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Efficiency of Induced Mutagenesis at Early Stages (Gametes, Zygotes, Proembryos) of Ontogenesis in *Nigella damascena* L.

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Summary. An original technique has been developed of treating gametes, zygotes and early embryos of *Nigella damascena* L. with chemical and physical mutagens. A delay in fertilization and a decrease in the rate of cell division of the embryo and the endosperm after mutagen treatment have been found. Our method of treating gametes, zygotes and proembryos with chemical and physical mutagens is, by all criteria, superior to that of treating dry seeds. Treatment applied at early stages of ontogenesis not only induced a much higher mutation ratio compared with dry seeds, but also gave a broader mutation spectrum. The 55 types of hereditary change obtained affect the structure of vegetative and reproductive organs. Mutations which change the structure of the reproductive organs of flowers are of specific interest.

The optimum dose for this object and the method of treatment which induces high mutation ratio (up to 96 % of families with changes) is 0.003 p (16 hrs) for ethylenimine and 0.005 p or 0.008 p (16 hrs) for nitrosomethylurea. Treatment of dry seeds turned out to be much less effective.

Introduction

An original technique has been developed of treating gametes, zygotes and early embryos of *Nigella damascena* L. with chemical and physical mutagens (Phan Phai 1971 a, b). Studies on mutagenesis in higher plants are usually carried out on dry or swollen seeds, i.e. a completely developed multicellular embryo is subjected to mutagens. Only mutations occurring in a few initial cells which afterwards form generative tissues may be realized during subsequent seed generations. In contrast, we decided to treat very early stages of embryogenesis. Studies of mutation induction on the embryo at early stages of ontogenesis have already been published (Mericle and Mericle 1961 a, b; Devreux and Scarascia 1962; Tramvalidis and Devreux 1964; Monti 1967; Bhaduri and Shome 1969). These authors used physical agents and showed that ionizing irradiation of early ontogenetic stages produced the largest number of highly fertile mutants. In order to reveal the specificity of the mutation process after treatment at early stages of embryogenesis, we applied both physical and highly active chemical mutagens.

The present communication deals with the data on seed formation in M_1 and mutation frequency in M_2 after treatment at the early stages of embryogenesis. The specific features of the developmental stage at which mutagens were applied resulted in a significant increase in the mutation ratio and also made it possible to obtain peculiar mutants which did not appear after treatment of dry seeds.

Material and Methods

Nigella damascena L. *(Ranunculaceae)* was used. It has 6 pairs of chromosomes which are distinguishable morphologically. *Nigella* is also convenient for cytological studies, for solving cytogenetic problems and for genetic purposes.

The following mutagens were used: X-irradiation (Xr), ethylenimine (EI), nitrosomethylurea (NMU), diethylsulphate (DES) and trimethylphosphate (TMP). Mutagens were applied to air-dried seeds, to gametes, zygotes and very early embryos (Table 1). Chemical mutagens were applied immediately after pollination; X-irradiation was carried out 4 hours after pollination.

120-140 plants for each variant were grown in a greenhouse. Only flowers of the main stem of M_0 plants of equal age were treated. In the experiments with EI and TMP, 140 flowers in each variant were treated, with NMU 100 flowers, and with DES 85 flowers. In the greenhouse 20-40 seedbuds were usually formed in each boll of M_0 plants; only in single plants did they number up to 80.

The treatment with chemical mutagens was performed immediately after pollination. Treatment was carried out in a special chamber made of organic glass. Chemical mutagens (5 ml) were injected with a syringe into each boll with seedbuds and washed out with water and a 5 % solution of sodium hyposulphite. In each series of radiation experiments 115 plants were irradiated on RUP-200, 4 hours after pollination of M_0 plants. A special experiment was run for comparison in which air-dried seeds were treated with aqueous solutions of mutagens. 100 plants of M_0 were grown as the control.

