Theor Appl Genet (1985) 70:259–264

The mutagenic effect of gamma rays on leaf protoplasts of haploid and dihaploid *Nicotiana plumbaginifolia*, estimated by valine resistance mutation frequencies *

E. Nielsen **, E. Selva, C. Sghirinzetti and M. Devreux
Biology Group, Directorate General XII, J.R.C. of European Communities, I-21020 Ispra, Italy

Received November 12, 1984; Accepted December 18, 1984
Communicated by D. von Wettstein

Summary. Leaf protoplasts isolated from haploid and dihaploid *Nicotiana plumbaginifolia* plantlets were treated with different doses of gamma-rays and their survival was determined by scoring for plating efficiency at each irradiation dose. A fixed number of surviving protoplast-derived colonies was then plated in the presence of inhibitory concentrations of L-valine and incubated until growing resistant calli could be scored and mutation rates calculated. Though haploid protoplasts were found to be a little more sensitive than dihaploids to the lethal effect of radiation, the two dose-response curves of gamma-rays that induced mutagenesis were very similar. The irradiation dose capable of causing a ten-fold increase of spontaneous mutation frequencies was about 500 rads with both haploid and dihaploid protoplasts.

Key words: Gamma rays mutagenesis – Valine resistance – *Nicotiana plumbaginifolia* – Haploid and dihaploid protoplasts

Introduction

In addition to having lethal effects, ionizing radiations are well known to induce mutations in populations of living cells. Therefore, it is of great importance to study both the quantitative relationship between the two phenomena, as well as the spectrum of the genetic changes induced.

Plant cells and protoplasts offer particular advantages for mutagenesis research insofar as they can be manipulated, cultured and cloned "in vitro" in the diploid as well as in the haploid state, and they can be eventually regenerated into fertile plants. It is, therefore, possible to carry out a genetic analysis of spontaneous or induced mutations, including the recessive ones, on large cell populations and on differentiated organisms regenerated from them (Bourgin 1982). Nevertheless, there is a paucity of information on specific studies on the dose-response relationships of mutagenic treatments of plant cell suspension cultures (Sung 1976; Colijn et al. 1979; Horsch and Jones 1980; Werry and Stoffelsen 1980), and especially on mesophyll protoplasts (Caboche and Muller 1980; Vunsh et al. 1982; Grandbastien et al. 1984).

Biochemical markers, easily selectable in "in vitro" conditions, are needed to determine spontaneous rates of mutations and their induced increase following different mutagenic treatments. Bourgin (1976) proposed resistance to toxic levels of L-valine as an appropraite marker for mutagenesis studies. He subsequently obtained valine-resistant plants from "in vitro" selected protoplast-derived cells of haploid *Nicotiana tabacum* (Bourgin 1978), and recently a dose-response of U.V. light-induced increase in the frequency of valine-resistant mutants has been described (Grandbastien et al. 1984).

U.V. light has also been used in survival and mutagenesis experiments performed on haploid or diploid protoplasts of *Nicotiana sylvestris* (N.s.) and of *Nicotiana tabacum* (N.t.) (Vunsh et al. 1982). A comparison of these four sources of U.V. light-treated protoplasts with respect to the establishment of valine-resistant mutant cell lines and plants was made. Although this study did not enable a statistical evaluation of mutation rates, it permitted an estimation of their relative order: nN.s. nN.t. 2nN.s. 2nN.t.

In the present study, we determined and compared the mutagenic effect of gamma rays on haploid and dihaploid *Nicotiana plumbaginifolia* protoplasts by estimating the induced increase of the mutation frequency to valine resistance. The aim of the work is to contribute to a better understanding of the processes involved in the induction and repair of ionizing radia-

* Contribution No. 2173 of the Biology Radiation Protection and Medical Research Programme, Directorate General XII of the Commission of the European Communities

** Present address: Dipartimento di Genetica e Microbiologia, "A. Buzzati-Traverso", Via S. Epifanio 14, I-27100 Pavia, Italy

tion damages and in the expression of mutations in complex eukaryotic cells.

