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A simple procedure for the isolation of streptomycin resistant plants in *Solanaceae*

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Summary. A system has been developed for rapid selection of streptomycin resistant mutants, as adventitious shoots arising from explants of several Solanaceous species. Efficient mutagenesis was achieved by incubating shoot culture-derived leaf strips with 1 or 5 mM nitroso-methylurea, for 90 or 120 min. In *Nicotiana tabacum* and *Lycopersicon peruvianum* these treatments resulted in white or variegated adventitious shoots from up to 3.5% of explants placed on medium promoting shoot regeneration. Chlorophyll deficiencies were only observed very rarely in *Solanum nigrum*. Streptomycin resistant shoots were obtained from leaf explants placed on medium containing 500 mg l^{-1} streptomycin sulphate, under which conditions explants are bleached and adventitious shoot development suppressed. Green adventitious shoots appeared at a frequency dependent both on the mutagenic treatment and on the species. The best response was with *S. nigrum* where $>70\%$ of the explants produced streptomycin resistant shoots, most of which retained their resistance on subsequent testing. Maternal inheritance of streptomycin resistance has been confirmed for several *N. tabacum* and *S. nigrum* mutants, and there is also evidence for paternal transmission in the latter species. The procedure has been successfully extended to other species, including *N. sylvestris* and *N. plumbaginifolia*, and also to obtain spectinomycin resistant mutants.

Key words: *Solanaceae* – Nitroso-methylurea – Chloroplast mutations – Streptomycin resistance

Introduction

The value of plastome-encoded antibiotic resistance markers for studies on organelle inheritance and interactions between nuclear and cytoplasmic genomes in higher plants have been alluded to by a number of workers (Maliga et al. 1973; Umiel 1979; Cséplö and Maliga 1982, 1984; Fluhr et al. 1985). Other plastid mutants which have generated interest include those resistant to herbicides inhibiting photosynthesis (Cséplö et al. 1985; Menczel et al. 1986), and chlorophyll deficiency resulting from either large-scale deletions of ptDNA (Day and Ellis 1984, 1985) or from point mutations (Hosticka and Hanson 1984; Sváb and Maliga 1986). The availability of in vitro-selected chloroplast markers in *Nicotiana* has been central to a number of recent developments within this genus. Chloroplast transfer through cybrid formation has benefited from the use of both streptomycin resistant donor chloroplasts (e.g. see Maliga et al. 1982) and plastome-encoded chlorophyll-deficient recipient protoplasts (Menczel et al. 1986). Detection of rare chloroplast recombination (Medgyesy et al. 1985) was dependent on a sophisticated selection scheme utilising a tobacco line (SR1-A15) in which the streptomycin resistant mutation is masked by an independent cpDNA-encoded pigment deficiency (Sváb and Maliga 1986). The SR1 mutation was also essential for the demonstration of paternal transmission of chloroplasts in *Nicotiana* (Medgyesy et al. 1986).

The extension of such investigations is dependent on the availability of suitable markers in a broader range of species. Apart from a report on *Onobrychis viciifolia* (Hamill et al. 1986), chloroplast-encoded antibiotic resistant mutants have to date been obtained mostly in *Nicotiana* species. Green callus or protoplast-derived cell cultures are generally used in a selection protocol based on retention or production of chlorophyll at drug levels normally causing bleaching (Maliga et al. 1973; Cséplö and Maliga 1984). The discovery of nitroso-methylurea (NMU) as an efficient mutagen for inducing plastome mutations (Hagemann 1982) resulted in a strategy for producing antibiotic resistant mutants after mutagenesis of tobacco seeds (Fluhr et al. 1985). Resistant mutants are selected as variegated seedlings on medium containing levels of the antibiotic normally causing bleaching. The most efficient selection scheme exploits early identification of "green islands" on cotyledons, and plant regeneration from them.

We would like to report here a simple procedure in which mutagenesis can be performed directly on leaf explants, from which resistant adventitious shoots can then be selected on drug-containing medium. The procedure has been successfully applied to *Lycopersicon peruvianum* and *Solanum nigrum* as well as *Nicotiana tabacum* and other *Nicotiana* species, and we feel it may be of wide application to species for which there is a good regeneration potential from cultured explants.

Materials and methods

Plant material. Seeds of *N. tabacum* var. Petit Havana were a pure line, maintained by selfing for at least four generations. Seeds of *L. peruvianum* (self-incompatibility genotypes S_1S_2 and S_4S_5), and *S. nigrum* were generously provided by K. Sree Ramulu and G.C. Douglas, respectively.

