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Use of Chemical and Physical Mutagens *In vitro*

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Introduction

In the twenty years since the first unequivocal reports (including inheritance data) on the selection of mutants *in vitro* [1, 2], a large number of mutants have been isolated, and reviews have frequently updated the progress, most extensively in a recent treatise [3]. The earliest reports made no use of deliberate mutagenesis, relying instead on the wide genetic variation exhibited by cultured cells, based on spontaneous mutation rates and possibly enhanced by genetic instabilities of rapidly dividing cells in culture [4]. The fact that the numbers worked in favour of selection of spontaneous mutants, when a dominant monogenic trait with a strong positive selection pressure was involved, probably delayed a critical evaluation of the role of chemical and physical mutagens in enhancing "mutation frequency". This was exacerbated by difficulties in reliable quantification of the latter term, given that single cell cultures were generally not in use, and colony forming potential (or plating efficiency) was usually profoundly influenced by viable cell density.

Whatever the precise reasons, many reports on *in vitro* selection of mutants have not used mutagens, and many others have had a "just for luck" attitude to their use. Mutagenic agents have been applied to cultures prior to selection, but little effort was made to optimise the treatment, or to evaluate its success in enhancing the yield of the desired mutant lines. Fortunately there have been sufficient exceptions to demonstrate how effective mutagenesis treatments can be. The most detailed and rigorous studies on both physical [5] and chemical [6] mutagenesis, benefited from the use of the efficient single cell plating procedures, and low density media, available for *Nicotiana* mesophyll protoplasts. While these investigations have clearly supported the value of mutagenic treatments for the isolation of biochemical mutants, other developments show a more critical dependence on their use.

The advent of efficient culture procedures for haploid protoplasts of several species led to the realistic methodical screening (total selection) of colonies for deficiency mutations, particularly auxotrophs [7, 8]. The laborious nature of this approach renders the use of mutagenesis procedures important to bring the number of colonies to be screened down to a manageable level. Additionally the increasing interest in minimising the callus step in *in vitro* selection, by selecting in organised cultures [9, 10], means that the number of selective units (cells with potential to develop into shoot primordia or embryos) is much lower

than in callus, protoplast, or cell suspension culture. Again mutagenesis may be important.

The choice of mutagenic agent is informed by the ease with which the plant material can be handled, the size and nature of the plant inoculum (protoplast, cell suspension, callus, explant etc.), the nature of the lesion sought, and the mechanisms of action of the individual mutagens. Negrutiu [11], in a long overdue review on all aspects of *in vitro* mutagenesis, gives careful consideration to these topics, with a particular emphasis on the action of the mutagens. In the hope of simplifying (perhaps oversimplifying!) the field to a level appropriate to the ensuing protocols, and a practically-based review, the current author will restrict his observations to a few general statements. The most frequently applied chemical mutagens, *in vitro*, are alkylating agents, ethyl-methanesulfonate (EMS), 1-methyl-3-nitro-1-nitrosoguanidine (MNNG), and the nitrosoureas, N-ethyl-N-nitrosourea (NEU), and N-methyl-N-nitrosourea (NMU). The last of these is particularly effective at inducing plastome mutations, both *in vivo* [12] and *in vitro* [9], but induces nuclear mutations as well [13]. The most commonly used physical mutagens are ζ-rays, x-rays and UV-radiation. The last of these is most widely available, easy to use, and can be extremely efficient for the induction of single gene mutations [5]. In this respect the spectrum of lesions achieved probably comes closer to those obtained with chemical agents, than ζ- or x-rays, which may produce a higher incidence of large deletions and other chromosomal abnormalities. The disadvantage of UV-radiation is its low penetration which may restrict its use to single cell cultures, such as protoplasts or pollen grains.

The use of chemical mutagens generally involves a short exposure to relatively high concentrations, followed by thorough washing to remove the mutagen. The extra washing steps may be to the detriment of the more delicate protoplast cultures, again making UV-radiation a more attractive alternative. NEU and NMU have short half lives in aqueous solution leading to protocols [14] which avoid the washing step by using lower concentrations and relying on the breakdown of the mutagen during culture.

