Theoretical and Applied Genetics 47, 109-114 (1976)

Anther Cultures of *Nicotiana tabacum* L. Mutants

J. Vagera, F.J. Novák and B. Vyskot

Czechoslovak Academy of Sciences, Institute of Experimental Botany, Praha, Experimental Station Olomouc (Czechoslovakia)

Summary. The theoretically expected and experimentally observed phenotypic ratios have been compared in populations of haploids derived from chlorophyll mutants of *Nicotiana tabacum* L. with a known genotypic constitution. The frequencies of mutant genotypes were significantly lower than the expected values, proving the existence of selection in a system of haploid embryoids developing in the anther.

The anthers from M_1 plants of a diploidized *Nicotiana tabacum* haploid cv. Samsun, treated with various concentrations of N-nitroso-N-methylurea and n-butylmethane sulphonate, were cultivated *in vitro*. The number of anthers which gave rise to haploids (embryogenic anthers) was stimulated by lower concentrations of both the mutagens. The stimulation at the level of M_1 sporophyte is explained by internal genetic heterogeneity induced by adequate mutagen concentration. The average number of haploids per embryogenic anther decreased in all the treatments. The frequency of haploid plants of the mutant phenotypes increased with increasing mutagen concentration.

Introduction

A haploid system in higher plants would be important for studying spontaneous and induced mutagenesis as well as for hybrid analysis. During the analysis of haploids derived from anther culture of hybrids deviations have been observed from the expected segregation ratios (Melchers and Labib 1970; Melchers 1972; Opatrný 1973), misrepresenting the results of a real gametic segregation of the factors. To verify the suitability of anther derived haploids for genetic analysis we compared the theoretical and observed phenotypic ratios in populations of haploids derived from heterozygous chlorophyll mutants of *Nicotiana tabacum* L. The conclusions of these model trials have a decisive influence on the interpretation of studies involving segregation in haploid progenies of plants with unknown genotypic constitution.

In further trials, which were related to our previous work (Vyskot et al., 1974), we studied the developments of haploids from anthers of M_1 plants obtained after the seeds had been treated with increasing concentrations of the mutagens. In haploid progenies of M_1 plants we estimated not only the frequencies of mutant phenotypes but also some quantitative characteristics of androgenesis previously omitted.

Materials and Methods

The chlorophyll mutants Sulfur (Su), Yellow seedling (ys) and White seedling (ws) of *Nicotiana tabacum* L., kindly provided by prof. L.G. Burk, served as initial material for analyses of haploids. The mutation Sulfur is incompletely dominant with a monogenic constitution and is lethal at the homozygous dominant stage. Heterozygous (Su/su) plants were utilized for anther culture. The monogenic mutation Yellow seedling is lethal at the homozygously recessive stage. The phenotype White seedling, produced by two genes with a duplicate non-cumulative function, is non-viable under normal conditions in the homozygous recessive condition. We used plants which were homozygously recessive in one of both the loci so that the mutation White seedling responded as a single recessive during segregation. According to the segregation of ys and ws mutants in anther cultures, the trial involved only donor plants with heterozygous genotypic constitution. Phenotypic frequencies in haploid progenies were studied using five plants from each of the three types of chlorophyll mutant. The culture contained 60 anthers from each plant.

In further trials the initial donor material was a diploidized haploid of *Nicotiana tabacum* L. cv. 'Samsun' obtained through regeneration from tissue culture of an androgenic haploid petiole. The seeds were treated with solutions of n-butylmethane sulphonate (BMS - 5 mM, 10 mM, 20 mM, 40 mM) and N-nitroso-N-methylurea (MNU - 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM) for 24 hrs at 23 - 25° C in the dark. The control was soaked in distilled water. The seeds were then rinsed in running water and sown on moist garden soil. Randomized sets of 20 plants in each variant (M_1 generation) were planted out in the experimental field and the anthers were taken from individual plants at the stage of uninuclear microspores. We have cultivated 40 anthers from each M_1 plant, i.e. 800 anthers per variant, with the exception of 40 mM BMS and 0.8 mM MNU (240 anthers and 600 anthers, respectively). The experiment was carried out at room temperature and under continuous illumination. The haploid plants were evaluated during the course of the trial (detection of chlorophyll mutations) and also in one lot after 13 weeks' cultivation (evaluation of quantitative characteristics of androgenesis). The evaluation involved 3,255 haploid plants. The chlorophyll mutations were classified according to the system proposed by Lamprecht (1960). In all the trials the anthers were cultivated on the medium of Nitsch (1969) with an addition of 0.1 mg/ℓ β - indoleacetic acid (IAA).