Materials and methods

1 Plant material

The species used in this study was *Nicotiana plumbaginifolia* Viviani (2n=20), from here onwards abbreviated as N.p. Haploid plantlets were obtained by the anther culture technique of Bourgin and Nitsch (1967), and a single clone was used for all experiments. Plantlets were axenically propagated by micro-cutting in 200 ml plastic containers with about 50 ml of MS medium (Murashige and Skoog 1962). Growing conditions were: 26 °C, 2,000 lux with a 12-h photoperiod.

In order to avoid genetic variability, a dihaploid homozygous clone was used. This was obtained by diploidization of haploid tissue which occurred spontaneously during callus proliferation and by subsequent plant regeneration and propagation. Ploidy level was checked cytologically in roots stained at metaphase with Feulgen.

2 Protoplast isolation, irradiation and culture

The procedure for the isolation of protoplasts from leaves essentially involved carborundum brushing, enzymatic incubation and density separation as described previously (Magnien et al. 1980). Protoplasts were diluted to 10^5/ml of culture medium K3 (Nagy and Maliga 1976), divided in as many aliquots as the irradiation doses to be delivered, and put in 200 ml sterile plastic containers. Irradiation was carried out in a ^{60}Co source "Gammacell 220" (Atomic Energy of Canada limited, Ottawa) which delivered an average dose rate of 600 rads/min. Each treated sample was then diluted 1:1 in culture medium and plated in 10 cm diameter plastic Falcon Petri dishes by gently pipetting 9 ml of protoplast suspension per dish. Incubation at 26 °C was carried out in the dark during the first 3 days, then in dim light.

3 Estimation of survival

After about 8 to 11 days of culture for dihaploids, and 12 to 15 days for haploids (depending on the gamma-ray dose absorbed), surviving protoplasts had given rise to small growing colonies with a morula-like appearance easily distinguishable from aspecific aggregates of dead protoplasts and protoplast-derived non-dividing cells.

The protoplast-derived colonies (PDC) were counted and plating efficiencies (and thus survival) could be calculated by simply dividing by the number of the protoplasts initially plated.

4 Selection for valine resistance

PDC from the plates of each irradiation dose were collected in centrifuge tubes and allowed to settle out. The supernatant was discarded and fresh K3 medium added so as to dilute the PDC to a density of 1.6×10^4/ml. Aliquots of 3 ml, corresponding to about 5×10^4 PDC, were then pipetted in 10 cm diameter plastic Falcon Petri dishes in which there was a bottom layer of 10 ml of solidified culture medium LS (Linsmayer and Skoog 1965). The solidified culture medium contained 0.2 M glucose as a carbon source, 0.1 mg/l NAA, 1 mg/l BAP, 1.2% agar Difco and 2.5 mM L-valine except in the control plates. Three ml aliquots of molten K3 medium (+1.2% agar) containing 5 mM L-valine, kept at 45 °C, were then poured onto the plates and quickly spread to form a soft top layer which included the PDC. The plates were incubated until presumptive resistant colonies reached a diameter of 2–5 mm (Fig. 1).

5 Confirmation of presumptive resistants

To further test the valine resistance of recovered colonies, 40–50 of them from each irradiation treatment were individually transferred to 25 wells Sterilin plastic plates containing the same selective medium as the bottom layer of the selection plates. Colonies which got through this second selective step and developed large green calli were considered to be valine-resistant.

Results

Gamma-ray induced lethality

The decrease in survival is the first visible effect of gamma irradiation on cells. This criterion was used for plant protoplasts to obtain dose-effect curves (Magnien et al. 1981, 1982).

Thus, the relative plating efficiencies for the different irradiation doses delivered to haploid and dihaploid N.p. protoplasts were calculated as percent of unirradiated controls and plotted as shown in Fig. 2. The radiosensitivity ratio between haploid and dihaploid protoplasts increases steadily in the region of lower doses, at which dihaploids appear able to endure the effect of gamma rays more than haploids, while it eventually reaches a plateau for higher doses.