The following protocols place an emphasis on the use of freshly isolated mesophyll protoplasts, or large tissue explants, chemical mutagens EMS, NMU or NEU (with or without washing steps), and the use of UV-radiation. The procedures using protoplasts are described for *Nicotiana plumbaginifolia* which is an amenable, widely used, model species. Haploid or diploid shoot cultures can be used as sources of protoplasts, but the former should be employed for total selection for auxotrophs. The same procedures should be applicable to other species provided suitable conditions for the isolation and maintenance of protoplasts are substituted. The leaf strip mutagenesis procedure described has been successfully used to obtain chloroplast mutants of five Solanaceous species: *Nicotiana plumbaginifolia*, *N. tabacum*, *N. sylvestris*, *Lycopersicon peruvianum*, and *Solanum nigrum* [9]. It is described for *S. nigrum* because the greatest frequencies have been achieved with this species, but culture media differences (the only point on which the protocol differs) are

indicated for the other species. Again the procedure should be amenable to other species for which efficient regeneration can be achieved from explants. The protocols are restricted to the preparation of the plant material, and the mutagenesis treatment. The downstream handling of cultures, to select mutants, is dealt with in succeeding chapters.

Finally, no consideration is given here to insertion mutagenesis, or "gene tagging". While beyond the scope of this chapter, this approach to mutagenesis is going to be extremely valuable for rapidly identifying genes associated with "loss of function" mutations, something which cannot be accomplished by conventional mutagenesis.

Procedures

Preparation and culture of protoplasts from shoot cultures of N. plumbaginifolia *Viviani*

The procedure is based on that of Maliga [15], and can also be used with shoot cultures of *N. tabacum*.

Steps in the procedure

1. Remove healthy, fully expanded, leaves from 4–6 week old axenic shoot cultures of *N. plumbaginifolia*. Finely slice the leaves with a scalpel and forceps and transfer to enzyme solution in sterile 100 ml Erlenmeyer flasks (15 ml per flask).
2. Incubate at 25 °C, in the dark, overnight (12–18 h). Some improvement in yield can be achieved by continuous slow rotary shaking (ca. 30 rpm), but this is not essential.
3. Swirl the flasks gently and filter through 60 μm nylon bolting cloth to remove partially digested material.
4. Transfer the suspension to sterile screw cap centrifuge tubes and spin at 500 rpm for 3 min.
5. Carefully remove the green surface layer of floating protoplasts, using a sterile Pasteur pipette, and transfer to fresh centrifuge tubes.
6. Fill the tubes with W5 solution, cap, mix thoroughly by gentle inversion, and spin at 500 rpm for 3 min.
7. Remove the supernatant and resuspend the pellet in a small volume (1–2 ml) of K_3 medium, containing 0.4 M glucose as osmoticum.
8. Pool the contents of the centrifuge tubes. Remove a sample and count intact protoplasts using a haemocytometer slide. Add sufficient K_3 medium (0.4 M glucose) to give a density of intact protoplasts of 10^5 ml^{-1}.
9. Mix gently by inversion and transfer to 5 cm Petri dishes (4–5 ml per dish). Seal with parafilm and incubate under low light intensity (ca. 100 lux).
10. After 8–10 days, when protoplasts have gone through 2–3 cell divisions, transfer the contents of each 5 cm dish to a 9 cm Petri dish and add 5 ml K_3 medium (0.4 M glucose). Culture as before.
11. After 7–10 days, remove half the contents of each dish to a fresh dish and add 5 ml K_3 medium (0.3 M glucose) to both.
12. After 7–10 days repeat step 11 using K_3 medium (0.2 M glucose).
13. After 7–10 days, if the colonies are at a high density but still quite small (< 1 mm diameter) repeat step 12.
14. Concentrate protoplast-derived colonies from step 12 or 13 by pooling the dishes and spinning in 50 ml sterile polypropylene centrifuge tubes at 500 × g for 3 min and discarding the supernatant.
15. Plate out colonies by adding to RMOP medium, containing 0.2 M glucose (instead of 2% sucrose) and 6.5 g l^{-1} Difco bactoagar, held molten at 40 °C. Add sufficient colonies to give a final density of 100–200 per dish when plated.

