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*Short communications*

# Influence of ploidy on plastome mutagenesis in *Nicotiana*

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**Summary.** A clear influence of ploidy was observed on the frequency of both spontaneous and nitroso-methylurea (NMU) induced, streptomycin-resistant, adventitious shoots developing on leaf explants of *Nicotiana tabacum* and *N. plumbaginifolia*. At nearly all NMU levels employed a significantly higher yield of resistant shoots was obtained from haploid compared with diploid leaf strips. At 1 mM NMU the differences were not significant and were absent when a high (1000 mg/l) selective concentration of streptomycin sulphate was used. The influence of ploidy is discussed in relation to the possible effect of plastome copy number on mutagenesis and sorting out of resistant plastids.

**Key words:** Mutagenesis – Nitroso-methylurea – Plastome – Ploidy – *Nicotiana*

## Introduction

A rapid, simple protocol for obtaining plastome-encoded streptomycin-resistant mutants of solanaceous plants has recently been reported (McCabe et al. 1989, 1990). This is based on the use of an efficient plastome-targetted mutagen, nitroso-methyl-urea (NMU, Hagemann 1982), and a highly regenerative leaf explant system in which the antibiotic causes bleaching and suppresses adventitious shoot initiation. The procedure works well for several species and in *Solanum nigrum* has also been used to select for spectinomycin and lincomycin resistance, as well as multiple markers for use in chloroplast transfer and recombination studies (Dix et al. 1990). The appearance of resistant shoots is dependent on a complex process of sorting out of resistant and sensitive plastome types during a sustained period of cell division at non-lethal levels of the selective agent.

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In this respect selection of these mutants is akin to that of rare plastome recombinants achieved through cell fusion (Medgyesy et al. 1985), a process that, in view of the large plastome copy number per cell, has been likened to population genetics at the cell level (Medgyesy 1990).

While this sorting out process is difficult to analyse, some useful indications can be achieved through comparisons between plants differing in plastid density. In haploid *Nicotiana tabacum* shoot cultures, mesophyll cells are characterised by a lower chloroplast density than those of diploid cultures of comparable age (E. Rice and P. Dix, unpublished). Previous investigations with diploid *N. tabacum* (McCabe et al. 1989) have shown stable retention of streptomycin resistance in shoots obtained through the leaf strip mutagenesis procedure, together with maternal inheritance of the trait. This therefore seems a suitable species for assessing the effect of ploidy on the generation of streptomycin-resistant shoots. Data from this study are presented in this report, together with a similar evaluation for a second species, *N. plumbaginifolia*.

## Materials and methods

*Plant material.* Seeds of *N. tabacum* var. Petit Havana and *N. plumbaginifolia* var. Viviani were pure lines maintained by selfing for at least six and ten generations respectively. Haploid plants of *N. tabacum* (NTH1) and *N. plumbaginifolia* (NPH28), the latter kindly provided by P. Maliga, were obtained through anther culture (Nitsch and Nitsch 1969) from plants raised from the same seed stocks used to provide diploid plants for the study.

Seeds were sterilised and germinated on RM medium as described previously (McCabe et al. 1989). The resulting diploid plantlets, and the haploid plants, were maintained as axenic shoot cultures by transferring nodal cuttings to fresh RM medium every 5–6 weeks. Shoot cultures, and all other cultures, were maintained at 25° C under an 18 h photoperiod. The ploidy status of experimental cultures was confirmed by root tip squashes after pretreatment with 0.05% colchicine (90 min), fixation with 3:1 ethanol/acetic acid and staining with propionic orcein.

*Mutagenesis.* Details of the mutagenesis procedure are provided in earlier reports (McCabe et al. 1989, 1990), the latter of which also provides detailed information on the safe handling and disposal of the mutagen. Briefly, leaf strips (0.2 × 1 cm) from 4-week-old shoot cultures were incubated in liquid RM medium supplemented with various levels of NMU (Sigma) on a rotary shaker (100 rpm) at 25° C for 120 min, washed three times in fresh culture medium and placed on the surface of shoot regeneration medium, RMB (Maliga 1984), containing 1 mg/l benzylaminopurine as the sole phytohormone, supplemented with 0, 500 or 1000 mg/l streptomycin sulphate (Sigma). Nine explants were used per 9 cm petri dish; dishes were sealed with parafilm and incubated for 8 weeks.