Seeds from changed and unchanged plants of M_1 were sown in families to obtain the M_2. 50 families

Table 1. Effect of mutagens on seed formation (in M_1) and mutation ratio (in M_2) after with air-dried seeds)

Material treated	Mutagen	Variant	Dose (r) concentration (%)	Number of treated seed-buds	Seeds obtained in %	Mutation ratio (%)	Ratio of families with mutations (%)
1	2	3	4	5	6 (% of 5)	7	8
-	-	Control (1)	- -	1385	70.32 ± 1.22	0.12 ± 0.05	6.0 ± 2.37
Gametes, zygotes, proembryos	X-irradiation	Xr_1	300 -	1715	37.26 ± 1.16	1.93 ± 0.28	38.0 ± 6.86
		Xr_2	600 -	1638	37.66 ± 1.26	1.51 ± 0.36	40.0 ± 6.92
		Xr_3	900 -	1724	31.78 ± 1.11	5.21 ± 0.44	58.0 ± 6.97
		Xr_4	1200 -	1419	26.42 ± 1.17	2.21 ± 0.30	46.0 ± 7.04
				6496	33.54 ± 0.58	3.21 ± 0.17	45.30 ± 7.04
	Ethylenimine	EI_1	0.003 (8h)	1165	55.53 ± 1.45	2.87 ± 0.33	56.0 ± 7.01
		EI_2	0.005 (8h)	1216	50.57 ± 1.43	4.13 ± 0.40	70.0 ± 6.47
		EI_3	0.008 (8h)	1468	32.62 ± 1.19	7.61 ± 0.54	72.0 ± 4.50
		EI_4	0.003 (12h)	1345	41.48 ± 1.34	8.35 ± 0.57	78.0 ± 5.85
		EI_5	0.005 (12h)	1625	37.96 ± 1.20	9.63 ± 0.61	86.0 ± 4.90
		EI_6	0.008 (12h)	1514	31.30 ± 1.19	11.84 ± 0.67	94.0 ± 3.35
		EI_7	0.003 (16h)	1395	33.55 ± 1.26	18.55 ± 0.82	96.0 ± 2.77
		EI_8	0.005 (16h)	1685	20.53 ± 0.98	6.48 ± 0.52	56.0 ± 7.01
		EI_9	0.008 (16h)	1842	17.64 ± 0.88	6.12 ± 0.51	42.0 ± 6.97
				13255	34.50 ± 0.41	8.35 ± 0.19	72.22 ± 6.33
	Nitroso-methylurea	NMU_1	0.008 (8h)	1275	36.23 ± 1.34	2.23 ± 0.30	46.0 ± 7.04
		NMU_2	0.01 (8h)	1139	36.69 ± 1.42	2.58 ± 0.31	46.0 ± 7.04
		NMU_3	0.005 (12h)	1315	32.31 ± 1.29	4.36 ± 0.41	52.0 ± 7.06
		NMU_4	0.008 (12h)	1428	27.10 ± 1.17	5.27 ± 0.45	60.0 ± 6.92

were sown in each variant, 50 seeds in each family. The total number of families studied in M_2 was 1800, the number of M_2 plants was 83003. 100 families, with 50 seeds in each, were sown as the control.

The mutation ratio was determined from the percentage of families with mutations and from the percentage of mutant plants.

Results and Discussion

a) Embryogenesis and the cytological effects of mutagen treatment

Nigella damascena L. has a unicellular archespore, and an embryo sac of the standard type with eight nuclei, three large unicellular antipodes and a small egg cell. Under greenhouse conditions at 25-28° C the normal course of pollination, fertilization and embryogenesis was as follows.

When the weather was favourable, *Nigella* pollen started germination on the stigma 10 min after pollination; 35-40 min after pollination the pollen tubes reached the style and entered the ovary. 50 min after the presence of sperm cells in the embryo sac their fusion with the egg cell and secondary nucleus (Fig. 1) was observed. Additional sperm cells (3-8 instead of 2) were often observed in the same embryo sac. After fertilization a short resting period was observed. 3 hrs after pollination the secondary nucleus was divided initiating endosperm development. After 4 hrs a two-cell embryo and binucleate endosperm were

treatment of early stages of embryonic development of *Nigella damascena* L. (compared