Swirl the flasks of medium plus colonies and pour into 9 cm Petri dishes (15–20 ml per dish).

16. Allow the agar to set, seal the dishes with parafilm and incubate in a culture room at 25 °C, 1000–1500 lux illumination, 16 h day.

Notes

1. Shoot cultures of *N. plumbaginifolia* are obtained by surface sterilising seed (30 s in 70% (v/v) ethanol, followed by 10 min in 20% (v/v) domestic bleach "Domestos", followed by two washes in sterile distilled water) and placing on RM medium in Petri dishes (20 ml per dish). When first true leaves are ca. 0.5 cm long, seedlings can be removed to RM medium in individual containers, to be used as a source of shoot cultures, maintained by transfer of single node cuttings to fresh RM medium every 4–6 weeks.
2. Protoplast yields vary greatly, even using fairly uniform shoot cultures. Typically one shoot culture shoot provides sufficient leaf material for one flask of enzyme solution and four such flasks provide $5–10 \times 10^6$ protoplasts, sufficient for small scale mutagenesis treatments.

10–13. The dilution steps may need to be modified depending on the efficiency of initiation of cell division (and hence the colony density) and the rate of colony growth. These factors vary between preparations and it is difficult to be precise about the interval between dilutions. Progress of cultures must be monitored daily. There should be substantial increase in colony size between dilutions, but any sign of browning of colonies should result in immediate dilution.

Solutions and media

Most solutions and media can be sterilised by autoclaving but those containing enzymes must be filter-sterilised through 45 or 22 μm filters. Final concentrations are given in Table 1. pH of all solutions should be adjusted to 5.6, by dropwise addition of 0.1 M KOH.

Enzyme solution must be freshly prepared. Other solutions and media can be stored for several weeks in the cold room, provided they remain clear, and are sealed to restrict evaporation.

- Enzyme solution: 0.5% Driselase, or alternatively 1% cellulase "Onozuka" R10 plus 0.5% Macerozyme R10 (all w/v), in K_3 medium (Table 1) containing 0.4 M sucrose (Table 2)
- W5 solution [15], containing (per l) 9.0 g NaCl, 1.0 g glucose, 18.4 g $CaCl_2 \cdot 2H_2O$, 0.4 g KCl, pH 5.6
- K_3 medium (Table 1) containing 0.4 M glucose (Table 2)
- K_3 medium (Table 1) containing 0.3 M glucose (Table 2)
- K_3 medium (Table 1) containing 0.2 M glucose (Table 2)
- RMOP medium: RM solution (Table 1) plus 100 mg/l m-inositol, 1 mg/l thiamine-HCl, 1 mg/l BAP, 0.1 mg/l NAA, solidified with 6.5 g/l Difco Bactoagar and containing 0.2 M glucose (Table 2)
- RM medium: RM solution (Table 1), solidified with 6.5 g/l Difco Bactoagar, for maintenance of shoot cultures

Table 1. Basal media for tissue and protoplast culture of *Nicotiana plumbaginifolia.* All components are listed in mg/l final concentration