Table 1. Evaluation of segregation ratios in populations of haploid plantlets in the *in vitro* anther culture derived from heterozygous chlorophyll mutants of *N. tabacum* L.

Donor plant No.	Total of regenerants obtained	Theor. segreg. ratio	Observed segreg. ratio	χ^2	P
1	159	79.5 : 79.5	89 : 70	2.27	0.30 - 0.10
2	121	60.5 : 60.5	70 : 51	2.98	0.10 - 0.05
3	171	85.5 : 85.5	97 : 74	3.09	0.10 - 0.05
4	144	72 : 72	82 : 62	2.78	0.10 - 0.05
5	112	56 : 56	65 : 47	2.89	0.10 - 0.05
Total	707	353.5 : 353.5	403 : 304	13.86	0.001
1	96	48 : 48	54 : 42	1.50	0.30 - 0.10
2	74	37 : 37	42 : 32	1.35	0.30 - 0.10
3	82	41 : 41	48 : 34	2.39	0.30 - 0.10
4	90	45 : 45	53 : 37	2.84	0.10 - 0.05
5	72	36 : 36	41 : 31	1.39	0.30 - 0.10
Total	414	207 : 207	237 : 177	8.70	0.01 - 0.001
1	91	45.5 : 45.5	56 : 35	4.85	0.05 - 0.02
2	121	60.5 : 60.5	74 : 47	6.02	0.02 - 0.01
3	102	51 : 51	61 : 41	3.92	0.05 - 0.02
4	96	48 : 48	58 : 38	4.17	0.05 - 0.02
5	108	54 : 54	63 : 45	3.00	0.10 - 0.05
Total	518	259 : 259	312 : 206	21.69	0.001

Results

In anther cultures derived from heterozygous *N. tabacum* plants of the mutations Sulfur, Yellow seedling and White seedling the frequency of mutant phenotypes was lower than of green plants. Although in most cases the biometric evaluation of haploid progenies, derived from individual mother plants, was in agreement with an expected gametic ratio 1:1, there was a significant disagreement in all three mutant types when the total values, obtained by summarizing results of segregation in 5 plants of the same mutation, were considered (Tab.1). The gene frequencies of mutant alleles Su, ys and ws decreased from the initial value of 0.50 (at the level of pollen grains) to that of 0.43, 0.43 and 0.40, resp. (at the level of haploid plants in the *in vitro* anther cultures). The coefficients of selection of mutant alleles reached the values of 0.25, 0.25 and 0.36 resp., fitness 0.75, 0.75 and 0.64, resp.

Haploid plantlets derived from M_1 plants emerged during the third week of anther culture and continued to appear until the 13^{th} week. The highest number of haploids per anther was 55, the highest number of true leaves per haploid plant was 14. The concentrations of the mutagens influenced the frequency of haploid formation in all the studied parameters. The efficiency of the method of *in vitro* anther culture can be well demonstrated by taking the average number of haploid plants obtained per cultivated anther (Fig.1). The concentration 5 mM BMS stimulated the number of haploid plants per cultivated anther and increased variability. Average numbers of haploid plants per cultivated anther and their variances decreased with increasing concentrations of BMS. The application of 0.1 mM MNU had no effect on the frequency of androgenesis but decreased its variability. There was a continual decrease in the average number of haploids per cultivated anther with higher concentrations of MNU. The average number of haploid plants per embryogenic anther decreased in all the treatments (Fig.2). The average number of embryogenic anthers per M_1 plant was also dependent on mutagen concentration. It was stimulated at a concentration of 5 mM BMS but decreased with increased concentrations. A similar relationship was recorded with MNU (Fig.2). All the applied concentrations of both the mutagens increased the variance.

The number of embryogenic anthers increased threefold, twofold and by 50 % in the variants with 5 mM BMS, 10 mM BMS and 20 mM BMS, respectively, when compared with the control. The concentration 0.1 mM MNU increased the number of anthers which gave rise to haploids by 75 %.

