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Valine Resistant Plants Derived from Mutated Haploid and Diploid Protoplasts of *Nicotiana sylvestris* and *N. tabacum*

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Summary. Protoplasts were derived from haploid and diploid *Nicotiana sylvestris* and *N. tabacum*. Exposure of the protoplasts to mutagenic doses of ultraviolet (U.V.) radiation prior to two selection rounds in the presence of 4 mM (or 5 mM) and 8 mM of valine, respectively, was required to obtain cell lines with persistent valine resistance. Such lines were obtained from haploid and diploid *N. sylvestris* protoplasts as well as from haploid protoplasts of *N. tabacum* but not from (1.8×10^7) diploid *N. tabacum* protoplasts. The ratio between number of verified valine-resistant cell lines and the initial number of U.V. exposed protoplasts enabled the estimation of the following order of mutation frequency: haploid *N. sylvestris* > haploid *N. tabacum* > diploid *N. sylvestris*. Plants which retained the valine resistance and transmitted it to their sexual progeny were derived from the resistant cell lines.

Key words: Haploid protoplast – Diploid protoplasts – Metabolic mutants – U.V. sensitivity – *Nicotiana*

Introduction

Results concerning the effect of mutagenizing agents like ultraviolet (UV) or X-irradiation on cultured plant cells are accumulating at an increased rate. Claims of obtaining mutant cell lines are also being frequently reported but until recently only in a very few cases was a systematic study conducted to assess the relationship between ploidy levels and irradiation doses (Galun and Raveh 1975; Krumbiegel 1979) and cases in which mutants, selected at the cell level, produced a mutant plant were rarely documented. One such reported mutant was a valine resistant *N. tabacum* plant isolated by Bourgin (1978). Mesophyll protoplasts of higher plants, which are capable of dividing and giving rise to mature flowering plants offer a good system for mutant isolation (Maliga 1980). The availability of androgenic haploid plants increases the probability of obtaining recessive mutants (Maliga et al. 1982) and indeed very recently mutant *Hyoscyamus* (Strauss et al. 1981; Gebhardt et al. 1981) and *Nicotiana* plants (Sidorov et al. 1981) were isolated using haploid mesophyll protoplasts.

In this study we evaluated and compared the U.V. irradiation sensitivities of haploid and diploid *Nicotiana sylvestris* protoplasts as well as of amphihaploid and amphidiploid (hence forth abbreviated to haploid and diploid, respectively) *N. tabacum* protoplast. We also compared these four protoplast sources in respect to the establishment of valine resistant cell lines and plants following U.V. irradiation.

Materials and Methods

1 Plant Material

Nicotiana tabacum cv. Xanthi and *N. sylvestris* plants were grown as described (Zelcer and Galun 1976). Haploid androgenetic plants of both species respectively were obtained by culture of surface sterilized anthers, containing microspores at the uninucleate stage, on Nitsch (1969) medium supplemented with 0.25% charcoal. The haploid plantlets were transferred to fresh Nitsch medium for rooting and, after about 20 days, planted in peat pots and transferred to the greenhouse. Thus, four sources for protoplasts were made available: diploid and haploid *N. tabacum* cv. Xanthi and diploid and haploid *N. sylvestris;* these are subsequently abbreviated as Nt(2n), Nt(n), Ns(2n) and Ns(n), respectively.

2 Protoplast Isolation

Protoplasts were isolated as described (Zelcer et al. 1978) except that enzyme concentrations were lowered to 0.05% Macerozyme, 0.125% Driselase and 0.25% Cellulase. The enzymes were dissolved in mannitol salt solution (MSS) for *N. tabacum* or in sucrose salt solution (SSS) for *N. sylvestris* as previously reported (Aviv and Galun 1980). Incubation time was 16–18 h at 26 °C.