*Chloroplast number per cell.* This was determined according to the method of Possingham and Smith (1972). Leaf discs were excised with a cork borer in a 3.5% glutaraldehyde solution on a sheet of dental wax, transferred to glass vials and rotated slowly in the glutaraldehyde solution for 2 h. The solution was then decanted and the discs washed three times in a 0.05% EDTA solution (pH 9) before incubation in the same solution for 2 h. Leaf discs were then squashed under a cover slip and mean chloroplast number per cell was determined by counting 20–30 cells in total from three leaf discs excised from a single plant.

## Results

### *N. tabacum*

The data on the appearance of resistant shoots on medium containing 500 or 1000 mg/l streptomycin sulphate are given in Fig. 1, where both the percentage of explants with resistant shoots, and the mean number of shoots per explant, are recorded. At 500 mg/l streptomycin sulphate, significantly higher values were obtained with haploid leaves. In the case of yield of resistant shoots (Fig. 1C) this represented a five- to tenfold increase, except where 1 mM NMU was employed where the increase was only about 50% and was not significant. Only 0.5 and 1 mM NMU treatments led to resistant shoots on 1000 mg/l streptomycin sulphate. After 0.5 mM NMU treatment the same trend is observed as on

**Table 1.** Mean chloroplast number per cell in *Nicotiana tabacum* shoot culture

| Ploidy | Cell type | Mean chloroplast no./cell |
|---|---|---|
| 2*n* | Palisade mesophyll | 67.2 ± 5.7 |
| | Guard cell | 8.5 ± 0.3 |
| *n* | Palisade mesophyll | 19.4 ± 0.6 |
| | Guard cell | 5.9 ± 0.2 |

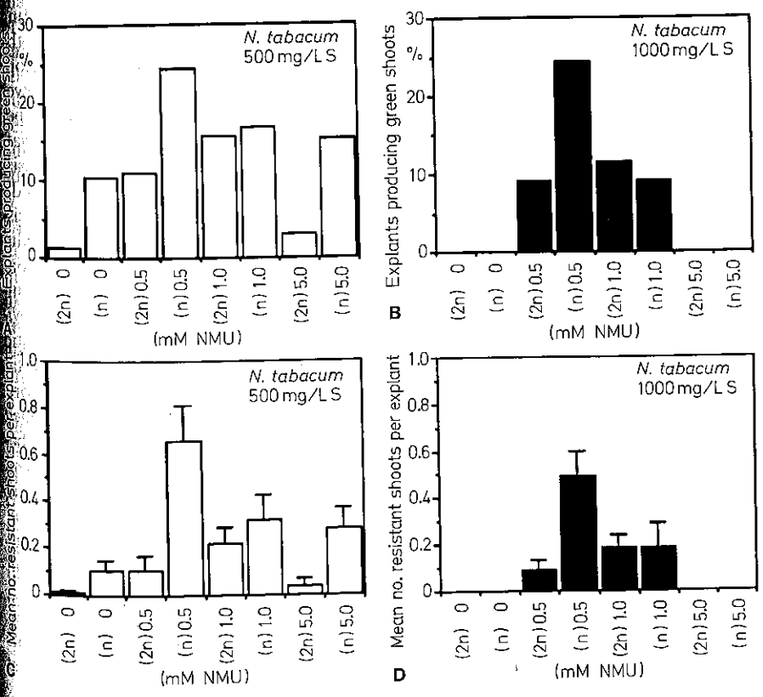


**Fig. 1A–D.** The occurrence of green adventitious shoots on *Nicotiana tabacum* leaf explants placed on medium supplemented with 500 (**A, C**) or 1000 (**B, D**) mg/l streptomycin sulphate after mutagenesis with nitroso-methylurea (NMU). The percentage of explants producing shoots (**A, B**) and the mean number of resistant shoots per explant (**C, D**) are recorded. Mutagen concentrations and ploidy levels (*n* or 2*n*) are indicated on the horizontal axes and *bars* represent the standard error of the mean. All observations were made after 8 weeks