Gamma-ray induced mutagenesis

Preliminary experiments, designed to choose the concentration of valine suitable for selecting resistants, showed that, under the experimental conditions described, the growth of both n and 2n N.p. PDC were completely suppressed by a valine concentration of 2.5 mM.

The scoring of the plates with such selective valine concentrations in which rare PDC had been able to grow small calli, might have allowed a direct estimation of mutation rates. However, to get confirmation of their resistant character, a representative sample of 40–50 clones from each set of plates was submitted to a second selective step. They were removed and transferred individually to fresh medium having the same valine concentration as used in the first step, and their regrowth was checked. As shown in Table 1, about 40–60% of the retested clones, irrespective of their origins, from either differently irradiated or unirradiated protoplasts, were confirmed to be valine resistant.

These values were used for the calculation of the mutation rate.

Since PDC submitted to selection were derived from isolated single protoplasts and did not dissociate or fuse during culture or plating, we considered the

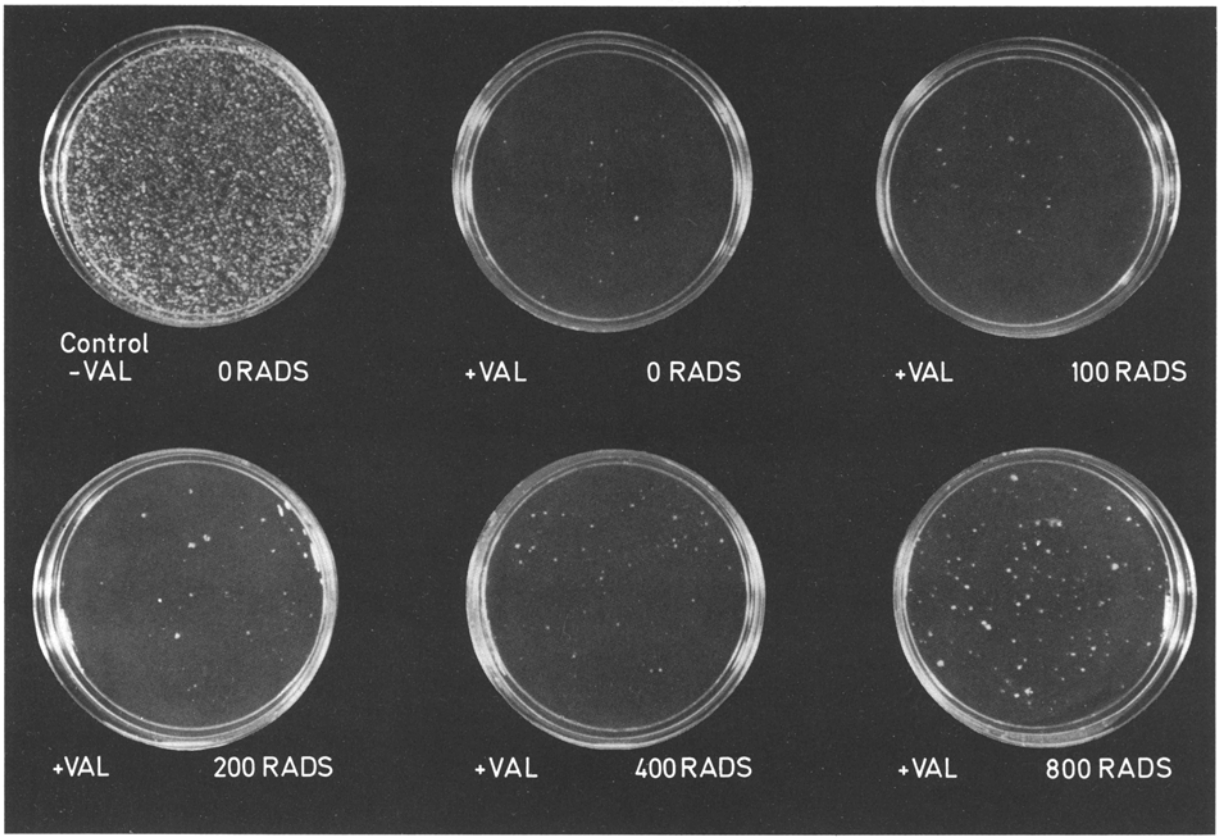

Fig. 1. Presumptive valine-resistant colonies grown on selective plates (containing 2.5 mM L-valine) in which had been seeded about 50,000 small colonies (PDC) derived from haploid N.p. protoplasts surviving treatment with different gamma-ray doses

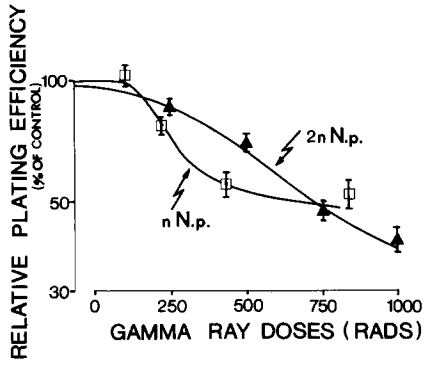

Fig. 2. Relative survival of haploid (□) and dihaploid (▲) N.p. protoplasts after irradiation with different doses of gamma-rays. 100% corresponds to the actual plating efficiencies of the unirradiated controls (51% and 54% for haploids and dihaploids, respectively)

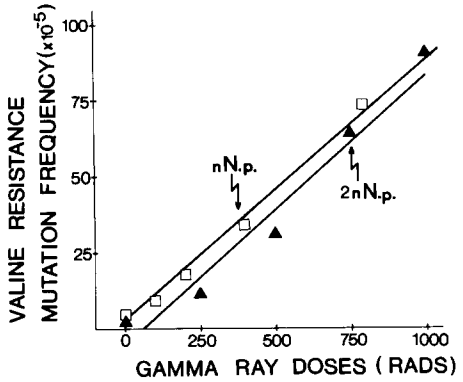