After 13 weeks' culture the mean number of true leaves on haploid plants was significantly higher in all

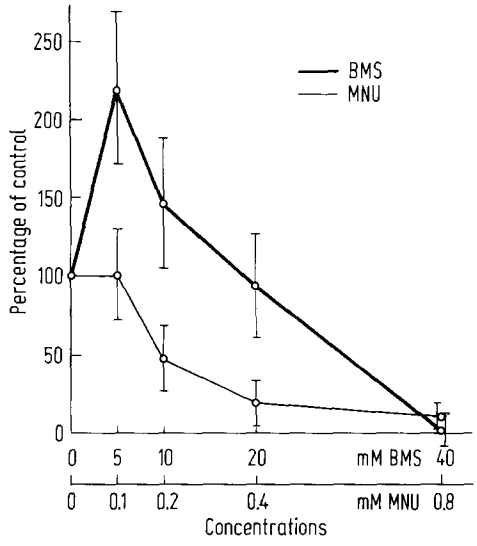

Fig. 1. Average number of androgenic haploids per cultivated anther in the control and treated variants (Φ = $P < 0.05$)

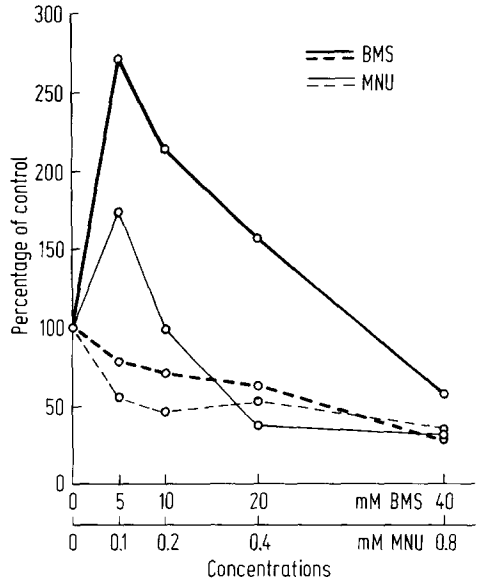

Fig. 2. Average number of embryogenic anthers per M_1 plant (full line) and that of haploid plants per embryogenic anther (dashed line) in the control and treated variants

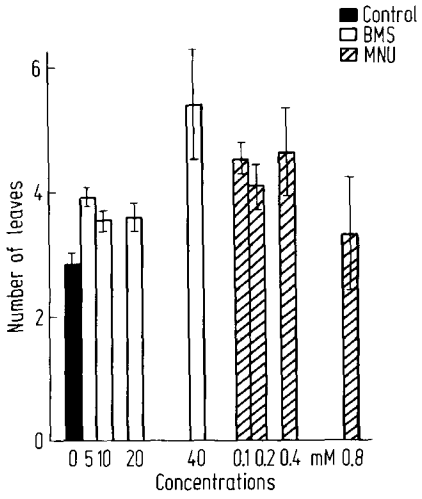

Fig. 3. Average number of true leaves on haploid plantlets after 13 weeks' cultivation in the control and treated variants (Φ = $P < 0.05$)

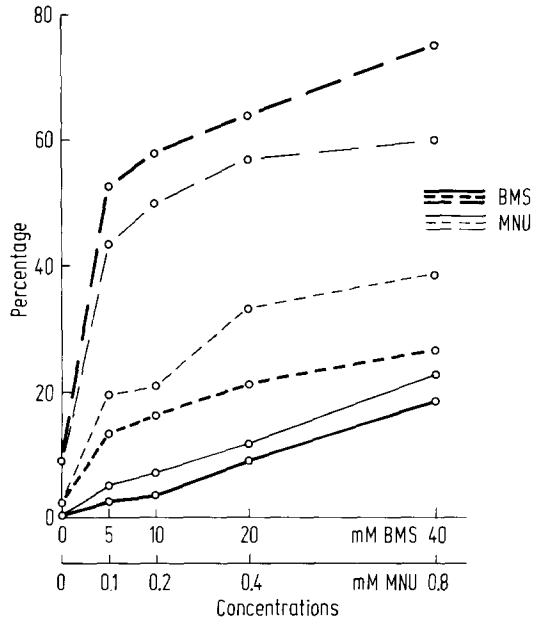

Fig. 4. Frequency of androgenic haploids of mutants phenotype (full line), frequency of embryogenic anthers segregating haploid mutants (short-dashed line) and of M_1 plants with embryogenic anthers and haploid mutants (long-dashed line) in the control and treated variants

the treated variants (except for 0.8 mM MNU) than in the control. The application of the mutagens also increased the variance, except in the case of 40 mM BMS. In relation to the MNU and BMS concentrations, the mean numbers of true leaves showed the same tendency - a slight decrease followed by an increase (Fig. 3).