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3 Irradiation of Protoplasts

The protoplasts were washed once with MSS or SSS (*N. tabacum* or *N. sylvestris*, respectively) and resuspended in SSS (10^5 cells/ml) in 10 cm plastic petri plates. The plates were then transferred to a dark laminar hood. The lids were removed and the protoplasts were exposed to ultraviolet (U.V.) light by an Hanovia 44562 lamp at an incident dose rate of 32 erg · mm^{-2} s^{-1}. The distance of the irradiation source from the protoplast level was 35 cm. The lids were then returned and the plates were immediately covered with aluminium foil and kept in the dark for 2 to 4 h to avoid photorepair. The protoplasts were then plated in culture medium as detailed below. Survival was evaluated by counting colonies after 3 weeks of culture.

4 Plating and Culture of Protoplasts

Untreated and mutagenized protoplasts were centrifuged (80×g, 5 min), resuspended in 22% sucrose and centrifuged again. The upper green band of protoplasts was resuspended and plated on either NT medium (Nagata and Takebe 1971) for *N. tabacum* or on NM medium (Nagy and Maliga 1976) for *N. sylvestris;* in both cases the media contained 0.8% agar. Plating was usually performed in 5 cm petri plates and protoplast densities were 2.10^4 to 5.10^4 cells/ml. Other culture conditions and regeneration of plants from calli were as previously described (Zelcer et al. 1978). To evaluate the effects of valine and U.V., protoplasts and colonies per unit volume, were counted on the day of plating and after 3 weeks, respectively. These counts served for calculation of survival rates.

5 Selection for Valine Resistance

Mesophyll protoplasts from several leaves were used in each experiment. Most of the protoplasts were exposed to U.V. irradiation before being plated on solidified NT or NM media (as detailed above) while the rest of the protoplasts were plated without irradiation. After one week the plates were impregnated with 4 mM or 5 mM L-valine in liquid MS medium (Murashige and Skoog 1962). Impregnation was performed by adding to each petri dish 5 ml of the valine containing medium; after 60 min the latter medium was removed and culture was continued for another three weeks. The cultures were then diluted (1 : 3 to 1 : 6) with MS medium containing 8 mM valine. Calli which grew in this medium were transferred after about 4 weeks to the same valine containing medium and after an additional 2 to 3 weeks the growing calli were considered as apparently valine resistant.

Control calli, obtained without valine screening, were also secured from each experiment.

6 Seed Test for Valine Resistance

Valine resistance of emerging seedlings was evaluated according to Bourgin (1978). Basically, surface sterilized seeds were put on solidified Nitsch medium containing one of several L-valine concentrations (between 0.25 and 2.0 mM). Seedlings are more sensitive than the respective protoplasts and calli; for accurate results it is crucial that no more than 10 seedlings per 5 cm petri dish are seeded.

7 Ploidy Level

Chromosome counts were performed with root tips which were treated with 0.1% colchicine for 3 h followed by overnight fixation in 2% acetocarmine in 45% acetic acid.

As leaves of haploid *N. tabacum* and *N. sylvestris* are considerably narrower than their respective diploids, ploidy level was determined in most cases by leaf shape. Occasional checks indicated that chromosome counts conformed with ploidy evaluation based on leaf shape.

Results

Valine Sensitivity of Newly Cultured N. tabacum and N. sylvestris Cells

The survival of newly cultured Nt(2n), Nt(n), Ns(2n) and Ns(n) cells was evaluated by flooding petri plates, one week after protoplast plating, with MS medium containing various L-valine concentrations. Details on this impregnation procedure were given in Material and Methods. Ns(2n) cells showed highest sensitivity; less than 10 percent of these cells survived after 4 mM valine treatment. Ns(n) and Nt(n) were less valine sensitive and Nt(2n) showed least valine sensitivity (Fig. 1). The 4 mM or 5 mM valine treatment was thus used in the first stage selection for valine resistance.

