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An Effective Chemical Mutagenesis Procedure for *Petunia hybrida* Cell Suspension Cultures

C.M. Colijn, A.J. Kool and H.J.J. Nijkamp
Department of Genetics, Biological Laboratory, Free University, Amsterdam (the Netherlands)

Summary. By using mercury(II)-chloride ($HgCl_2$) and Dl-6-fluorotryptophan (6FT) as positive selection conditions we were able to show that N-methyl-N'-nitro-N-nitrosoguanidine (NG) is an effective mutagen for *Petunia hybrida* suspension cells.

A number of the 205 calli resistant to $HgCl_2$ and 17 calli resistant to 6FT were isolated. The highest mutation frequency was 1.0×10^{-5} and 2.0×10^{-6} for $HgCl_2$ and 6FT, respectively. A preliminary characterization of the mutants is presented.

A significant increase in the number of drug-resistant calli was only obtained at NG-concentrations (5-40 μg/ml) that had no observable effect on the survival of the mutagenized cultures.

Key words: Mutagenesis – *Petunia hybrida* cell lines – Drug resistant mutants

Introduction

A number of chemicals, also used for bacterial mutagenesis, have been applied to the isolating of mutants from plant cell cultures and protoplasts. For a recent review see Widholm (1977a). In only a few cases are data presented that show an increase in mutation frequency after mutagenic treatment (Nishi et al. 1974; Sung 1976; Widholm 1976). However in most cases the effect of the mutagenic treatment on the mutation frequency was not clear. This might be explained from the fact that little information is available on the concentration of mutagen that is most effective for the isolation of mutants from plant cell cultures. We decided, therefore, to develop an effective mutagenesis procedure by determining the correlation between mutagen concentration, survival of the cells and the yield of mutants.

Haploid plant material is probably the best starting material for mutant isolation although in quite a number of cases haploid cell lines are not required e.g. for the isolation of cis- and trans dominant mutants. In diploid organisms, mutants of wild type alleles can be obtained when the particular allele is present as a heterozygous gene pair. Furthermore, no one has actually shown that there is a difference in mutation frequency between diploid and haploid cell cultures. In addition, cell cultures started from haploid material usually lose their haploid character very fast, ending up as mixture of aneuploid and polyploid cells (Vasil and Nitsch 1975). Therefore, we preferred to use the more stable, diploid cell cultures.

In this paper we describe the development of positive selection procedures to facilitate the scoring of mutants. Furthermore we show that NG can be successfully used for the isolation of drug-resistant mutants from *Petunia hybrida* suspension cultures.

Materials and Methods

Cell Culture

Cell suspension cultures, initiated from leaves of a diploid *Petunia hybrida* PZ5050D_1 (obtained from Prof. Dr. F.K. Bianchi, Laboratory of Genetics, University of Amsterdam, The Netherlands) were grown in MS medium (Murashige and Skoog 1962), supplemented with 1 μg of 2,4-dichlorophenoxyacetic acid (2,4-D) per ml, under continuous light (Philips no. 33, 200 lux) at 26° C and 60% relative humidity on rotary shakers (125 rev/min).

Mutagenesis

Three days after subculturing, the cell suspensions (130 ml), each containing 1.5×10^5 cells/ml, were used for mutagenic treatment. They were incubated for 4 hours at 26° C with various concentrations of mutagen and then washed 3 times with MS medium. After growth for 2 days in MS medium, survival of the cells was scored by using the dye phenosafranine (Widholm 1972): samples (0.2 ml) of the cell cultures were mixed with 0.2 ml of phenosafra-

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nine solution (0.1% in MS medium) and incubated at 25°C for 5 min. The supernatant was then removed and the cells were suspended in 0.4 ml of MS medium. To determine the percentage of stained, dead cells an average of 4000 cells were counted under the light microscope.

After another two days of culture, the mutagenized cells were plated at a density of 4.5×10^5 cells/plate on MS medium supplemented with drugs and incubated under continuous light (Philips no. 33, 350 lux) at 26°C and 60% relative humidity. After 4-6 weeks the plates were screened for mutant growth.