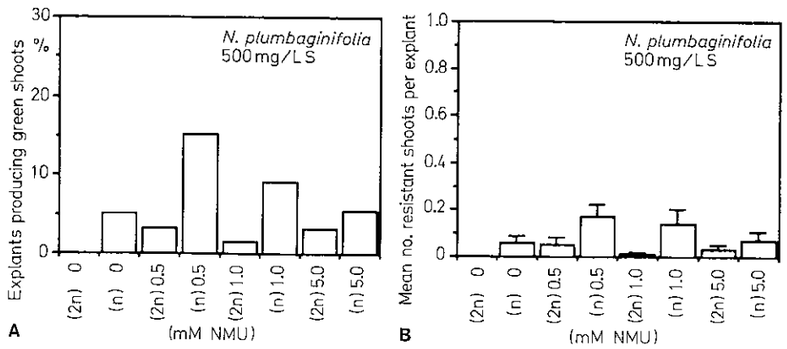


**Fig. 2A and B.** The occurrence of green adventitious shoots on *Nicotiana plumbaginifolia* leaf explants on medium supplemented with 500 mg/l streptomycin sulphate, after mutagenesis with NMU. The percentage of explants producing shoots (**A**) and the mean number of resistant shoots per explant (**B**) are recorded. Further details are as in legend to Fig. 1

500 mg/l streptomycin sulphate, but this disappears in the case of the 1 mM treatment.

Chloroplast numbers in palisade mesophyll, and guard cells are recorded in Table 1.

*N. plumbaginifolia*

The effect of ploidy on the appearance of streptomycin-resistant shoots in *N. plumbaginifolia* is shown in Fig. 2. Although the occurrence of resistant shoots is less frequent overall, the same trend is observed when comparing haploid and diploid leaves. Results are only presented for 500 mg/l streptomycin sulphate as resistant shoots for this species only appeared very rarely, in any of the treatments, at the higher (1000 mg/l) level.

*Effect of mutagenesis on adventitious shoot initiation*

Only the highest NMU concentration (5 mM) had any noticeable effect on multiple adventitious shoot initiation on non-selective medium (no streptomycin). At this level there was some reduction in density of shoots on diploid leaf strips, although all leaf strips continued to exhibit a morphogenic response. The effect on haploid leaves was much more pronounced however with adventitious shoots developing from only 57% and 79% of leaf strips of *N. tabacum* and *N. plumbaginifolia* respectively.

## Discussion

The results confirm the general efficiency of the mutagen NMU for the induction of mutations to antibiotic resistance. This has been the mutagen of choice for most recent work on the production of chloroplast mutants (e.g. Fluhr et al. 1985; McCabe et al. 1989, 1990; Jansen et al. 1990; Dix et al. 1990), although an interesting exception is the report of To et al. (1989), in which efficient plastome mutagenesis was achieved with N-methyl-N′-nitro-N-nitroso-guanidine in protoplast cultures of *N. plumbaginifolia*.

The principal observation from the current data is the far greater yield of resistant shoots from haploid compared with diploid leaves. This effect is most striking in the non-mutagenised treatments where spontaneous mutants on the diploid leaf strips are extremely rare (a single resistant shoot for *N. tabacum*, and none for *N. plumbaginifolia* in the present experiment). The extent of the enhancement is too great to be accounted for by the slightly smaller size (and hence larger number per explant) of haploid cells, and the results suggest that, at least in these species, induced mutagenesis may not be necessary if haploid cultures are employed.