With respect to chlorophyll mutations the treated variants contained the following phenotypes: *albina, viridalba, albina-xanthescens, albina-virescens, xantha, xantha-virescens, chlorina virescens, chlorotica* and *viridissimus*. In the control a chlorophyll chimera with *albina, xantha, chlorina* and *chlorotica* segments ap-

Table 2. Numbers of haploid plants, embryogenic anther and chlorophyll mutants

Variants	Number of embryogenic anthers	Total of haploid plants obtained	Number of chlorophyll mutants
Control	47	439	1
5 mM BMS	128	956	25
10 mM BMS	98	637	21
20 mM BMS	71	414	38
40 mM BMS	8	22	4
0.1 mM MNU	82	439	24
0.2 mM MNU	47	208	15
0.4 mM MNU	18	92	11
0.8 mM MNU	13	48	11

peared, while there was no chlorophyll chimera in the treated variants. The frequency of haploid plantlets of a mutant phenotype increased with increasing mutagen concentration. There was a linear relationship between mutagen concentration and mutation frequency. The mutagen concentration also influenced the increase in the frequency of embryogenic anthers with mutant plants, and the proportion of M_1 plants whose anthers gave rise to androgenic mutants (Fig.4). A part of the albino types remained in the culture, while a major part became green some time later. The highest and lowest frequencies of mutant phenotype plants were induced by 0.8 mM MNU and 5 mM BMS, respectively (Tab.2). Because production of androgenic haploids in chimerical plants was low, the relationship between the frequency of chimeras in M_1 and that of haploid chlorophyll mutants could not be determined. The haploid genomic state of the plants obtained from anther cultures in both parts of the trial was verified at random by meiotic analysis. In all cases we found a haploid chromosome number (n = 24).

Discussion

In order to use the technique of *in vitro* anther cultivation for basic studies in plant genetics and breeding, it is essential to determine if competition between the various genotypes of pollen grains occurs. The mean number of pollen grains per anther is in the order of 40,000. Sunderland (1970) observed that in the *in vitro* culture only about 5 % of pollen grains were embryogenic, i.e. the anther contained about 2,000 "potential plantlets". The actual number of haploids obtained from one anther is, however, essentially lower, e.g. in our trials with *Nicotiana tabacum* cv. 'Samsun' there were 2 plants per cultivated anther (Vyskot and Novák 1974). This shows that only about 0,005 % pollen grains give rise to haploid plants. During embryonic development some gametes with unfavourable alleles or allele combinations may be eliminated by selection and thus the real gametic segregation of the factors may be misrepresented. In some studies the phenotypic ratios found in F_2 haplophase for various morphological characters, such as resistance to diseases etc., agreed with the expected segregation (Nakata 1971; Nakata and Kurihara 1972; Vyskot et al., 1974). Certain complications occur during the study of the expression of subvital and lethal alleles. In the present trials we have found a statistically significant decrease in the frequency of lethal alleles Su, ys and ws compared with the theoretically expected values.

In spite of a certain decrease in frequency, these plantlets (being subvital under *in vitro* conditions) were relatively numerous in anther cultures. It can be regarded as an experimental demonstration of the suitability of induced androgenesis for genetic analysis with the aim of obtaining rare haploid genotypes. The ascertained selection against the phenotypes with subvital expression of the alleles should be taken into account when interpreting the results obtained in the *in vitro* anther cultures for genetic analysis.

In other studies dealing with *in vitro* induction and detection of mutations at the level of androgenic plants, the mutagens were applied, as a rule, directly to anthers or haploid plantlets (Nitsch 1969; Tyrnov et al.