Results

Effect of Chemical Mutagens on the Survival of P. hybrida Cells

To make an analogy to microbial and animal systems we wanted to determine the correlation between yield of mutants and survival after treatment of the *P. hybrida* cells with chemical mutagens. In order to construct the required survival curves, we selected 6 chemicals that are used for bacterial and animal mutagenesis: acridine orange, ethidium bromide, ethyl methanesulphonate (EMS), methyl methanesulphonate (MMS), N-methyl-N-nitroso-N′-nitroguanidine (NG) and hydroxylamine. Their effect on the survival of *P. hybrida* cells was scored by the phenosafranine dye method rather than by plating of the cell cultures in order to avoid problems with the plating of low cell numbers (at high mutagen concentrations). The scoring of the percentage of survival from plated cells is usually inaccurate due to the presence of cell aggregates in these cell cultures. Furthermore, the plating method is not suitable for the detection of a low percentage of killing.

To show the reliability of the phenosafranine method which specifically stains dead cells, we used the dye fluorescein diacetate which specifically stains viable cells (Widholm 1972a): a mutagenized cell culture was treated with both fluorescein diacetate and phenosafranine and viewed by both the phase contrast and fluorescence technique. We observed that the cells stained by phenosafranine never showed fluorescence and that the cells that did fluoresce were not stained by phenosafranine.

Table 1. Survival of mutagenized *P. hybrida* cell cultures

Mutagen	Conc. range (μg/ml)	Survival (% of control)[a]
Acridine orange	100-1000	100-74.8
Ethidium bromide	100-1000	100-74.3
Ethyl methanesulfonate	100-1000	100
Methyl methanesulfonate	10-1000	100- 4
Nitrosoguanidine	10- 200	100- 5
Hydroxylamine	5-1000	100- 8.5

[a] The fraction of viable cells in the non-mutagenized cultures was 86 ± 5%

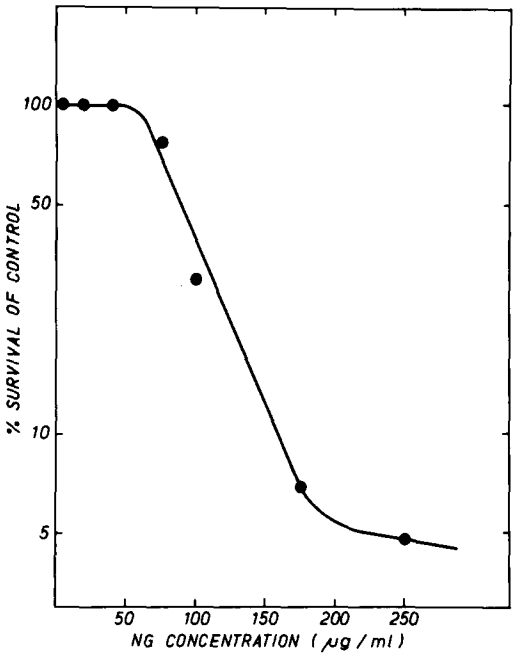

Fig. 1. Survival of *P. hybrida* cells after treatment with various concentrations of NG. The data are the average of the values from three separate experiments

Table 1 summarizes the percentage of survival of *P. hybrida* cells after treatment with various concentrations of mutagen. The cells appear to be quite sensitive to the mutagens NG, hydroxylamine and MMS and rather resistant to EMS, ethidium bromide and acridine orange. The latter mutagens, even at concentrations as high as 1000 μg/ml, have only a small effect on survival. For the proceeding mutagenesis experiments we arbitrary chose concentration levels for NG. A survival curve of *P. hybrida* cells treated with various concentrations of NG is shown in Figure 1.

Positive Selection Conditions

To determine the mutagen concentration that is most effective for the isolation of mutants we developed positive selection conditions to facilitate the scoring of the number of mutants. A frequently used method for positive selection is the growth of resistant calli on media containing drugs at concentrations that do not allow the growth of wild type cells. To set up such selection systems we have tested the growth of *P. hybrida* suspension cells after plating them on MS agar medium containing drugs at various concentrations.