Medium	K3[a] (15, modified from 16)	RM[b] (17)
NH_4NO_3	240	1650
KNO_3	2400	1900
$CaCl_2 \cdot 2H_2O$	900	440
$MgSO_4 \cdot 7H_2O$	250	370
KH_2PO_4		170
$NaH_2PO_4 \cdot 2H_2O$	120	
$(NH_4)_2SO_4$	130	
$FeSO_4 \cdot 7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
H_3BO_3	3.0	6.2
KI	0.75	0.83
$MnSO_4 \cdot 4H_2O$	6.7	22.3
$NaMoO_4 \cdot 2H_2O$	0.24	0.25
$ZnSO_4 \cdot 7H_2O$	2.3	8.6
$CoCl_2 \cdot 6H_2O$	0.025	0.025
$CaSO_4 \cdot 5H_2O$	0.025	0.025
m-inositol	100	100
nicotinic acid	1.0	
pyridoxine · HCl	1.0	
thiamine · HCl	10	
xylose	250	
sucrose	see Table 2	30000
glucose	see Table 2	
BAP[c]	0.2	
NAA[c]	1.0	
2,4-D[c]	0.1	
pH	5.6	5.8

[a] K_3 medium, excluding sugars (sucrose or glucose) and phytohormones[c], can be prepared at 10 × final concentration and stored in suitable aliquots (e.g. 50 or 100 ml) at − 20 °C. Medium can be prepared by thawing, diluting, and adding remaining components before adjusting pH.

[b] RM and RMOP media are normally prepared using MS salts (Flow Laboratories) at 4.6 g/l, which supply all the mineral salts listed in Table 1. When preparing from individual salts three stock solutions should be used: macroelements, first 5 in Table 1 (final concentration × 4), (Na_2EDTA + $FeCl_3 \cdot 6H_2O$) (× 200) and remaining microelements (× 100). Each litre of medium will contain 250 ml, 5 ml, and 10 ml, respectively, of these stocks which can be stored for up to 4 weeks in a cold room.

[c] Abbreviations of phytohormones: 2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = 1-naphthalene acetic acid; BAP = 6-benzylaminopurine. Hormones should be prepared as 1 mg/ml stock solutions, in 0.1 M KOH (2,4-D, NAA) or 0.1 M HCl (BAP), and stored in refrigerator.

Table 2. Concentrations of glucose or sucrose (g/l) to provide different molarities of K3 or RMOP required in the protocol

	Concentration (M)		
	0.4	0.3	0.2
Sucrose	136.8		
Glucose	72.0	54.0	36.0

Mutagenesis with low concentrations of N-ethyl-N-nitrosourea (NEU)

This is an extremely simple technique, taken from Marton *et al.* [14]. They reported an increase in frequency of chlorate-resistant (nitrate reductase-deficient) mutants, from haploid *N. plumbaginifolia* protoplasts, from 5.8×10^{-5} to 1.1×10^{-3} (greater than two orders of magnitude), using 0.3 mM NEU, a concentration giving 52% protoplast survival (compared to control), and sufficiently low to allow the natural decay of the mutagen, without washing. A lower (0.15 mM) concentration of NEU also gives substantial improvements in mutation frequency over controls (about 10 fold), and may lead to the recovery of fertile plants from a greater proportion of the mutants.

Steps in the procedure

1. Isolate *N. plumbaginifolia* protoplasts as described in preceding section.
2. When transferring freshly isolated protoplasts into K_3 medium (0.4 M glucose) for culture, add mutagen stock to give a final concentration of 0.15 or 0.3 mM before plating.
3. Culture mutagenised protoplasts, alongside non-mutagenised controls, as described in preceding section.

Notes

1. Safe handling of mutagen is paramount in all these procedures. It is important to avoid skin contact, and protective apron and gloves should be used, in addition to a respirator. Wash down all work areas after use, in case of spillage. Spillage in the laminar flow can be retained by working on absorbent paper backed with aluminium foil. Exclude other workers from the area while manipulations using mutagens are in progress, and use a vertical flow, or containment unit, if available.
2. Wash hands after use, and immediately wash any area of skin where contact with mutagen is suspected. Use gentle washing motions (avoiding excess rubbing), and soapy water.
3. Contaminated paper should be incinerated. Glassware, and other materials as well as leftover mutagen solutions, should be decontaminated overnight with 5% NaOH in the fume hood, and given a second soaking (longer than 1 h) with 5% NaOH before washing in tap water, followed by distilled water.
4. Concentrations of NEU may need to be modified if a species other than *N. plumbaginifolia* is used. A concentration giving about a 50% reduction in plating efficiency should be effective.