Fig. 3. Linear regressions of gamma-ray induced increases in the frequency of reconfirmed valine-resistant mutant colonies recovered from haploid (□) and dihaploid (▲) N.p. protoplasts

resistants recovered as originating from distinct mutational events.

Table 1 shows the spontaneous and induced frequencies of mutation to valine resistance of haploid or dihaploid N.p. protoplasts.

The reported figures represent the means obtained by pooling the data from two independent experiments on haploids and three independent experiments on dihaploids. The linear regressions plotted with such values are shown in Fig. 3.

Table 1. Summary of the results on gamma-ray induced lethality and mutagenesis on protoplasts of haploid and dihaploid *Nicotiana plumbaginifolia*. Numbers in brackets represent the percentages of isolated colonies confirmed to be valine-resistant upon retesting

Gamma-ray doses (rads)	Relative plating efficiency	PDC submitted to selection	Recovered colonies	Average frequency confirmed Val-res
	Haploid *Nicotiana plumbaginifolia*			
0	100%	1.5×10^{6}	126 (56%)	$4.4\pm 0.5\times10^{-5}$
100	103%	6.0×10^{5}	121 (46%)	$9.1\pm 1.4\times10^{-5}$
200	77%	8.5×10^{5}	248 (60%)	$18.3\pm 0.9\times10^{-5}$
400	55%	5.5×10^{5}	407 (48%)	$35.1\pm 2.8\times10^{-5}$
800	52%	5.5×10^{5}	684 (58%)	$72.1\pm 6.5\times10^{-5}$
	Dihaploid *Nicotiana plumbaginifolia*			
0	100%	1.0×10^{6}	97 (40%)	$3.1\pm 1.0\times10^{-5}$
250	86%	5.0×10^{5}	122 (47%)	$11.6\pm 2.1\times10^{-5}$
500	70%	5.0×10^{5}	277 (57%)	$31.8\pm 10\times10^{-5}$
750	47%	5.5×10^{5}	704 (50%)	$64.0\pm 4.8\times10^{-5}$
1,000	40%	2.5×10^{5}	513 (44%)	$90.3\pm 4.0\times10^{-5}$

It is noteworthy that a gamma-ray dose as low as 500 rads, which causes 50% killing of haploid and 30% of dihaploid protoplasts is sufficient to increase tenfold the frequency of appearance of valine-resistant mutants from protoplasts of both ploidy levels.