U.V. Sensitivity

For mutagenic treatment protoplasts of diploid and haploid *N. tabacum* and *N. sylvestris* were exposed to U.V. irradiation. The survival rates are indicated in Fig. 2. Ns(n) protoplasts (12 chromosomes) were most sensitive while Nt(2n) protoplasts (48 chromosomes) were most resistant to the U.V. irradiation; Ns(2n) and

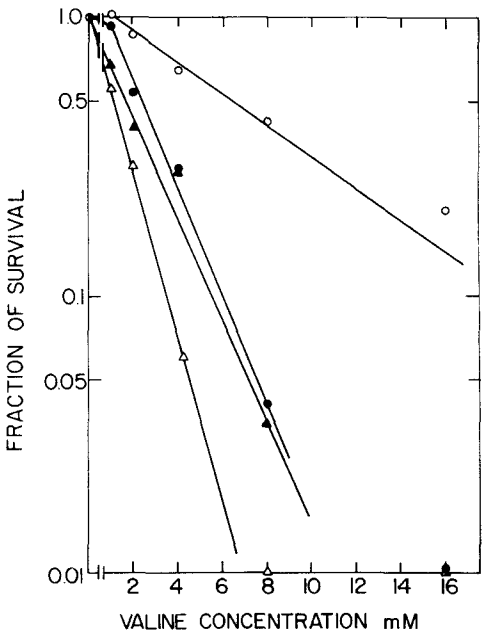

Fig. 1. The effect of valine concentrations on the survival of newly cultured protoplasts. Nt(2n) -○-; Nt(n) -●-; Ns(2n) -△-; Ns(n) -▲-

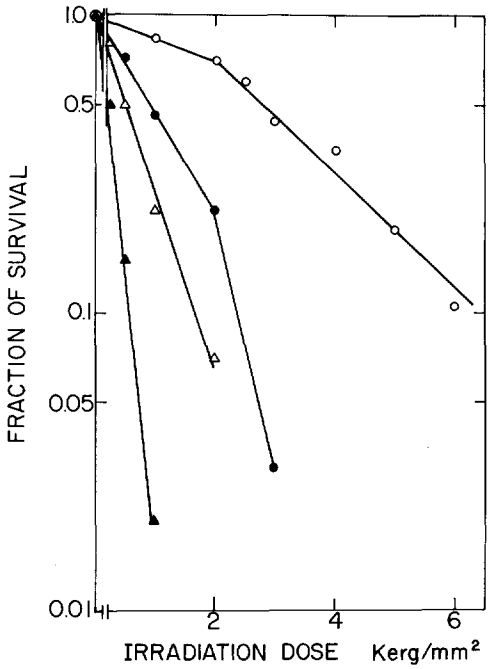

Fig. 2. Dose-response of diploid and haploid *N. tabacum* and N. sylvestris protoplasts to U.V. irradiation, Nt(2n) -○-; Nt(n) -●-; Ns(2n) -△-; Ns(n) -▲-

Nt(n) protoplasts (24 chromosomes) showed an intermediate sensitivity. Thus a positive correlation was indicated between chromosome number and U.V. resistance. U.V. doses resulting in about 0.5 survival were thus routinely applied as mutagenic treatment.

Selection of Mutants

1 Nicotiana tabacum

Two experiments led to valine resistant *N. tabacum* plants. Haploid *N. tabacum* protoplasts served as starting material in both experiments. One of these experiments is described schematically in Fig. 3. In this experiment 7.50×10^6 Nt(n) protoplasts were exposed to 1000 erg · mm^{-2} U.V. irradiation. About half of the treated protoplasts survived U.V. exposure and were processed through two stages of valine selections, resulting in 14 callus lines with apparent valine resistance. These lines were designated Nt^{VR}-1 to Nt^{VR}-14. In addition 4 calli derived from protoplasts which were not exposed to the U.V. irradiation (designated Nt^{VR}-15 to Nt^{VR}-18) also survived the two-stage valine exposures. Twelve calli derived lines from these 18 were retested for valine resistance. Seven of these lines showed consistent high resistance; another 5 lines showed lower resistance (Table 1). In all but one of the 12 callus lines which showed consistent valine resistance, shoot regeneration could be induced in the presence of 4 mM valine. (Table 1, Fig. 4). No shoot regeneration occurred in control calli in the presence of 4 mM valine.

