sepals; c) single flower, reduced sepals; d) double flower, non-reduced sepals.

Table 1 - F_2 segregation for sepal shape and floral morph in cross of single flower, elongated sepals (wild type) and double flower, oval sepals (mutant type) plants in *N. damascena*

F_1 phenotype	Total F_2 plants	F_2 phenotypes				Segregation ratio tested	χ2 (P value)
		single flower, non-reduced sepals	single flower, reduced sepals	double flower, non-reduced sepals	double flower, reduced sepal		
Single flower, non-reduced sepals	100	61	17	16	6	(3:1) × (3:1) = 9:3:3:1	0.29 (0.59)
Single flower, non-reduced sepals	68	40	9	13	6	(3:1) × (3:1) = 9:3:3:1	1.39 (0.24)

$\chi 2_{05}$ (d.f. 3) = 7.82.

tern, showing complete dominance of the wild type over the mutant in F_1, and a 3:1 segregation ratio in F_2 (Table 2).

The allelism test performed showed that these two genes, which determine the shortening of the sepals, are non-allelic. However, they have a number of similar pleiotropic effects. Both genes, without affecting plant height, cause shortening of true leaf segments. The bracts of both mutants are also shortened and more densely attached to the ripening boll than in the wild type. It should be noted that the *shs2* gene as compared with the *shs1* gene causes stronger changes (Fig. 3). Shortening the leaves and bracts changes the habit of the plant, making it more compact.

Both mutant genes affect not only true leaves, but also cotyledons, causing them to be dissected into lobes. The division of one or two cotyledons into two lobes is characteristic of the mutant with *shs2* gene (Fig. 4 b), while the multi-lobed state of both cotyledons is inherent for the mutant carrying *shs1* gene (Fig. 4 a).

The negative effect of both mutant genes on the main function of the flower, which is reproduction, was also noted. The mutants were successfully used in various crosses as a source of pollen~~,~~ but their involvement in hybridization as female parents was problematic. Sometimes such crossings were successful with the *shs2* mutant when using late flowers, but it was not possible to obtain seeds from the *shs1* mutant even after free pollination. This indicates serious disturbances in the female generative sphere of the flowers of both mutants.

Previously, in our studies with *Linum grandiflorum* Desf., a mutant with short petals, resembling a wild carnation flower, was identified (Lyakh, 2018). After mutagenic treatment of immature sunflower embryos, a mutant with shortened petals (ray flowers) was also obtained (Soroka and Lyakh, 2009). In both cases, as for Nigella, ethyl methanesulfonate was used. The mutation identified in sunflower had a strong pleiotropic effect, affecting the stem, leaf, and even cotyledons. At the same time, true and cotyledon leaves had, in contrast to the elongated, rounded end of the leaf blade.

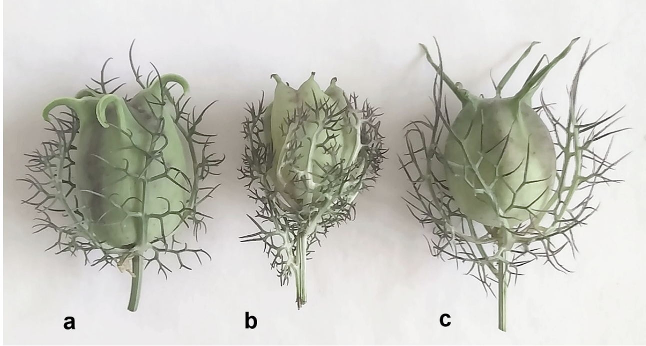

Fig. 3 - Bracts and capsules of two *Nigella damascena* mutants with reduced sepals compared to the wild type: a) *shs1* mutant, b) *shs2* mutant, c) wild type (single flower).

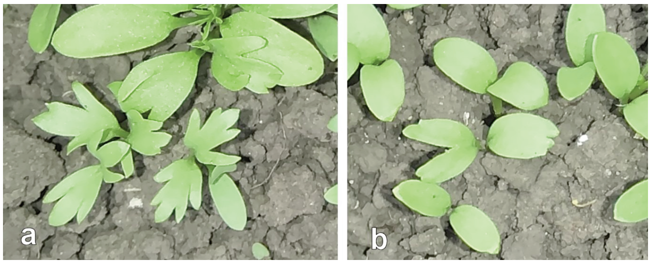

Fig. 4 - Cotyledons of two *Nigella damascena* mutants: a) *shs1* mutant, b) *shs2* mutant.

Two-locus genetic control of petal shape was revealed in *Linum grandiflorum* and sunflower, where a shortened petaled plant is a double recessive homozygote (Soroka and Lyakh, 2017; Lyakh, 2018). In turn, a simpler genetic system is known that controls the shape of plant organs, in particular leaves. Thus, it was found that the shape of the leaflet in cowpea is monogenously controlled, with the lanceolate leaflet shape dominant over the ovoid one (Nwofia, 2014). The same monogenic control of leaf shape, but with a co-dominant interaction of

Table 2 - F_2 segregation for sepal shape in cross of single flowered plants with non-reduced (wild type) and reduced sepals (mutant type) in *N. damascena*

F_1 phenotype	Total F_2 plants	F_2 phenotypes		Segregation ratio tested	χ2 (P value)
		non-reduced sepals	reduced sepals		
Non-reduced sepals	62	51	11	3:1	1.74 (0.19)
Non-reduced sepals	59	50	9	3:1	2.94 (0.09)

$\chi2_{05}$ (d.f. 1) = 3.84

alleles, was found in caladium (Deng and Harbaugh, 2006).

As noted above, both mutations caused a partial reduction in the size of sepals of *Nigella* flowers. There is an opinion that the size of the flower organs is controlled by one genetic program, while the number of flower organs is determined by another genetic system, independent of the first one. At the same time, they both regulate the size of the generative organ itself, the flower (Weiss *et al.*, 2005). Our data on the independent combination of genes that determine the number of sepals and their size support the above judgment.

Of the two mutations of reduced sepals identified in *Nigella*, only one (*shs1* gene) affected the shape of the flower, turning an ordinary double flower into a rose-like flower with a decorative value.

4. Conclusions

The preservation and reproduction of plants with such a flower shape for ornamental use in the usual way is problematic due to the inferiority of the female generative sphere.

A partial way out of this problem could be the use of the pleiotropic effect detected at the cotyledon leaf stage. Then the screening of the offspring of heterozygous plants that are the part of a self-pollinated family, carrying the rose-like flower gene, and a subsequent elimination of seedlings with wild-type cotyledons, will not only preserve but also allow to use this unique genotype in ornamental floriculture.

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