Gametes, zygotes, proembryos	Nitroso-methylurea	NMU_5	0.01	(12h)	1359	25.09 ± 1.17	6.31 ± 0.50	72.0 ± 6.34
		NMU_6	0.005	(16h)	1576	24.36 ± 1.13	8.76 ± 0.58	82.0 ± 5.43
		NMU_7	0.008	(16h)	1558	24.77 ± 1.09	10.33 ± 0.63	88.0 ± 4.59
		NMU_8	0.01	(16h)	1611	22.59 ± 1.03	4.87 ± 0.44	44.0 ± 7.01
					11261	28.12 ± 0.42	5.55 ± 0.16	61.25 ± 6.89
	Diethylsulphate	DES_1	0.05	(8h)	1167	61.26 ± 1.42	1.88 ± 0.27	44.0 ± 7.01
		DES_2	0.1	(8h)	1255	54.74 ± 1.40	2.79 ± 0.33	40.0 ± 6.92
		DES_3	0.05	(12h)	1345	43.79 ± 1.35	3.28 ± 0.36	50.0 ± 7.07
		DES_4	0.1	(12h)	1264	39.63 ± 1.37	5.91 ± 0.48	60.0 ± 6.92
		DES_5	0.05	(16h)	1057	41.34 ± 1.51	7.32 ± 0.52	60.0 ± 6.92
		DES_6	0.1	(16h)	1418	29.97 ± 1.21	7.38 ± 0.54	66.0 ± 6.69
					7506	44.68 ± 0.56	4.83 ± 0.17	53.33 ± 7.06
	Trimethyl-phosphate	TMP_1	0.01	(8h)	1427	16.46 ± 0.98	3.13 ± 0.34	38.0 ± 6.86
		TMP_2	0.01	(12h)	1589	18.06 ± 0.96	7.21 ± 0.53	70.0 ± 6.47
		TMP_3	0.01	(16h)	1705	12.78 ± 0.80	5.27 ± 0.47	50.0 ± 7.07
					4721	15.67 ± 0.52	5.15 ± 0.26	52.55 ± 7.06
Air-dried seeds	X-ir-radia-tion	$Xr\text{-}DS_1$	9000	-	-	-	-	2.03 ± 0.29	28.0 ± 6.02
		$Xr\text{-}DS_2$	12000	-	-	-	-	1.25 ± 0.25	16.0 ± 5.18
						average		1.68 ± 0.20	22.0 ± 5.56
	Ethylenimine	$EI\text{-}DS_3$	0.025	(12h)	-	-	-	3.50 ± 0.38	32.0 ± 6.58
		$EI\text{-}DS_4$	0.025	(18h)	-	-	-	9.03 ± 0.60	46.0 ± 7.04
		$EI\text{-}DS_5$	0.050	(12h)	-	-	-	6.71 ± 0.54	44.0 ± 7.01
		$EI\text{-}DS_6$	0.050	(18h)	-	-	-	2.65 ± 0.36	26.0 ± 6.20
						average		4.12 ± 0.21	37.0 ± 6.83

formed. After 6 hrs the four-cell embryo and tetranucleate were formed. 7 hrs after pollination, divisions in the embryo and the endosperm became more intensive and 10-12 hrs after pollination the multicellular embryo and endosperm were oberserved. After two days seed-lobes could be observed in the embryo. After 6 days radicle, root-cap and plumule were seen.

It was found that chemical mutagens drastically depressed fertilization and the rate of embryonic development. The strongest effect was produced by EI and TMP, particularly at high doses. Unfertilized egg-cells were observed up to 12 hrs after pollination, while multicellular embryos (more than 8 cells) appeared only 20 hrs after pollination. Many seed buds of the plants of these variants died at various developmental stages. The least pronounced effect was produced by DES: in its presence the developmental rate became only 2-4 hrs slower than in the control. NMU and X-irradiation produced intermediate effects.

Thus our procedure at short exposure time (8 hrs) provided mutagen treatment of gametes, zygotes and of the first developmental stages of the embryo (at low concentrations of mutagens). At 16 hrs exposure, mutations should be also induced in a relatively multicellular embryo. As X-irradiation was applied 4 hours after pollination, mainly fertilized egg-cells and two-cell embryos were treated.