Mesophyll protoplasts derived from plants which were regenerated from some of the valine resistant callus lines were tested for valine resistance; protoplasts isolated from 4 such valine-resistant plant lines showed high plating efficiency and callus production in the presence of 5 mM valine (Table 1, Fig. 5). Seeds obtained after self-pollinations of plants regenerated from

Table 1. Characteristics of valine resistant variants derived from haploid *N. tabacum* protoplasts. Calli were either highly resistant (+ +) or resistant (+) showing normal or reduced growth rates (respectively) to 8 mM valine; protoplasts of plants derived from resistant calli were either resistant (+) or sensitive to 5 mM valine, high resistance (+ +) and resistance (+) of seeds indicates normal seedlings in the presence of 1.0 and 0.5 mM valine, respectively; sensitive seeds (–) resulted in defective seedlings in 0.5 mM valine; n.t. not tested, n.p. no plants

Line designation	Callus resistance	Regeneration capability in presence of 4 mM valine	Valine resistance of protoplasts from regenerated plants	Chromosome number of regenerated plants	Valine resistance in seeds from regenerated plants
Nt^{VR}-1	+ +	yes	+	48	+ +
Nt^{VR}-2	+ +	yes	–	48	+
Nt^{VR}-3	+	yes	+	24/48	+ +
Nt^{VR}-4	+	yes	+	24/48	n.t.
Nt^{VR}-5	+ +	yes	+	48	n.t.
Nt^{VR}-6	+ +	yes	n.t.	48	n.t.
Nt^{VR}-9	+ +	yes	–	n.t.	n.t.
Nt^{VR}-12	+ +	yes	–	24/48	n.t.
Nt^{VR}-14	+	yes	n.t.	24	n.p.
Nt^{VR}-15	+	no	–	n.t.	n.t.
Nt^{VR}-16	+ +	yes	–	48	–
Nt^{VR}-17	+	yes	n.t.	n.t.	n.t.