Discussion

The experiments described above were designed to evaluate the mutagenic effect of gamma rays and to compare their effectiveness on plant cells at different ploidy levels. This was achieved by measuring the mutation rate of a genetic marker: resistance to toxic levels of L-valine. The main reason for this choice is that several biochemical studies that characterized this marker have been carried out.

Mechanisms of valine toxicity have been studied in bacteria (Umbager 1969; De Felice 1977) and plant systems (Miflin 1969; Miflin 1973; Borstlap 1972; Borstlap 1981). It was shown in these studies that the final steps of the biosynthetic pathways of valine (and leucine) and isoleucine share the same enzymes (Bryan 1976) and that regulator feedback inhibition of the first of them, acetohydroxyacid synthase, is exerted by leucine and valine in most plant species (Miflin and Cave 1972). Thus, an excess of leucine and valine, but even of only one of them, may lead to the inhibition of isoleucine biosynthesis and consequently of cell growth.

Valine-resistant tobacco plants selected from U.V. treated haploid protoplasts were submitted to genetic and biochemical analysis (Bourgin 1982). This led to their classification into two types. Mutants of the first type show a low-level resistance which is transmitted as a single Mendelian dominant character possibly attributable to acetohydroxyacid synthase less sensitive to valine feedback inhibition. Those of the second type have a high-level resistance which is transmitted as a digenic recessive character and is due to a reduced permeability of cells to valine and other amino acids.

Up to now characterization studies of this kind are not possible with our valine-resistant clones of N.p. because the regeneration of plants from the isolated calli has not yet been achieved, even after repeated transfers, onto non-selective media which quickly induce the shooting of wild-type control calli.

Special conditions might be required by mutant calli to form buds and we are still assaying new nutrients and/or hormone combinations and concentrations. In any case, if it persists, this apparent difficulty in the regenerative process might be attributed to an interference of valine-resistance mutations with the differentiation processes of N.p. rather than to a secondary deleterious effect of the gamma-ray treatment. In fact, even the spontaneous valine-resistant calli deriving from unirradiated protoplasts have not yet regenerated any buds. Loss of morphogenetic capacity has also been described for some, but not all, valine-resistant cell lines selected from N.t. and N.s. protoplasts (Vunsh 1981).

The spontaneous frequencies of mutation to valine resistance of haploid or dihaploid N.p. protoplasts, determined as described in the above sections, were both found to be rather high (respectively 4.4×10^{-5} and 3.1×10^{-5}) and not too different from each other. This might be due to the presence in N.p. genome of many loci that, following a mutational event, can offer valine resistance. A consistent number of such mutations should be of the dominant type since the frequency of spontaneous valine-resistants is not much higher in haploids, from which both dominant and recessive mutants can be recovered, than in dihaploids. On the other hand, as in dihaploids every gene is present in two copies, the probability that any mutation occurs is twice that of haploids. Thus, the dominant mutations should arise in dihaploids at twice the rate of those in haploids.

Our data emphasize the need of retesting the resistance of the colonies grown in selection plates. Lack of confirmation of valine resistance has already

been reported to occur in part, 33–76% of the N.t. clones passed a first selective step (Grandbastien et al. 1984). In our case, about 40–60% of the selected colonies, irrespective of the irradiation dose absorbed by the protoplasts from which they derived, could not be reconfirmed as valine-resistants. Such clones may not represent simply a background of wild-type valine sensitive cells escaping selective pressure since their proportion could not remain constant, but should greatly decrease, increasing the mutagenic treatment dose. A likely explanation is that the non-confirmed clones are true mutants with low level resistance that, once isolated from the other plated PDC, are no longer able to bear the selective level of valine. Another possibility is that the mutagenic treatment is somehow able to increase, together with the frequency of true mutants, even that of "pseudo mutants", as reported by Horsch (1979).