Table 1 shows the data on seed formation of M_0 plants after treating seedbuds with various mutagens.

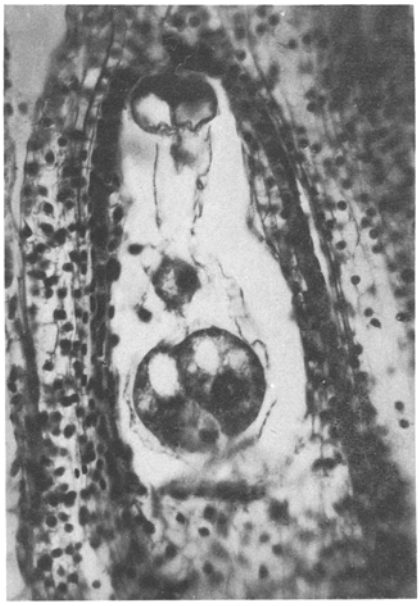

Fig. 1. Embryonic sac of *Nigella damascena* L. in the moment of mutagen treatment before fertilization

They demonstrate a delay in fertilization after chemical mutagen treatment. In order to find out the duration of mutation induction after mutagen treatment, we checked the number of chromosome aberrations for 80 hrs after the beginning of the treatment. The mutagens caused a significant retardation of onset of mitosis and prolonged the mitotic cycle. The induction of chromosome aberrations proceeds for at least 80 hrs after treatment with chemical mutagens. A positive correlation between the number of chromosome aberrations and the depression of plant growth and development has been observed. The frequency of cells with chromosome aberrations found in anaphase I of meiosis significantly increases after mutagen treatment. As a result the amount of sterile pollen increases and plant fertility decreases (Phan Phai, unpubl.).

b) Mutation induction

As Table 1 (rows 7 and 8) indicates, the offspring of plants treated with mutagens at early developmental stages showed twice as many mutations as after the treatment of dry seeds (both according to averaged values obtained with all doses of a given mutagen and according to maximum mutation ratio). The average ratio of families with mutations after X-irradiation and EI-treatment of gametes, zygotes and proembryos was 45.30 % and 72.22 %, when dry seeds were treated it was 22.0 % and 37.0 % respectively.

These data also show that chemical mutagens are more effective than physical ones in all experiments. According to the ability of inducing mutations in M_2 the mutagens used in our experiments can be arranged in the following sequence EI > NMU > TMP > DES > Xr. This sequence does not depend on the method of registration.

Treatment with chemical mutagens applied at early stages of embryogenesis resulted in a high mutation ratio in M_2. The highest variability was observed for EI_7 - 96.0 ± 2.77 and NMU_7 - 88.0 ± 4.59 %, i.e. a change was revealed almost in each family.

After EI treatment of dry seeds mutations were revealed in less than a half of all the families studied (EI - DS_4: 46.0 ± 7.04).

Among the experimental variants with X-irradiation, irradiation of zygotes and proembryos with the dose of 900 r turned out to be the most effective (percentage of families with mutations after Xr_3 was 58.0 ± 6.97 %). With the increase of the dose to 1200r mutation ratio decreased (Xr_4 - 46.0 ± 7.04 %). After irradiation of dry seeds the maximum ratio of families with mutations was only 28 % (Xr - DS_1: 28.0 ± 6.02).

The analysis of literature shows that in most cases chlorophyll mutations are not related to large chromosome aberrations, they are due to point mutations or microaberrations (Gustafsson 1938; Blixt 1961; Gostimsky 1966, 1971). Therefore the proportion of chlorophyll mutations can to some extent serve as a measure of mutagen ability to induce point mutations.

The investigation of chlorophyll mutation ratio showed that the highest value was observed, when chemical mutagens were applied at early stages of ontogenesis. The maximum amount of families with chlorophyll mutations was induced by EI (EI_7-24.0±6.04 %) and NMU (NMU_7 - 20.0 ± 5.65 %). With increased doses some tendency towards the drop of the ratio was observed (EI_9 and NMU_8 - 8.0 ± 3.82 %).