Of the 29 drugs tested, only 10 compounds gave complete growth inhibition of *P. hybrida* cells when used at 'moderate' concentrations (5-100 μg/ml). The concentrations listed in Table 2 are the minimal concentrations

Table 2. Drugs that inhibit growth of *P. hybrida* cells

Drug	Growth inhibiting concentration (μg/ml)	Drug	Growth inhibiting concentration (μg/ml)
Chloramphenicol	100	Methyl-DL-Leucine	> 500
Cycloheximide	5	Dl-α methyl-Serine	> 100
2,4-D	50	Dl-α methyl-tyrosine	> 100
7-Methyl-DL-Tryptophan	10	L-Proline	> 500
5-Methyl-DL-Tryptophan	100	L-Valine	> 500
DL-6-Fluorotryptophan	10	DL-p-Fluorophenylalanine	700
5-Bromo-2'-deoxyuridine	100	n Methyl-DL-Alanine	750
5-Fluorodeoxyuridine	10	Hydroxy-L-Proline	750
8-Azaguanine	10	5 Methyl-Cytosine	750
Mercury(II)-chloride	5	α Methyl-DL-Methionine	500
		Kanamycin	1000
Tetracyclin	1000	Trimetoprim	600
Oxytetracyclin	200	D-Cycloserine	200
Streptomycin	7500	Puromycin	≥ 250
Ampicillin	> 1000		
Erythromycin	4000		

of drug that has to be added to the MS agar medium to prevent the growth of plated *P. hybrida* cells up to a period of 4 months of incubation. Of these potentially useful drugs we arbitrary chose mercury(II)-chloride and Dl-6-fluorotryptophan for the positive selection of drug-resistant mutants.

Isolation of Drug-resistant Cell Lines

Cell cultures of *P. hybrida* were mutagenized with various concentrations of NG, washed, resuspended in fresh MS medium and incubated for 4 days to allow for phenotypic expression of possible mutations. In this period of 4 days the fresh weights of both the non-mutagenized control cultures and the cultures treated with a low concentration of NG (5 μg/ml) increased about 80%. All cultures were

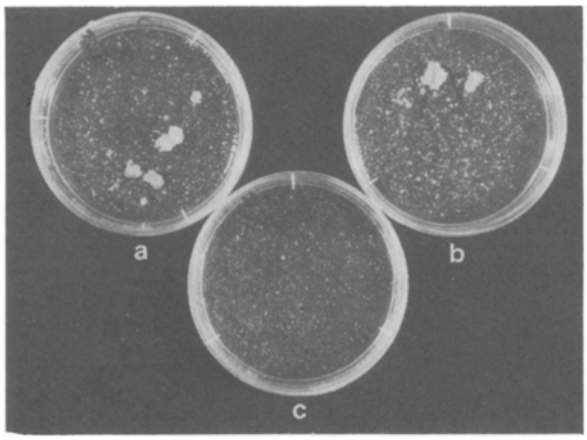

Fig. 2a-c. Growth of mutagenized *P. hybrida* cells on $HgCl_2$-containing (20 μg/ml) agar plates. Plate **a** and **b** contain mutagenized cells, control plate **c** contain non-mutagenized cells

Table 3. Number of $HgCl_2$-resistant calli obtained after NG mutagenesis

NG conc. (μg/ml)	Survival[a] (% of control)	Experiment 1		Experiment 2		Experiment 3	
		Number of resistant calli	Frequency[b]	Number of resistant calli	Frequency[b]	Number of resistant calli	Frequency[b]
0	100 ± 7.0	1	1.3×10^{-7}	2	2.8×10^{-7}	0	$< 1.3 \times 10^{-7}$
5	100 ± 3.0	68	9×10^{-6}	32	4.3×10^{-6}	9	1.4×10^{-6}
40	102 ± 7.1	25	3.5×10^{-6}	71	1.0×10^{-5}	3	4.2×10^{-7}
75	77 ± 7.5	n.d.	–	2	3.3×10^{-7}	0	–
100	30 ± 11.2	0	–	0	–	0	–

[a] The percentage of survival represents the average value from the 3 separate experiments
[b] The frequency was calculated by dividing the number of resistant calli by the total number of plated viable cells

subsequently plated on MS medium containing either $HgCl_2$ (20 μg/ml) or 6FT (40 μg/ml) and incubated at 26°C. After 4-6 weeks the number of grown, resistant calli was scored. Figure 2 shows the growth of resistant calli on $HgCl_2$-containing medium. The number of resistant calli that grew on the $HgCl_2$-containing plates are summarized in Table 3. Despite the fact that the rate of survival for a given NG concentration is quite constant in the 3 listed experiments, the total number of resistant calli and the concentration of NG that yields the highest number of resistant calli varies from experiment to experiment. The reason for this is not clear. Nevertheless, it is obvious that the NG treatment results in a considerable increase in the number of $HgCl_2$-resistant calli. Compared to the non-mutagenized cultures this increase can be as much as 70-fold. In all experiments a significant increase in the number of $HgCl_2$-resistant calli was only obtained at NG concentrations that did not observably influence the survival of the *P. hybrida* cells.