It has been reported (Grandbastien et al. 1984) that the spontaneous mutation rate of valine resistance of haploid N.t. protoplasts is in the range of 10^{-6} or even lower. The possible differences in genome structure and expression between the two species occurring naturally, or induced by different responsiveness to "in vitro" culture conditions, might be at the basis of such lower mutation rates of N.t. as compared to those of dihaploid N.p. (due to the amphiploid nature of the tobacco genome, haploid N.t. might be compared with diploid N.p.). A possibility is also that for isolating N.p. resistants we used fairly different culture and selective conditions (at least as far as culture media, cell density and time of valine addition) from those used for N.t. resistants. This may have led us to rescue a class of mutants which, on the contrary, is killed in the case of N.t.

To verify this point it would be of interest to set up experiments of isolation of valine-resistant mutants of the two species under identical experimental conditions.

The data on the gamma-ray induced increase of valine-resistance mutation rate, confirm that ionizing radiations are powerful mutagenic agents of plant cells. In fact, the mutagenesis efficiency is very high and the induced mutation rates increase linearly with the irradiation doses on cell populations of both ploidy levels. The slopes of the two linear regressions are very similar, pointing out that valine-resistance mutations are induced by gamma rays approximately at the same rate in both haploid and dihaploid protoplasts.

From a comparison of the data on the killing effect with those of the corresponding mutagenic effect induced by each irradiation dose delivered, one can clearly deduce that in plant cells it is not necessary to use doses of gamma rays inducing high killing rates to obtain satisfactors increases in the frequency of mutation. Undesirable secondary mutations as well as deleterious effects on the morphogenetic capacity can thus be minimized, reducing the mutagen dose.

This had been shown to be true also in the case of such other kinds of mutagenic treatments as U.V. (Grandbastien et al. 1984) or N-ethyl-N-nitrosourea (Marton et al. 1982).

The comparison of the efficiencies and the optimization of the use of mutagens may now be envisaged on plant protoplasts employing the above described experimental system *Nicotiana plumbaginifolia*-valine resistance as a routine test.

Acknowledgements. The authors wish to thank Mr. Xavier Dalschaert for his skilful technical assistance and Dr. Margaret Merlini for a review of the manuscript.

References

Borstlap AC (1972) Changes in the free amino acids of *Spirodela polyrhiza* (L.) Schleiden during growth inhibition by L-valine, L-isoleucine or L-leucine. A gas chromatographic study. Acta Bot Neerl 21:406–416

Borstlap AC (1981) Interactions between the branched-chain amino acids in the growth of *Spirodela polyrhiza.* Planta 151:314–319

Bourgin JP, Nitsch JP (1967) Obtention de *Nicotiana* haploides à partir d'étamine cultivées in vitro. Ann Physiol Veg 9:377–382

Bourgin JP (1976) Valine-induced inhibition of haploid tobacco protoplasts and its reversal by isoleucine. Z Naturforsch 31c:337–338

Bourgin JP (1982) Selection of tobacco protoplast-derived-cells for resistance to amino acids and regeneration of resistant plants. In: Kleinhofs T, Lurquin PT (eds) Proc NATO Adv Study Inst "Genetic engineering in eukaryotes". Plenum Press, New York, pp 195–214

Bryan JK (1976) Amino acid biosynthesis and its regulation. In: Bonner J, Varner JE (eds) Plant biochemistry. Academic Press, New York London, pp 525–560

Caboche M, Muller JF (1980) Use of a medium allowing low cell density growth for "in vitro" selection experiments: isolation of valine-resistant clones from nitrosoguanidine-mutagenized cells and gamma-irradiated tobacco plants. In: Sala F, Parisi, Cella R, Ciferri O (eds) Plant cell cultures: results and perspectives. Elsevier/North-Holland Biomedical Press: Amsterdam New York Oxford, pp 133–138