When dry seeds were treated not only less families with chlorophyll defects were observed but also their proportion in the total amount of all mutations decreased. Thus, after EI treatment of proembryos chlorophyll mutations in some variants (EI_6 and EI_7) comprised 1/4 of all mutations, while after EI treatment of dry seeds their proportion did not exceed 15 %. The same tendency was observed after X-irradiation.

Among chemical mutagens used with gametes, zygotes and proembryos TMP induced the least amount of chlorophyll mutations (13.22 + 6.59 %). Thus an inverse relationship between the ability of mutagens to induce chromosome aberrations (Phan Phai 1971a) and chlorophyll mutation ratio was observed.

10 types of chlorophyll mutations were found after the applying of chemical mutagens at early stages of ontogenesis: albina, viridis, chlorina, virescens, xantha, xantha-virescens, vario-micromaculata, yellow-variegated, brown and blackening plants. Mutagens inducing more chlorophyll defects turned to induce also the widest mutation spectrum (EI and NMU). Of chemical mutagens the most narrow spectrum of chlorophyll mutations was induced by TMP (it was almost on the same level as that after X-irradiation).

Specific differences in the occurrence of some types of chlorophyll mutations induced by various mutagens are of great interest. After EI effect a reliable increase of mutation "chlorina" was observed, whereas in NMU experiments a high peak of mutation "xantha" was recorded.

Only after appropriate genetical analysis it can be said whether a prevailing mutation of certain loci or phenotypically similar realization of mutations of different genes was involved.

The effect of chemical and physical mutagens on gametes, zygotes and proembryos besides chlorophyll mutations induced also 45 types of morpho-physiological changes. 4 types of the latter are concerned with cotyledons (tricotyledonous, rounded- and elongated-cotyledonous, and with changed arrangement of cotyledons); 3 types affect leaves (small, hairlike, changed first and second leaves); 11 types affect plant habitus (with fasciated stem, weakly branching, strongly branching, with short internodes, with thin stem, with long internodes, weakly developed, dwarf, full-grown); 3 types pink coloration (pink leaves and stem, brown, black); 17 types flowers (long or short stamens and stigmae, elongated ovary, transformation of spath leaflets into petals or follicles with open ovules, with transformation of petals into leaflets (Fig.2), double-flowering (Fig.3), with increased number of nectaries (Fig.4), with transformation of stamens into leaflets with open ovules (Fig.5), with transformation of various flower elements or all of them into leaves and etc. (Fig.6); 4 types bolls (small, large, changed, chimeric coloration of bolls), 3 types coloration of seeds (white-black, brown and yellow).

Chemical mutagens induced the broadest spectrum of mutants, an increase of the number of various mutation types under the effect of alkylating compounds being accompanied by an increase of the ratio of fertile types of mutants. The broadest spectrum of morpho-physiological changes was obtained in M_2 after EI treatment of gametes, zygotes and proembryos (43 types). NMU was the second by the number of types of morpho-physiological changes (37 types), whereas DES and TMP were significantly less potent (27 and 21 types respectively). After X-irradiation the spectrum of morpho-physiological changes was more narrow (18 types).

The narrowest spectrum of morpho-physiological changes was obtained after the treatment of dry seeds (only 13 types after EI treatment and 8 types after irradiation). No mutation affecting the structure of generative organs was observed throughout all the experiments on dry seeds.

In many cases several mutations were obtained in M_2 within one family. The number of families with multiple changes increased with the increase (up to a certain limit) of the mutagen dose. It was the greatest after the treatment of gametes, zygotes and proembryos with EI (EI_7 - 37.50 ± 6.99 %) and NMU (NMU_7 - 29.54 ± 6.88 %). After the treatment of dry seeds the proportion of families with multiple changes was significantly lower ($Xr - S_1$: 14.28 ± 9.35 %).

Recently several authors found a positive correlation between the number of chromosome aberrations and depression of plants' growth and development (Caldecott 1961; Khvostova 1965; Khvostova and Elshuni 1966).