Comparable results were obtained for the isolation of 6FT-resistant calli. Table 4 presents the combined data of 3 separate experiments. Fluorotryptophan-resistant calli were only obtained at NG concentrations that did not cause a significant killing of the *P. hybrida* cells. Furthermore, NG mutagenesis appeared to be essential for the isolation of 6FT-resistant calli since none of the control plates of all three experiments contained any spontaneous 6FT-resistant callus.

Preliminary Characterization of the Drug-resistant Cell Lines

The possibility existed that not all calli which grew on the drug-containing selection plates were truly resistant: partial inactivation of the drug or a locally decreased concentration of the drug in the vicinty of a fast growing resistant callus could also permit the growth of adjacent non-resistant cells. Therefore, all calli grown on the $HgCl_2$- and 6FT-containing selection plates were re-tested: small pieces (about 25 mm^3 in size) were taken from several parts of the resistant calli, placed on fresh drug-containing medium and grown until they reached a size of 3 cm^3. This procedure was repeated 5 times, which corresponded to about 35 generations. About 80% of the originally isolated calli from the $HgCl_2$-containing selection plates survived this procedure and all of the calli from the 6FT-plates appeared to be truly 6FT-resistant.

This repeated transfer of small parts of the calli to fresh drug-containing plates should also have eliminated possible 'escapes' which could have been present in the resistant calli as the single cell origin of the resistant calli on the selection plates could not be ascertained since the mutagenized cell cultures contained also small cell aggregates (up to 40 cells per clump).

The growth of resistant calli on the selection plates may also result from habituation or adaptation (physiological induction of gene expression resulting in the drug-resistant phenotype). Although this possibility is not very likely since only very few resistant calli arose from the non-mutagenized cell cultures, we have tested this possibility by growing the $HgCl_2$- and 6FT-resistant cells for a minimum of 35 generations in the absence of the selective drugs. All cell lines that we tested remained drug-resistant and growth was identical to the original resistant lines that were continuously grown in the presence of drugs.

Different mutations can result in phenotypically identical cells. Also, a given mutation can have several pleiotropic effects. Therefore we have performed a number of experiments to investigate the possible existence of several phenotypic classes amongst the resistant cell lines. To find out whether all resistant calli exhibit the same level of resistance, we tested the growth of 9 6FT-resistant calli on agar plates containing various concentrations of 6FT. One callus was able to grow on 100 μg/ml, 2 calli on 80 μg/ml, 2 calli on 60 μg/ml and 4 calli grew on 40 μg of 6FT per ml. Amongst the $HgCl_2$-resistant calli, a similar spread in the level of resistance (up to 40 μg/ml) was observed.

In isolated carrot cell lines resistant to 5-methyltryptophan (5MT) the resistance is due to an altered anthranilate synthetase that is less sensitive to feedback inhibition by tryptophan or 5MT. This lessened control caused in a 27-fold increase in the level of free tryptophan in the cells (Widholm 1972b). In addition to the increased levels of free tryptophan, about one-half of the 5MT-resistant cell-lines were also auxin-autotrophic (Widholm 1977a; Sung 1975). At this moment, we have not yet studied the levels of free tryptophan in our 6FT-resistant cell lines. However, 7 of the 9 tested 6FT-resistant cell lines appeared to be also auxin-autotrophic (Table 5), suggesting a mutation with similar pleiotrophic effects as the one observed in the 5MT-resistant carrot cell lines.

Assuming that a biochemical mechanism similar to the one discovered for the resistance of the 5MT-carrot lines is responsible for the resistance of the *P. hybrida* cell lines to 6FT, one could expect that, like in the carrot lines (Wid-

Table 4. Number of 6FT-resistant calli obtained after NG mutagenesis

NG conc. (μg/ml)	Survival (% of control)	Number of resistant calli	Frequency
0	100 ± 7.0	0	$< 1.4 \times 10^{-7}$
5	104 ± 8.0	15	2.0×10^{-6}
20	104 ± 5.2	2	2.9×10^{-7}
40	106 ± 6.0	0	–
100	36 ± 8.3	0	–

Table 5. Properties of a number of isolated 6FT-resistant *P. hybrida* cell lines