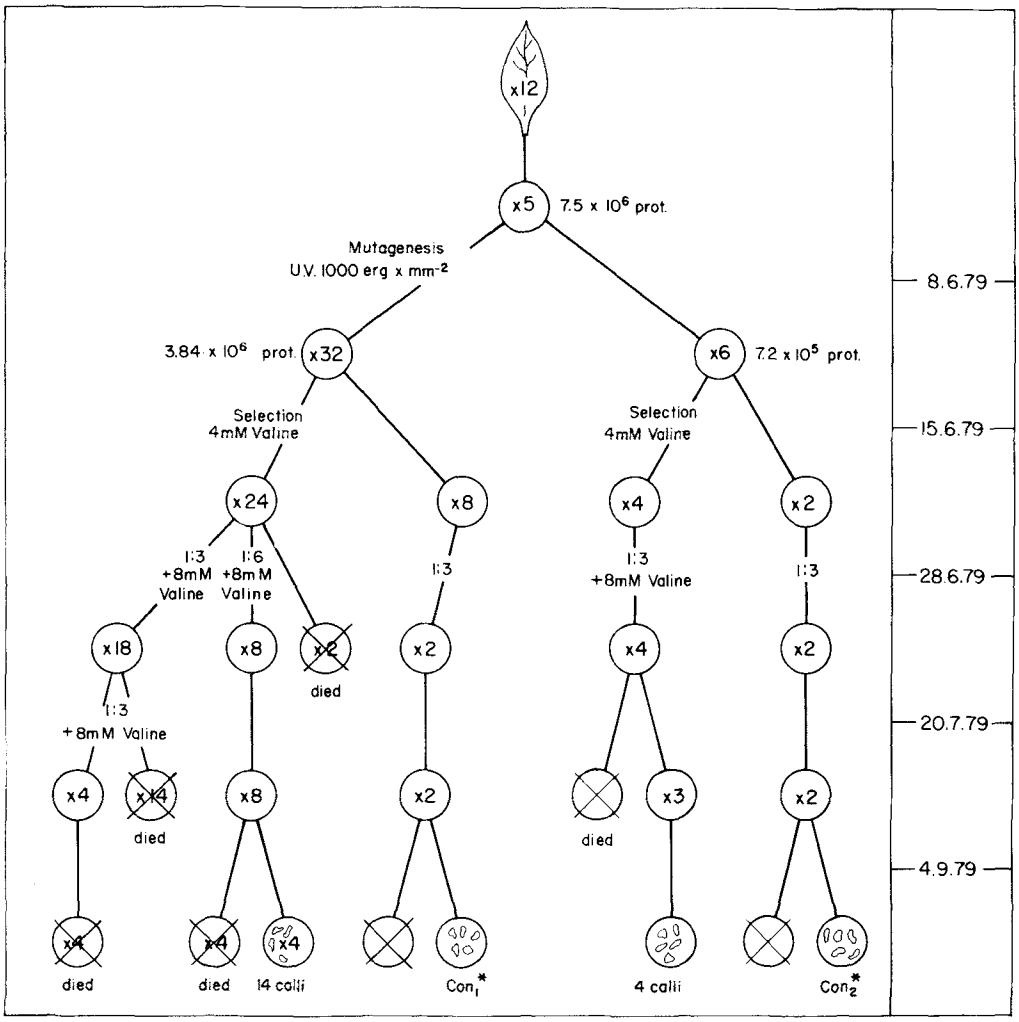

Fig. 3. Scheme of selection procedure for the isolation of valine resistant cell lines. The scheme, typical of the strategy followed in this study, represents an actual experiment with Nt(n) protoplasts. Numerals in circles denote number of plates

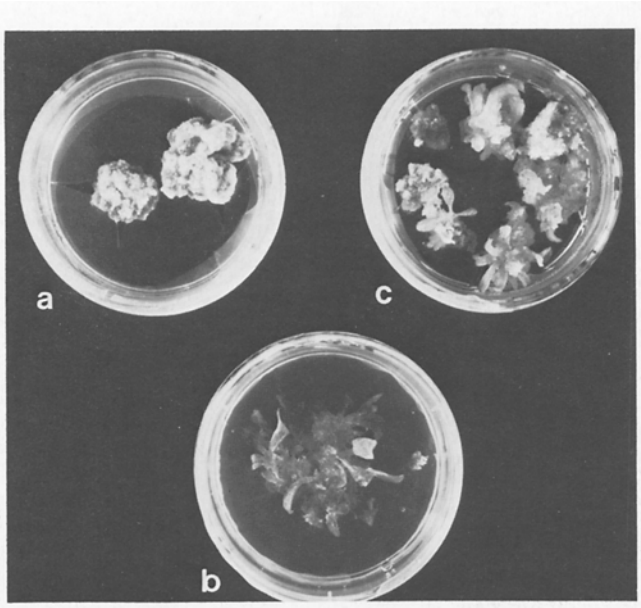

Fig. 4a – c. Differentiation capability of calli from a Nt(n) plant (**a, c**) and from cell line Nt^{VR}-12 (**b**) in the presence of 4 mM valine (**a, b**) or without valine (**c**)

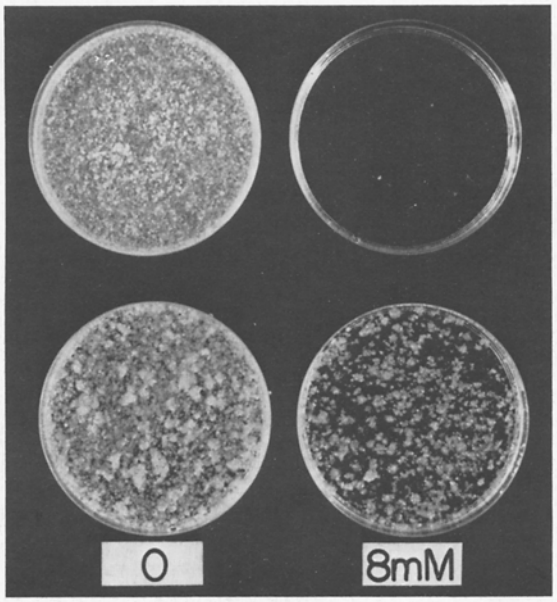

Fig. 5. Valine response of protoplasts from a normal Nt(n) plant (upper plates) and from a plant derived from the valine resistant line Nt^{VR}-5 (lower plates)