1973; Devreux and Laneri 1974; Mondeil 1974; Stolarz 1974). Vyskot et al. (1974) detected haploid mutants from anthers of M_1 plants (after N-nitroso-N-methylurea treatments). For the present study we chose a similar procedure, which allowed comparison of the sporophyte and gametophyte and eliminated undesirable effects produced by the immediate action of the mutagen on microspore metabolism. The first interesting fact was an increase in the absolute number of haploid plants in the variants treated with lower mutagen concentrations. Our experiments show that the diploid sporophyte plays a decisive role in stimulation of the average number of androgenic plants per cultivated anther. The number of embryogenic anthers was stimulated while the average number of haploids in the embryogenic anther decreased. This leads to the conclusion that the induced lethal mutations which come into existence may have a negative influence on the number of haploids in the anther.

The stimulating effect of proper doses of ionizing radiation at the level of diplophase has been studied e.g. by Sax (1955, 1963) etc. in intact plants, and by King (1949), Holsten et al. (1965) and Bajaj et al. (1970) in tissue cultures. The stimulation was also recorded after application of chemomutagens (Vagera 1972) etc. The stimulation of androgenesis can be explained by an increase in internal genetic heterogeneity in M_1 (Andreev 1963) due to the application of an adequate mutagen concentration. At the same survival frequency, BMS and MNU induce more point mutations and fewer chromosomal aberrations than does ionizing radiation (Gichner 1968). The phenotypic effect of the increase in internal genetic heterogeneity, caused by the induction of recessive point mutations in M_1, should be particularly expressed in a diploidized haploid with regard to its homozygosis. Maruyama and Crow (1975) demonstrated the importance of heterozygous or homozygous genetic background in affecting the viability during induction of mutations in *Drosophila melanogaster*. Under the same conditions of cultivation in the control variant with *Nicotiana tabacum* L. cv. 'Samsun', we have obtained a smaller mean number of haploids per cultivated anther than in our previous work (Vyskot et al., 1974). However, in our previous work heterozygous material was utilized, while in this trial a diploidized haploid was used, indirectly supporting our conclusion of a potential stimulation mechanism. Our results indicate that adequate BMS concentrations can be recommended for stimulating androgenesis, perhaps even with other plant species.

An increase in the average number of true leaves in haploids derived from the treated variants corresponded with a smaller number of plant lets in an embryogenic anther, i.e. with their spatial and nutritional advantages. Under such conditions the growth and development of androgenic plants are dependent primarily on their density in the embryogenic anther, and secondarily on the applied mutagen concentration. It should be pointed out, however, that the concentration applied is decisive in determining the mean number of haploid plants per androgenic anther.

The induced chlorophyll defects were phenotypically identical with common chlorophyll mutations. The amphidiploid nature of *Nicotiana tabacum* L. does not exclude the multiple presence of genes with similar effects. It would also influence the phenotype of some chlorophyll mutations. Some of the original mutations of the *albino* phenotype did not change their own phenotype in the course of the whole cultivation; a major part could be characterized as *albina-virescens* a later stage. A similar development was observed in the original phenotypes *xantha* and *chlorina*.

The results of the first part of the present work show that mutant embryoids with subvital phenotypic expression were partly eliminated due to the action of selection pressure. It is very probable, therefore, that the frequency of mutant plantlets due to selection decreased even in the cultures derived from M_1 plants, providing the technique of *in vitro* anther cultivations was the same.

Acknowledgements

The authors thank Dr. Z. Landa and Dr. Z. Opatrný for their valuable comments, Ing. M. Adamusová, Mrs. J. Eliášová and Mr. I. Klement for technical assistance and Miss J. Potomková for preparing the English text.

Literature

Andreev, V.S.: The genetic mechanism of radiostimulation of plants (in Russian). In: Predposevnoje obluczenije semjan selskochozjajstvennych kultur. Moskva: A. N. SSSR 1963

Bajaj, Y.P.S.; Saettler, A.W.; Adams, M.W.: Gamma irradiation studies on seeds, seedlings and callus tissue cultures of *Phaseolus vulgaris* L. Radiat. Bot. 10, 119-124 (1970)

Devreux, M.; Laneri, U.: Anther culture, haploid plant, isogenic line and breeding researches in *Nicotiana tabacum* L. Vienna: IAEA (1974)