The reason for the elevated number of mutants in haploid explants could relate to an inherently higher tendency to mutation in the plastome of haploid cells, or to a more efficient sorting out of the resistant and sensitive plastids under the selection pressure. In the absence of any documented evidence for differences in the organisation of DNA in plastids from haploid and diploid cells, or for recessive plastome mutators (Epp 1973) in *Nicotiana* species, differences in efficiency of sorting out seem to provide the more likely explanation. If the target cells, i.e. those capable of embarking on a developmental pathway to adventitious shoots, contain fewer chloroplasts, one would anticipate fewer mutations per cell. Our suggestion is that this is more than compensated by the more rapid stabilisation of homoplastidic resistant cells through segregation during subsequent cell division under positive selection for streptomycin resistance. The importance of selection for directional channelling of this segregation process was neatly demonstrated in a recent report in which the fate of lincomycin- and streptomycin-resistant plastids was followed in *Nicotiana* somatic hybrids with a mixture of the two plastid types (Moll et al. 1990). In the case of plastome mutagenesis, where one is selecting in favour of a tiny minority of mutant plastid DNA molecules, it can be envisaged that these must reach a certain threshold proportion before the selection pressure is capable of pulling them through, and that this is more readily reached in haploid cells with a lower plastid density. The situation is of course further complicated by the two tiers of competition (intra-plastidic and inter-plastidic) between resistant and sensitive types.

We believe that the inverse relationship between ploidy and the recovery of streptomycin-resistant shoots is a significant observation, but interpretation of it must



tempered by the limitations to quantification posed by the selection system. The selective unit is a large complex tissue only a proportion of the cells of which are competent for morphogenesis in response to the triggers supplied in the culture medium. The origin of the adventitious shoots is unclear, and has not been resolved by histological and ultrastructural investigations (A. Timmons and P. Dix, in preparation), hampered by a loss of tissue distinction before emergent shoot primordia became apparent. Differences in chloroplast number in mesophyll cells might not reflect differences in plastid number in the progenitor cells for the resistant shoots, and we have no information on plastome copy number per plastid in these cells. Furthermore, the sorting out process is certain to be influenced by the substantial changes in numbers of plastids and plastome copies on transition from a differentiated to a meristematic cell (Thomas and Rose 1983; Mullet 1988).

A protoplast-based system for mutagenesis and selection of plastome mutants, of the type employed by several groups (Cseplö and Maliga 1984; Hamill et al. 1986; To et al. 1989; Jansen et al. 1990) would lend itself to better estimation of frequency of recovery of resistant lines, and comparisons using haploid and diploid protoplasts might be informative. However, genetic instability during the callus phase may result in erratic changes in ploidy status which would complicate such an analysis. For example, Jansen et al. (1990), commencing with diploid protoplasts of *Lycopersicon periwianum*, found 22 out of 25 streptomycin-resistant shoots, regenerated after at least 6 months of selection, to be diploid. To et al. (1989), on the other hand, only recovered haploid plants from 2 (both of which derived from progenitors that had not been subjected to mutagen treatment) out of 20 streptomycin-resistant colonies selected using initially haploid protoplasts of *N. plumbaginifolia*. Their selection and regeneration protocol appears to be rapid, but is not fully chronicled. Ploidy estimations have not been carried out on resistant plants in the present study, but in a separate investigation (P. Dix, unpublished) 12 adventitious shoots arising from leaf explants of haploid *N. tabacum*, were all found to be haploid. Generally a greater stability of ploidy can be expected in adventitious shoots developing directly from explants, than in those recovered following a callus step. Stability is unlikely to be complete however. For example, diploid axillary shoots frequently develop from haploid *N. plumbaginifolia* shoot stocks maintained through nodal cuttings.

We have no ready explanation for the erosion of the ploidy effect in *N. tabacum* after a particular mutagenesis treatment (1 mM NMU), except that there may, after all, be some differential sensitivity of haploid and diploid cells to certain mutagenesis treatments. Some reduction of the effect is also found in *N. plumbaginifolia* after exposure to 5 mM NMU, but this can be explained by the inhibition of adventitious shoot initiation, greater in haploid than diploid leaf strips, caused by this high concentration of the mutagen.

In conclusion, although haploid leaf strips give a higher yield of streptomycin-resistant shoots, NMU is also sufficiently effective with diploid leaf strips to favour their use for routine obtention of plastome markers, in view of the convenience of immediately recovering fertile, diploid plants.

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