Our data show that growth, development and fertility of M_2 plants correlate with the number of chromosome aberrations in embryo cells and radicle meristem. However mutation ratio in M_2 is not directly proportional to that in M_1. Variants obtained at high mutagen doses which induced a strong disturbance of chromosome structure in embryo cells and greatly delayed the growth and development of M_1 plants showed a low percentage of mutations in M_2. A decrease of the number of families with multiple mutations and late mutations was also observed in these variants.

The analysis of mutation ratio in M_2 induced after the treatment of zygotes, proembryos and dry seeds

Fig.2.

Fig.3.

Fig.4.

Fig.5.

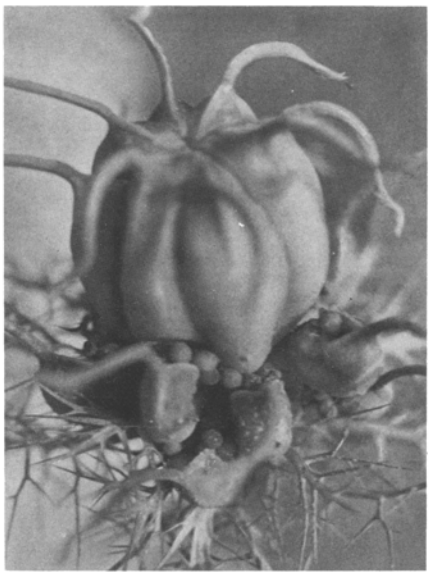

Fig.6.

Fig.2. Normal flower of *Nigella damascena* L. Figs.3-6. Mutants of flower generative organs after the treatment of gametes, zygotes and proembryos with different mutagens

with different mutagens showed by all recording methods that chemical mutagens were superior to physical ones. This conclusion well agrees with the evidence of other authors obtained on other objects (Ehrenberg, Gustafsson, and Lindquist 1961; Eiges 1965; Sermin and Voytovitch 1965).

It is well known that chromosome aberrations and visible mutations are the two types of changes which can occur independently. To obtain more viable mutations it is necessary to decrease the number of large chromosome aberrations responsible for the disturbance of genic balance and cell death.

The great number of point mutations induced by chemical mutagens seems to be concerned with their greater ability to induce relatively "soft" changes of different loci. There are some data concerning greater selectivity of their effect on separate loci. It is also known that chemical mutagens induce less common and more varied mutations, which may also be connected with the total increase of mutation ratio (Ehrenberg 1960; Khvostova, Mozhaeva and Eiges 1963; Rapoport 1971).

When comparing chlorophyll mutation ratio with the ratio of other morpho-physiological mutations obtained in our experiments it was found that the contribution of chlorophyll mutations to overall mutability was higher after the treatment at early stages of ontogenesis.

Morpho-physiological mutations induced by chemical mutagens seem to be to a lesser extent concerned

with chromosome aberrations than the irradiation induced ones. This seems to account for the fact that more fertile mutations were observed after chemical mutagens than after irradiation.

Our method of treatment gametes, zygotes and proembryos with chemical and physical mutagens is by all criteria superior to that of treating dry seeds. Treatment applied at early stages of ontogenesis not only induced much higher mutation ratio as compared with dry seeds but also gives a broader mutation spectrum. The obtained 55 types of hereditary changes are affecting the structure of vegetative and generative organs. Mutations changing the structure of flower generative organs are of specific interest. Further investigation of developmental genetics of these mutations will contribute to the solution of a number of theoretical (study of the origin of flower and of some of its structures in *Angiospermae*) and practical problems. *Nigella damascena* L. can be used as a model for studying possible increase of seed productivity in bisexual plants.

It is important to increase the available spectrum of mutations, because it enables the study of structure of character; i.e. it permits to determine the number of genes, responsible for the development of the character studies, the sequence of their function in ontogenesis, peculiarities of the interaction of these genes. Apprehension of the fine structure of the character will permit construction of organisms with desirable characters and properties.

Our data show that the method of treating gametes, zygotes and proembryos is very effective in inducing mutations. The optimum dose for the given object and method of treatment which induces high mutation ratio (up to 96 % of families with changes) is EI - 0.003 % (16 hrs), NMU - 0.005 % and 0.008 % (16 hrs). The method of treating dry seeds turned to be much less effective.

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