Coljn CM, Kool AJ, Nijkamp HJJ (1979) An effective chemical mutagenesis produced for *Petunia hybrida* cell suspensions. Theor Appl Genet 55:101–106

De Felice M, Squires C, Levinthal M, Guardiola J, Lamberti A, Iaccarino M (1977) Growth inhibition of *Escherichia coli* K 12 by L-valine: a consequence of a regulatory pattern. Mol Gen Genet 156:1–7

Grandbastien MA, Bourgin JP, Caboche M (1985) Valine-resistance, a potential marker in plant cell genetics. Optimization of U.V. mutagenesis and selection of valine-resistant colonies derived from tobacco mesophyll protoplast. Genetics (in press)

Horsch RB (1979) Somatic plant genetics, a model system using cultured plant cells of *Haplopappus gracilis.* Thesis, University of California, Riverside, pp 191

Horsch RB, Jones GE (1980) The selection of resistance mutants from cultured plant cells. Mutat Res 72:91–100

Linsmayer EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18:100–127

Magnien E, Dalschaert X, Romengous M, Devreux M (1980) Improvement of protoplast isolation and culture technique from axenic plantlets of wild *Nicotiana* species. Acta Genet Sinica 7:235–240

Magnien E, Dalschaert X, Coppola M (1981) Dose-effect relationships, r.b.e. and split-dose effects after gamma-ray and fast neutron irradiation of protoplasts from wild *Nicotiana* species. Int J Radiat Biol 40:463–474

Magnien E, Devreux M, Dalschaert X, Coppola M (1982) Unexpected dose dependance of the radiosensitivity-ratio between haploid and diploid protoplasts. In: Fujiwara (ed) Plant tissue and cell culture. Proc 5th Int Cong Plant Tissue Cell Culture. Jpn Assoc Plant Tissue Culture, Tokyo, pp 457–458

Marton L, Manh Dung T, Mendel RR, Maliga P (1982) Nitrate reductase deficient cell lines from haploid protoplast cultures of *Nicotiana plumbaginifolia*. Mol Gen Genet 186:301–304

Miflin BJ (1969) The inhibitory effect of various amino acids on the growth of barley seedlings. J Exp Bot 28:299–329

Miflin BJ, Cave PR (1972) The control of leucine, isoleucine and valine biosynthesis in a range of higher plants. J Exp Bot 23:511–616

Miflin BJ (1973) Amino acid biosynthesis and its control in plants. In: Milborrow BV (ed) Biosynthesis and its control in plants. Academic Press, London New York, pp 49–69

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Nagy JI, Maliga P (1976) Cultures induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z Pflanzenphysiol 78:453–455

Sung ZR (1976) Mutagenesis of cultures plant cells. Genetics 84:51–57

Umbarger HE (1969) Regulation of the biosynthesis of the branched-chain amino acids. Curr Top Cell Regul 1:57–76

Vunsh R (1981) Induction and selection of biochemical mutants from plant protoplasts and cell suspensions. PhD Thesis, Rehovot (Israel), Weizmann Institute of Science

Vunsh R, Aviv D, Galun E (1982) Valine resistant plants derived from mutated haploid and diploid protoplasts of *Nicotiana sylvestris* and *Nicotiana tabacum*. Theor Appl Genet 64:51–58

Weber G, Lark KG (1980) Quantitative measurements of the ability of different mutagens to induce an inherited change in phenotype to allow maltose utilization in suspension cultures of soybean, *Glycine max*. Genetics 96:312–322

Werry PATJ, Stoffelsen KM (1980) Theoretical and practical aspects of radiation induced mutagenesis in plant cells. In: Sala F, Parisi B, Cella R, Ciferri O (eds) Plant cell cultures: results and perspectives. Elsevier, Amsterdam, pp 115–120