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Seeds were surface sterilised using 10% domestic bleach ("Domestos") for 10 min, followed by 3 × 5 min washes with sterile distilled water, then germinated on the surface of RM medium consisting of MS (Murashige and Skoog 1962) salt solution plus 30 g l^{-1} sucrose, pH adjusted to 5.7 and solidified with 7 g l^{-1} Difco Bacto-agar. Sterile plants were maintained by nodal cuttings transferred every 5–6 weeks to the above medium.

Mutagenesis and plant regeneration. Sterile seeds (after imbibition for 18 h in sterile distilled water at 25° C) or leaf strips (0.2 × 1–1.5 cm) obtained from shoot cultures were incubated for 90 or 120 min in liquid culture medium in which 1, 5 or 20 mM nitroso-methylurea (NMU) had been dissolved, on a rotary shaker (100 rpm) at 25° C, and were subsequently washed three times with fresh medium. Mutagenised seeds were germinated as described above. Other explants were placed on the surface of culture medium with or without 500 mg l^{-1} streptomycin sulphate, solidified with 0.7% Difco Bacto-agar. Culture media used were as follows: *N. tabacum*, RMB medium (Maliga 1984) consisting of MS salts (Flow Laboratories) plus (in 1 l) 30 g sucrose, 100 mg meso-inositol, 10 mg thiamine-HCl, 1 mg benzyladenine; *L. peruvianum* and *S. nigrum*: R3C8 medium (Meredith 1979) consisting of MS salts plus (in 1 l) 30 g sucrose, 100 mg meso-inositol, 1 mg thiamine-HCl, 0.5 mg nicotinic acid, 0.5 mg pyridoxine-HCl, 0.2 mg indole acetic acid, 2 mg zeatin. All media were adjusted to pH 5.7. Cultures were maintained in 9-cm plastic petri dishes, sealed with parafilm, under an 18 h photoperiod at 25° C.

Shoots arising on explants were rooted by transfer to RM medium. After root formation they were potted in soil and transferred to the greenhouse. For the first 2 weeks they were protected from desiccation by covering with transparent plastic film.

Inheritance tests. N. tabacum seeds were surface sterilised and mixed with aqueous agar solution (0.2%) which was pipetted (3 ml) on to the surface of 10 ml solidified (0.8% agar) RM medium, with or without 1000 mg l^{-1} streptomycin sulphate in 5-cm petri dishes. Bleaching or greening was scored after 2 weeks of culture under the same conditions as the leaf strip cultures. Inheritance tests with *S. nigrum* were performed on leaf strips excised from individual sterile seedlings. The procedure followed was identical to the original selection system except that it was performed at both 500 and 1000 mg l^{-1} of streptomycin sulphate.

Results

N. tabacum

a) Seedlings. To confirm the effectiveness of NMU in inducing chloroplast mutations, 2- to 3-week-old seedlings were transferred to fresh RM medium with or without 500 mg l^{-1} streptomycin sulphate. After a further 28 days, seedlings were scored for variegated true leaves. The results are recorded in Table 1. Completely green or albino shoots were raised by transfer of sectors from variegated seedlings to RMB medium, and these were rooted after 6–8 weeks on RM medium. Inheritance was investigated in three of the green plants raised from streptomycin resistant sectors after NMU treatment, and was maternal in all cases (data not shown).

Table 1. The occurrence of variegated *Nicotiana tabacum* seedlings after nitroso-methylurea (NMU) mutagenesis, on medium with or without 500 mg l^{-1} streptomycin sulphate. More than 250 seedlings were tested for each treatment

NMU concentration (mM)	Duration of exposure (min)	Number of variegated seedlings (%)	
		− streptomycin	+ streptomycin
0	90	0	0
1	90	0	0
5	90	18.2	0
20	45	84.2	0.9
20	90	95.0	1.3

Table 2. Production of albino and streptomycin resistant adventitious shoots from *Nicotiana tabacum* leaf strips after NMU mutagenesis

NMU concentration[a] (mM)	No. tested on RMB medium + streptomycin sulphate	Adventitious shoot production[b]	Green shoots on RMB medium + streptomycin sulphate	Albino shoots on RMB medium (200 tested in all cases)
0	210	+ + +	0	0
1	225	+ + +	1	1
5	215	+ + +	5	7
20	225	+	0	0