Gichner, T.: Chemomutagens (Review) (in Czech). Praha: UVTI 1968

Holsten, R.D.; Sugii, M.; Steward, F.C.: Direct and indirect effects of radiation on plant cells; their relation to growth and growth induction. Nature 208, 850-856 (1965)

King, G.S.: Direct and transmitted X-ray effects on growth of tobacco callus *in vitro*. Amer. J. Botany 36, 265-270 (1949)

Lamprecht, H.: Classification system of leaf colour mutants. Agri Hort. Genet. 18, 135 (1960)

Maruyama, T.; Crow, J.F.: Heterozygous effects of X-ray induced mutations on viability of *Drosophila melanogaster*. Mutation Res. 27, 241-248 (1975)

Melchers, G.: Haploid higher plants for plant breeding. Z. Pflanzenzüchtung 67, 19-32 (1972)

Melchers, G.; Labib, G.: Die Bedeutung haploider höherer Pflanzen für Pflanzenphysiologie und Pflanzenzüchtung. Ber. Dtsch. Bot. Ges. 83, 129-150 (1970)

Melchers, G.; Labib, G.: Somatic hybridisation of plants by fusion of protoplasts. I. Selection of light resistant hybrids of "haploid" light sensitive varieties of tobacco. Molec. Gen. Genet. 135, 277-294 (1974)

Mondeil, F.: Irradiation de microspores en culture d'anthères: Essai d'une nouvelle technique d'obtention de mutations immédiatement décelables et fixables (application à *Nicotiana tabacum*). Ann. amélior. plant. 24, 1-11 (1974)

Nakata, K.: Competition among pollen grains for haploid tobacco plant formation by anther culture. I. Analysis with leaf color character. Jap. J. Breed. 21, 29-34 (1971)

Nakata, K.; Kurihara, T.: Competition among pollen grains for haploid tobacco plant formation by anther culture. II. Analysis with resistance to tobacco mosaic virus (TMV) and wildfire diseases, leaf color and leaf-base shape characters. Jap. J. Breed. 22, 92-98 (1972)

Nitsch, J.P.: Experimental androgenesis in *Nicotiana*. Phytomorphology 19, 389-404 (1969)

Opatrný, Z.: Androgenesis *in vitro* in anther cultures of chlorophyll mutants of *Nicotiana tabacum*. Biol. Plant. 15, 286-289 (1973)

Sax, K.: The effect of ionizing radiation on plant growth. Amer. J. Bot. 42, 360-364 (1955)

Sax, K.: The stimulation of plant growth by ionizing radiation. Radiat. Bot. 3, 179-186 (1963)

Stolarz, A.: Induction of androgenesis in pollen grains of *Secale cereale* L. cv. Strzekeciński jare in the *in vitro* culture (in Pol.). Hodowla Roslin, Aklimatyzacja i nasiemnictwo 18, 217-220 (1974)

Sunderland, N.: Pollen plants and their significance. New Scientist 16, 142-144 (1970)

Tyrnov, V.S.; Sukhanov, V.M.; Khokhlov, S.S.: Prospects of using haploid plants for breeding by means of selection of mutants in Angiosperms (in Russian). Genetika 9, 38-46 (1973)

Vagera, J.: Studies in quantitative genetics. II. Micromutations and macromutations induced in einkorn wheat (*Triticum monococcum* L.) by the effect of N-nitroso-N-methylurea, butylmethane sulphonate and X-rays. Folia Fac. Sci. Nat. Univ. Purkynianae Brunensis, Biologia 35, XIII, 21-84 (1972)

Vyskot, B.; Novák, F.J.: Genotypic foundation of the frequency of androgenesis *in vitro* and characteristics of stomates in haploid plants. Scripta Fac. Sci. Nat. Univ. Purkynianae Brunensis, Biologia 1, 4, 25-34 (1974)

Vyskot, B.; Novák, F.J.; Havránek, P.: A genetic analysis at the level of haploid sporophytes of *Nicotiana tabacum* L. Z. Pflanzenzüchtung 72, 245-251 (1974)

Received September 23, 1975
Communicated by H. Stubbe

Dr. Jiři Vagera
Dr. František J. Novák
Dr. Boris Vyskot
Czechoslovak Academy of Sciences
Institute of Experimental Botany
Experimental Station Olomouc
Sekolovská 6
77200 Olomouc (Czechoslovakia)