Solutions

– Mutagen solution: 10 mM (1.17 mg/ml, adjusted to take account of acetic acid added by manufacturer as a stabiliser) NEU (Sigma), in K_3 medium (Table 1) with 0.4 M glucose.

Mutagenesis with high concentrations of EMS, NEU, or NMU

Alkylating agents are most effective when cells are undergoing DNA replication, and mutagenesis of cell suspension cultures is generally carried out with cultures in the exponential phase of growth. Efficient mutagenesis can also be achieved with freshly isolated mesophyll protoplasts (believed to be in G1) however, with the attraction that the risk of generating chimeric colonies is minimised. This is the protocol described here. More efficient mutagenesis may be achieved by culturing the protoplasts for 36–48 h in K_3 (0.4 M glucose) before re-collecting and using the same protocol. This should enrich the population in S-phase cells, but to really optimise this will require additional series of experiments, particularly if a species other than *N. plumbaginifolia* is used.

The following protocol is a general one, applicable to several different alkylating agents, and includes a preliminary test to determine effective concentrations of the mutagen.

Steps in the procedure

1. Isolate mesophyll protoplasts from shoot cultures of *N. plumbaginifolia*, as described previously.
2. When transferring freshly isolated protoplasts to K_3 medium (0.4 M glucose) add mutagens at a range of concentrations from filter-sterilised stock solutions (except EMS – see Note 1) prepared in the same medium. As a rough guide suitable concentration ranges would be: 0.1–3% v/v EMS, or 0.3–10 mM (NEU or NMU). The effective concentration may be influenced by cell density which should be standardised at 10^5 protoplasts/ml.
3. After 60 min incubation under gently rotary shaking (ca. 30 rpm), sediment the protoplasts by centrifugation at 500 rpm for 3 min. Carefully remove the mutagen solution with a sterile Pasteur pipette, and resuspend the protoplasts in K_3 (0.4 M glucose) to a final density of 10^5/ml and plate in 5 cm Petri dishes. Seal and incubate under low light intensity (ca. 100 lux) for 8 days.
4. Score the cultures for percentage cell division and determine the effect of each mutagen concentration on division frequency. Select a mutagen concentration giving 10–50% division compared to the non-mutagenised control (taken as 100%).
5. Repeat steps 1–3 using the chosen mutagen treatment only, and continue culturing the protoplasts as described previously.

Notes

1. EMS is a volatile liquid, hence the recommended concentration range is given as % (v/v). The bottle should be opened in a fume hood and the required amount added to K_3 medium by automatic pipette, using autoclaved disposable tips.
   Solutions contaminated with EMS should be inactivated by gradual addition to a large excess of 3 M KOH in 95% ethanol, heated under reflux. The mixture should be refluxed and stirred for 2 hours before cooling, diluting with tap water, and disposal down the drain, chased by a large volume of tap water.

The safe disposal of NEU and NMU, and general comments on the safe handling of these mutagens, are provided in notes 1–3 of the preceding section.

2. The arbitrary duration of mutagen treatment (60 min) works well in most cases but may need to be modified downward (eg. to 30 min) if dividing cells are used. Longer (90—120 min) treatments can also be used for NMU and NEU.
3. EMS is poorly miscible with water. It is therefore a better mutagen for use with cell suspension cultures, than protoplasts, where more vigorous agitation (100 + rpm) can be used to ensure proper mixing.

Solutions

– All solutions required for protoplast isolation and culture are described in the first section and Table 1.

Leaf strip mutagenesis of Solanum nigrum *to obtain chloroplast-encoded antibiotic resistant mutants*

This simple procedure is based on the use of NMU as an efficient plastome-targeted mutagen. It is particularly effective with *S. nigrum*, but also gives good results with *Lycopersicon peruvianum*, *Nicotiana tabacum*, *N. sylvestris*, and *N. plumbaginifolia* [9, 18]. Attempts to adapt it to *Brassica* species have so far failed (O'Neill and Dix, unpublished).