[a] The duration of NMU treatment was 90 min in all cases
[b] The estimation of adventitious shoot production was made using an arbitrary score where + + + is shoot formation at a comparable frequency to untreated controls, and + indicates only occasional shoots

Table 3. Inheritance of streptomycin resistance[a] of mutant NTS1 of *N. tabacum* obtained through NMU (5 mM) mutagenesis of leaf strips

	Number of green seedlings	Number of bleached seedlings
NT[b](SELF)	0	217
NTS1(SELF)	205	0
NT♀ × NTS1♂	0	189
NTS1♀ × NT♂	232	0

[a] The tests were performed on medium containing 1000 mg l^{-1} streptomycin sulphate
[b] NT is *N. tabacum* var. Petit Havana, from which NTS1 was derived

b) Leaf strips. Excised leaf strips were exposed to each of the three concentrations of NMU for 90 min. The effect on subsequent development of adventitious shoots, albino shoots, and streptomycin resistant shoots is shown in Table 2. No variegated shoots were observed.

Four of the streptomycin resistant shoots were grown to maturity and crossed with wild-type *N. tabacum* var. Petit Havana. Seedlings were tested for resistance as described in the Materials and methods. Maternal inheritance was exhibited by all four mutants. The data for one of them, line NTS1, are shown in Table 3. For this mutant,

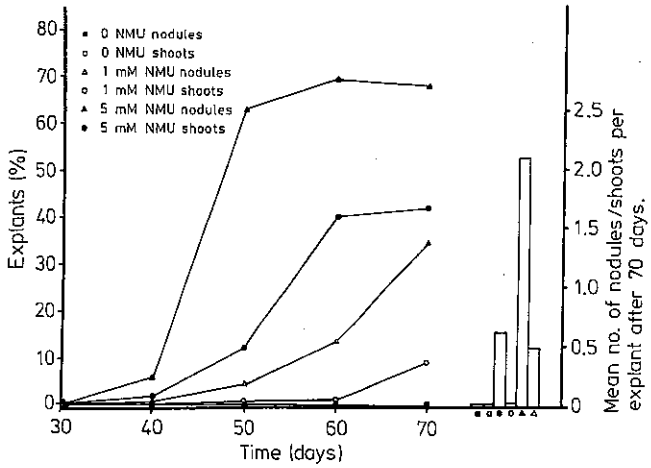

Fig. 1. Production of streptomycin resistant mutants on leaf strips of *Solanum nigrum*. Explants producing green nodules (see Fig. 2a) and shoots (Fig. 2b) were counted at various time intervals after transfer of nitroso-methylurea (NMU)-treated and control leaf strips on regeneration medium containing 500 mg l^{-1} streptomycin sulphate. After 70 days the mean numbers of green nodules or shoots per explant were determined

Table 4. Progeny leaf strip tests of streptomycin resistant mutants SNS20, SNS28, and SNS29 of *S. nigrum* (SN) obtained after leaf strip mutagenesis with 5 mM NMU (90 min)

	No. seedlings tested	No. showing resistance[a]		No. showing segregation[b]	
		Streptomycin concentration (mg l^{-1})			
		500	1000	500	1000
SN(SELF)	13	0	0	0	0
SNS20(SELF)	15	15	15	0	0
SN♀ × SNS20♂	5	0	0	2	0
SNS20♀ × SN♂	5	5	5	0	0
SNS28(SELF)	10	10	10	0	0
SN♀ × SNS28♂	3	0	0	0	0
SNS28♀ × SN♂	3	3	3	0	0
SNS29(SELF)	10	10	10	0	0
SN♀ × SNS29♂	24	0	0	0	0
SNS29♀ × SN♂	7	7	7	0	0

[a] Resistance is defined as retention of chlorophyll and green adventitious shoot production on medium containing 500 or 1000 mg l^{-1} streptomycin sulphate

[b] Segregation is defined as the production of variegated adventitious shoots

callus initiated from leaves of 4-week-old seedlings was also tested for streptomycin resistance (data not shown). This confirmed the maternal inheritance of streptomycin resistance and no evidence was found for paternal transmission of the trait.