Cell line	Level of resistance to 6FT[b]	5MT resistance[c]	Auxin independence[d]	$HgCl_2$ resistance[e]
P. hybrida-AK 1000[a]	10	–	–	–
P. hybrida-AK 1103	40	–	–	–
P. hybrida-AK 1109	40	–	–	+
P. hybrida-AK 1101	40	+	±	–
P. hybrida-AK 1104	40	+	+	–
P. hybrida-AK 1105	60	±	±	–
P. hybrida-AK 1108	60	+	+	+
P. hybrida-AK 1102	80	+	±	–
P. hybrida-AK 1106	80	+	+	–
P. hybrida-AK 1107	100	+	+	+

No growth: –, slower growth than AK 1000 on normal medium: ±, normal growth: +
[a] *P. hybrida*-AK 1000 is the wild type, parental cell line used in these mutagenesis experiments
[b] Concentration (in μg/ml) of 6FT in the agar medium that permitted normal growth
[c] Growth on agar medium containing 5MT (100 μg/ml)
[d] Growth on medium lacking hormones
[e] Growth on agar medium supplemented with $HgCl_2$ (20 μg/ml)

holm 1977b), all or some of the 6FT-resistant petunia lines are also resistant to other tryptophan analogs, especially 5MT. We observed that of the tested 6FT-resistant lines about 70% were also resistant to 5MT (Table 5).

Some of the 6FT-resistant calli showed still another phenotype: three out of 9 6FT-calli were also resistant to $HgCl_2$, suggesting an altered permeability or uptake in these three 6FT-resistant calli.

Up till now we have paid little attention to the characterization of the $HgCl_2$-resistant lines. Preliminary experiments with $^{203}HgCl_2$ showed that in the 2 tested $HgCl_2$-resistant mutants, the uptake of $HgCl_2$ was identical to that of wildtype calli. Indications for a possible modification of the $HgCl_2$ into non-toxic compounds were not obtained.

Discussion

In this study we have shown that NG-mutagenesis of *P. hybrida* cell cultures results in a strong increase in the number of $HgCl_2$-resistant or 6FT-resistant calli only at NG-concentrations that do not cause a significant killing of the mutagenized petunia cell cultures. This result can not be explained from the fact that we used the dye phenosafranine instead of plating to determine the percentage of survival: at NG-concentrations up to 100 μg/ml, the NG-survival curve obtained with phenosafranine runs quite parallel with the NG-survival curve obtained from plating the mutagenized cells on drug-free medium and then counting the number of grown calli. An explanation for the phenomenon that mutants were only isolated at low NG-concentrations could be that at high NG-concentrations (more than 40 μg/ml) the mutated cells get so 'sick' that they are no longer able to grow.

We concluded that the $HgCl_2$- and the 6FT-resistance of these cells is caused by mutation rather than by an epigenetic alteration because: (i) the NG-treatment greatly increased the number of $HgCl_2$-resistant calli, and 6FT-resistant calli could only be obtained by using the mutagen NG; (ii) the isolation-frequency of drug-resistant calli never exceeded 1.0×10^{-5}; (iii) the drug-resistant characters of these cells were stably inherited by their daughter cells for more than 35 generations, both in the presence as well as in the absence of the selective drug; (iv) amongst the 6FT-resistant calli a number of different phenotypic classes existed, as concluded from: a) the different levels of resistance, b) the cross-resistance of some calli to 5MT, c) the cross-resistance to $HgCl_2$, and d) the fact that a number of calli were also auxin-autotrophic. Additional evidence for the mutant nature of our drug resistant cell lines could be obtained by showing the passing of the mutant character through the seeds. However, at the moment it is difficult to regenerate *P. hybrida* cell cultures into plants. Since in the isolated mutant cell lines chloroplast biogenesis can still be induced by using 6-benzylaminopurine (1 mg/l), in our laboratory the described NG-mutagenesis procedure will be used e.g. for the study on the genetic regulation of the chloroplast biogenesis.

In conclusion we have shown that the developed NG-mutagenesis procedure is effective for the isolation of (at least drug-resistant) mutants from *P. hybrida* cell suspension cultures. A similar approach, as presented in this paper, should be equally successful for the isolation of mutants from suspension cultures of plant species other than *Petunia*.

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C.M. Colijn
Department of Genetics
Free University
De Boelelaan 1087
Amsterdam-Buitenveldert (the Netherlands)