Steps in the procedure

1. Remove leaves from axenic shoot cultures, and cut into strips (5–15 mm × 2–3 mm). Add 200 strips to 100 ml mutagen solution in a 250 ml Erlenmeyer flask.
2. Incubate on a rotary shaker (ca. 50 rpm).
3. Decant the mutagen solution, and wash the leaf strips four times with 100 ml RM solution, or sterile distilled water, pH adjusted to 5.6.
4. Transfer leaf strips, lower surface downwards to the surface of selective medium, 5 strips per 9 cm Petri dish.
5. Seal the dishes with parafilm and incubate in a culture room (1,500 lux, 16 h photoperiod) until green (resistant) adventitious shoots appear on bleached leaf strips (40–60 days).

Notes

1. For safe handling and disposal of NMU, and NMU-contaminated materials see notes for preceding sections.
2. The same procedure can be used for *Lycopersicon peruvianum* and *Nicotiana* species, but in the latter case a better selective medium is RMB:RM (Table 1) plus 100 mg/l m-inositol, 1 mg/l thiamine-HCl, 1 mg/l BAP, and selective levels of the antibiotics.

Solutions

- RM solution (Table 1)
- Mutagen solution: RM solution containing 5 mM NMU from a freshly prepared stock solution (80 mg NMU in 20 ml RM). For 100 ml mutagen solution 12.9 ml stock solution is added to 87.1 ml RM solution
- Selective medium: RM solution (Table 1) with sucrose reduced to 20 g/l, and the addition of (per l) 100 mg meso-inositol, 1 mg thiamine-HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine-HCl, pH 5.6, solidified with 0.65% (w/v) Difco Bactoagar, plus selective levels of antibiotics added from filter-sterilised stock solutions (100× final concentration, prepared in distilled water), to autoclaved, molten medium, prior to pouring. Selective levels are as follows: Streptomycin sulphate: 500 mg/l, spectinomycin: 50 or 100 mg/l, or lincomycin hydrochloride: 100 mg/l

UV mutagenesis of N. plumbaginifolia *protoplasts*

This protocol closely follows the procedure developed by Grandbastien *et al.* [5] for tobacco protoplasts. The protocol describes the determination of a suitable UV dose, based on reduction in colony-forming ability, which can subsequently be used for mutagenesis and selection. This dosage is likely to be in the order of 1,000 ergs/mm^2.

Steps in the protocol

1. Isolate *N. plumbaginifolia* protoplasts and culture at 10^5/ml in K3 (0.4 M glucose) medium.
2. After 24 h, expose the protoplasts to UV doses in the range 200–2000 ergs/mm^2. Place dishes under the UV source and remove the lids before turning the source on.
3. Culture the treated protoplasts in total darkness for 48 h.
4. Carry out all remaining culture steps as before, taking care to use uniform dilution steps for all treatments.
5. 4 weeks after plating protoplast-derived colonies in solid medium, count the growing colonies and determine the effect of the mutagen treatment on the colony-forming ability of protoplasts.
6. Select a UV-dose giving 10–50% colony forming of non-mutagenised controls, for subsequent mutagenesis treatments carried out as above.

Notes

1. Safe handling of UV is straightforward. Safety glasses should be worn and direct exposure of the skin should be avoided.
2. If unable to calibrate the UV source, suitable treatments can still be determined by varying either the distance from the lamp or the period of exposure, and determining the effect on colony-forming ability.
3. Effect of UV dose on protoplasts can be determined at an earlier stage of culture, while protoplast-colonies are still in liquid K_3 medium, but care should be taken to exclude residual (1–2) cell divisions, of many UV-treated protoplasts, which are unable to go on to form colonies.
4. The same procedure can be used with both haploid and diploid protoplasts.

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