S. nigrum

In *S. nigrum*, streptomycin resistant mutants first appear as green nodules (Fig. 2a), most of which subsequently differentiate into leafy shoots (Fig. 2b). The incidence of such mutants is rare in the absence of a mutagenesis treatment, but is high after exposure to 1 or 5 mM NMU for 90 min. The latter treatment results in resistant nodules in almost 70% of the leaf strips and a mean value of >2 nodules per leaf strip (Fig. 1). Resistant shoots were rooted on RM medium without streptomycin, and resistance was confirmed in further leaf strip tests on R3C8 containing 500 mg l^{-1} streptomycin sulphate. Most showed continued resistance, although expression was reduced in about 35% of the lines, mostly those selected late (>70 days after mutagenesis).

Crosses have been performed with three of the lines. In view of the small numbers of seed obtained, progeny tests were performed not on whole seedlings but on leaf strips excised from individual seedlings. The results are shown on Table 4. For SNS29 the appearance of the leaf strip tests is illustrated in Fig. 2c. The leaves of variegated shoots, found in tests with two seedlings obtained with SNS20 as the paternal parent, are illustrated in Fig. 2d. When these shoots are transferred to RM medium without streptomycin the leaves revert to a normal green colour.

L. peruvianum

Longer (120 min) incubations with NMU were more effective in *L. peruvianum*, and the best yield of streptomycin resistant shoots was achieved using 1 mM NMU. Results for both genotypes (S_1S_2 and S_4S_5) are given in Table 5. In contrast to *N. tabacum* and *S. nigrum*, many of the initial green shoots (including all those obtained without mutagenesis) proved sensitive on repeated transfer to fresh medium containing streptomycin. By the third subculture, however, resistance had stabilised in most of the lines and all shoot cultures established on RM medium without streptomycin from the fifth subculture proved resistant in subsequent leaf strip tests. Inheritance data are not yet available for the *L. peruvianum* lines.

Albino and variegated shoots were obtained at a low frequency from mutagenised leaf strips incubated on medium without streptomycin. In S_1S_2, a single albino and a single variegated shoot were obtained after treatment with 1 mM NMU, while in S_4S_5, two albino and one variegated shoot were obtained with 1 mM and an additional variegated shoot with 5 mM NMU. Stable, chlorophyll-deficient shoot cultures have been established from these mutants.

Discussion

NMU mutagenesis of tobacco seeds results in a very high incidence of variegated seedlings and a low frequency of streptomycin resistant sectors on the seedlings' true leaves. This phenomenon, previously reported by Fluhr et al. (1985) and confirmed in the current paper, is reversed when a leaf strip system is used for both mutagenesis and selection. The relatively high frequency of recovery of streptomycin resistant shoots, while chlorophyll deficiency mutants are rare, is probably a feature of the leaf strip selection system rather than any change in the targeting of mutagenesis. Fluhr et al. (1985) have already reported the induction of streptomycin resistant cells appearing as green islands in up to 90% of cotyledons after NMU mutagenesis of tobacco seeds. The sublethal levels of streptomycin employed here combined with the protracted process of adventitious shoot initiation, appears to maximise the opportunity for sorting out of resistant and sensitive plastids. The absence of variegated shoots under selective conditions at-