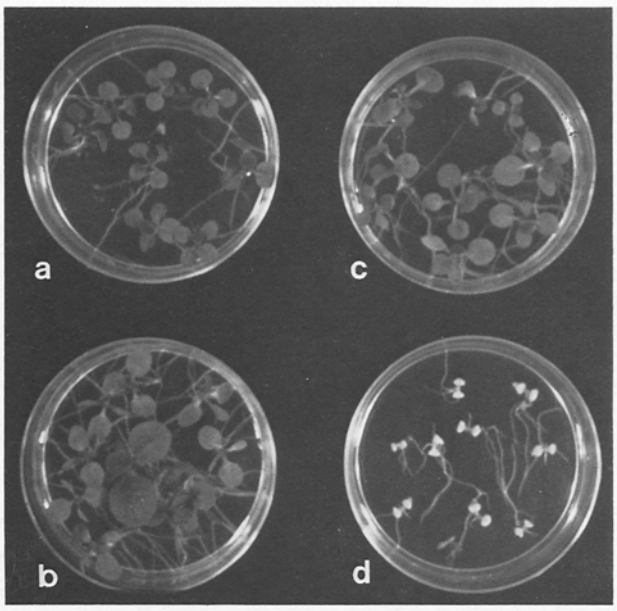

Fig. 6 a – d. Germination of seeds from a valine resistant plant (**a, b**) derived from line Nt^{VR}-1 and from *N. tabacum* (**c, d**) on medium lacking (**a, c**) or containing (**b, d**) 0.5 mM valine

part of the valine resistant callus lines showed resistance to valine (Table 1, Fig. 6). In most of the regenerated plants the chromosome number was doubled, but other plants either retained the haploid chromosome number or contained cells with the haploid and cells with the diploid chromosome number (Table 1). Tests for valine resistance was continued in 3 M_2 plants which were grown from resistant seeds. Leaf mesophyll protoplasts of two plants, Nt^{VR}-1 and Nt^{VR}-3 (Fig. 7), showed high valine resistance while protoplasts from a third M_2 plant, Nt^{VR}-2, showed only partial resistance.

In a second experiment the same mutagenesis and selection steps were applied. About half of the 1.44×10^6 Nt(n) protoplasts withstood the U.V. treatment and 11 calli survived after the first and second selections in the presence of toxic valine levels. Only one of these lines expressed valine resistance in the mesophyll protoplasts of regenerated M_1 plant. Seeds were obtained after spontaneous diploidization and self pollination. These seeds as well as protoplasts of the resulting M_2 plants were valine resistant.

Several additional experiments in which a total of 1.80×10^7 Nt(2n) protoplasts were exposed to a mutagenic treatment (3000 erg · mm^{-2}) did not result in cell lines which passed the valine selection procedure.

2 Nicotiana sylvestris

We exposed 5.2×10^5 Ns(n) protoplasts to U.V. (500 erg · mm). The protoplasts were then processed through the valine selection procedure and 20 callus lines were thus obtained. Calli of these lines were regenerated into plants in the presence of 5 mM valine. Furthermore the growth rates of these calli, in the presence of 8 mM valine, was similar to the growth of control calli in the absence of valine. The valine resistant lines were designated Ns^{VR}-1 through Ns^{VR}-20 (Table 2). Protoplasts of plants regenerated from nine such lines were retested for valine resistance; in addition we evaluated the valine resistance of seeds which were produced by valine resistant M_1 plants. In all the tested cases, protoplasts obtained from M_1 plants which were regenerated from resistant cell lines showed valine resistance, but the degree of resistance differed as indicated in Table 2. Unselected calli derived from Ns(n) protoplasts did not survive in 2 mM valine.