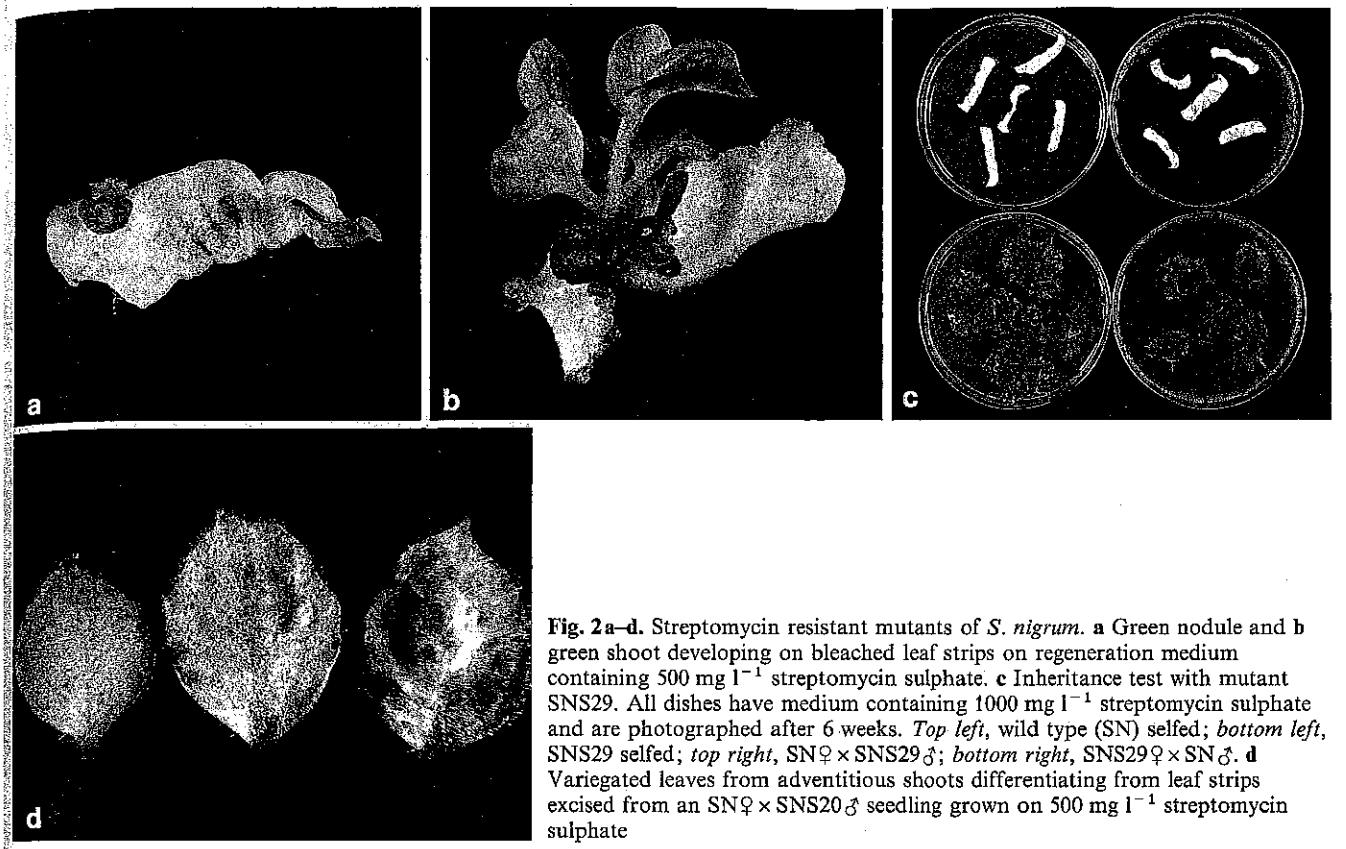

Fig. 2a–d. Streptomycin resistant mutants of *S. nigrum*. **a** Green nodule and **b** green shoot developing on bleached leaf strips on regeneration medium containing 500 mg l^{-1} streptomycin sulphate. **c** Inheritance test with mutant SNS29. All dishes have medium containing 1000 mg l^{-1} streptomycin sulphate and are photographed after 6 weeks. *Top left*, wild type (SN) selfed; *bottom left*, SNS29 selfed; *top right*, SN♀ × SNS29♂; *bottom right*, SNS29♀ × SN♂. **d** Variegated leaves from adventitious shoots differentiating from leaf strips excised from an SN♀ × SNS20♂ seedling grown on 500 mg l^{-1} streptomycin sulphate

Table 5. Occurrence and stability of streptomycin resistant shoots on leaf strips of *Lycopersicon peruvianum*

Genotype	NMU concentration (mM)	No. leaf strips tested	Resistant shoots[a]		
			On explant	Subculture[b]	
				3rd	5th
S_1S_2	0	83	23	0	0
	1	131	38	17	16
	5	75	11	5	5
S_4S_5	0	75	15	0	0
	1	75	22	19	17
	5	79	4	3	3

[a] Medium was R3C8 and contained 500 mg l^{-1} streptomycin sulphate throughout

[b] Subcultures were performed at 4–6 week intervals

tests both to the unicellular origin of the adventitious shoots, and to the completion of the sorting out process at an early stage in the differentiation of shoot primordia. The low frequency of chlorophyll deficient shoots arising from a mutagenised leaf compared to embryonic cells could be due to a lower efficiency of mutagenesis in the former, but may also relate to a selective disadvantage of the mutant plastids during the processes of initiation and development of adventitious shoots.

We have shown NMU to be effective in inducing genetic change within a range of *Solanaceae* species. It has been equally effective with *Antirrhinum* (Hagemann 1982) and we have used it to obtain albinos in strawberry (Malone and Dix, in preparation). In addition to its apparently wide species range, NMU is also effective when applied to a number of different types of plant material, from seeds to leaf explants. Our inheritance data support the preferential targeting to plastid DNA, although NMU also induces many nuclear mutations (Hagemann 1982; Hosticka and Hanson 1984). Nitrosoguanidine, another alkylating agent has been shown to act most effectively on dividing cells, both in yeast (Carter and Dawes 1978) and higher plants (King 1984). While our leaf strip system does not lend itself to comparisons between cycling and quiescent cells, efficient NMU-induced plastome mutagenesis of the latter is strongly suggested. A possible relationship between the cell cycle and ratios of nuclear and cytoplasmic mutations would merit further study.

Streptomycin resistant plants were retrieved regularly from all three species described here, in addition to *N. sylvestris* and *N. plumbaginifolia* for which data are not presented. Recovery of resistant lines, however, was substantially more frequent from *S. nigrum*. Difficulties in quantifying the morphogenetic response in leaf explants preclude any realistic estimation of mutation frequency. While it is possible to count macroscopic shoots on control explants, detailed histological investigation would be needed to determine the total number of shoot primordia. Even this would not be a fair indication of the number of cells with a potential for differentiation into shoots if the development of their neighbours is suppressed, for example by streptomycin. Despite these difficulties in quantification *S. nigrum* does seem to have a particularly high induced mutation rate. It has been speculated (Arntzen and Duesing 1984) that the high number of chloroplast-encoded atrazine resistant plants which have appeared over the years, may be due to *S. nigrum* having a nuclear mutator gene similar

to that of *Oenothera* (Epp 1973). The results we have obtained with *S. nigrum* may add to the circumstantial evidence for the presence of such a gene. On the other hand, Hosticka and Hanson (1984) have observed big differences in the frequency of variegated seedlings from NMU-mutagenesised seeds, between varieties of a single species (*L. esculentum*).

While the inheritance of streptomycin resistance was dictated by the maternal parent in the seven tested lines, the appearance of resistant cells in the progeny of the SN♀ × SNS20♂ cross suggest a paternal origin. Spontaneous mutation to streptomycin resistance can be excluded, since this is rare in *S. nigrum* leaf strips and invariably results in green, not variegated, shoots. Paternal inheritance of atrazine resistant chloroplasts in *S. nigrum* has been observed by Gasquez et al. (1981). The strong positive selection pressure applied to the leaf explant in the present work may facilitate the retention of the paternal chloroplasts in adventitious shoots. A mixed plastid population must, however, have been present in individual leaf cells of the seedlings prior to removal of explants for testing. The development of variegated shoots is clearly dependent on streptomycin concentration. Leaf strips from these seedlings were bleached and adventitious shoot regeneration was suppressed, on 1000 mg l^{-1} streptomycin sulphate, under which conditions maternal transmission is expressed.

We have used the *S. nigrum* variant lines to select for resistance to spectinomycin; additionally we have performed mutagenesis on atrazine resistant biotypes and selected for antibiotic resistant lines, providing a range of chloroplast markers and multiple markers (data to be presented elsewhere). This range of markers enhances the investigation of the genetics of such processes as chloroplast recombination after both intra- and intergeneric cell fusions. The availability of mutants plus the ease of selection in vitro will facilitate study of paternal transfer of chloroplasts in higher plants.

Particular attractions of the leaf strip system for mutant selection lie in its potential applicability in situations where seed mutagenesis is inconvenient, such as in vegetatively propagated species, sterile hybrids, and species with large seeds or impermeable seed coats in which it is difficult to standardise a protocol for imbibition and seed mutagenesis. In addition the rapid fixation of the mutation in M_1 is an attraction compared to seed mutagenesis where the first step is usually the production of variegated seedlings. Genetic crosses or protoplast fusion are, however, still necessary to assign the mutations to the chloroplast genome.

Finally, there is no reason to suppose that selection in organogenic explants should be restricted to chloroplast-encoded traits. Provided a suitable mutagenesis treatment is applied, there are good prospects for using this approach to obtain mutants of crop plants with improved agronomic traits, such as resistance to diseases, environmental stresses, and herbicides, while minimizing the gross chromosomal changes frequently associated with other approaches to selection in vitro (Dix 1986). A comparable procedure has also been developed (Horsch et al. 1985) for the selection of antibiotic resistant plants after *Agrobacterium tumefaciens* mediated transformation of leaf discs.

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