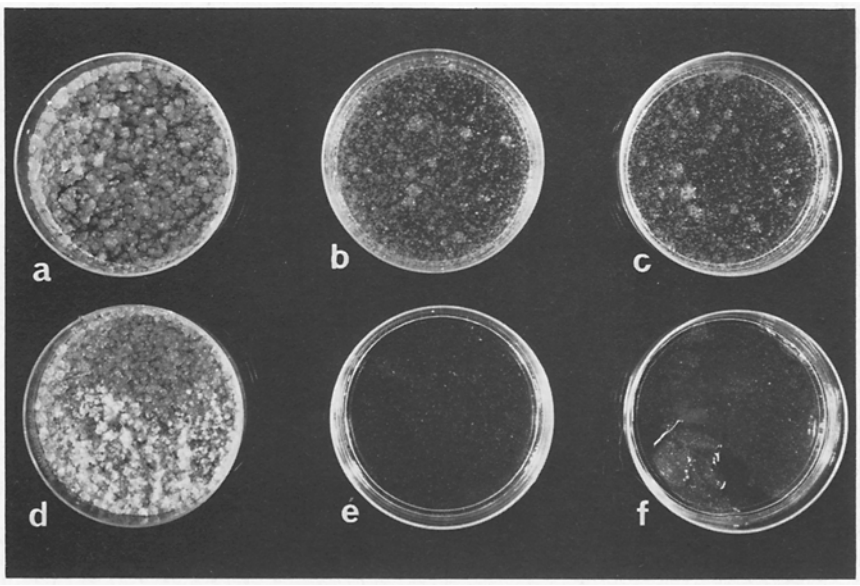

Fig. 7 a – f. Valine resistance of protoplasts derived from the selfed progeny (M_2) of a valine resistant plant. Plants from line Nt^{VR}-3 were selfed and protoplasts were obtained from a M_2 plant (**a, b, c**); in parallel protoplasts were obtained from wild type *N. tabacum* (**d, e, f**). The protoplasts were plated on control media (**a, d**) and on media containing 5 mM (**b, e**) or 10 mM (**c, f**) valine

Table 2. Characteristics of valine resistant variants derived from haploid *N. sylvestris* protoplasts. All M_1 calli showed high valine resistance (+ +) meaning normal growth in the presence of 8 mM valine; protoplasts from 9 M_1 plants, derived from valine resistant calli were either highly resistant (+ +) or partially (+) resistant to 4 mM valine; protoplasts from other 11 M_1 plants were not tested (n.t.). Seed resistance was evaluated in the presence of 0.25 to 2.0 mM valine; high resistance (+ +) and resistance (+) indicates normal seedlings in the presence of 1.0 and 0.5 mM valine respectively; sensitivity (–) indicates defective seedlings in the presence of 0.5 mM valine

Line designation	Callus resistance	Regeneration capability in presence of valine	Valine resistance of protoplasts from regenerated plants	Chromosome number of regenerated plants	Valine resistance in seed from regenerated plants
Ns^{VR}-1	+ +	yes	n.t.	24	–
Ns^{VR}-2	+ +	yes	n.t.	24	n.t.
Ns^{VR}-3	+ +	yes	n.t.	24	n.t.
Ns^{VR}-4	+ +	yes	+ +	24	+ +
Ns^{VR}-5	+ +	yes	n.t.	24	+
Ns^{VR}-6	+ +	yes	n.t.	24	–
Ns^{VR}-7	+ +	yes	n.t.	24	–
Ns^{VR}-8	+ +	yes	n.t.	12	n.t.
Ns^{VR}-9	+ +	yes	+ +	24	+
Ns^{VR}-10	+ +	yes	+	24	–
Ns^{VR}-11	+ +	yes	+	12	n.t.
Ns^{VR}-12	+ +	yes	+	24	–
Ns^{VR}-13	+ +	yes	n.t.	24	+ +
Ns^{VR}-14	+ +	yes	+	24	–
Ns^{VR}-15	+ +	yes	n.t.	24	n.t.
Ns^{VR}-16	+ +	yes	+ +	24	–
Ns^{VR}-17	+ +	yes	n.t.	24	–
Ns^{VR}-18	+ +	yes	+	12	n.t.
Ns^{VR}-19	+ +	yes	+	24	–
Ns^{VR}-20	–	yes	n.t.	24	n.t.

Plants regenerated from 3 resistant callus lines (Ns^{VR}-8, Ns^{VR}-11 and Ns^{VR}-18) retained the original haploidy. These as well as diploid plants regenerated from some other callus lines did not produce seeds. Plants of four callus lines (Ns^{VR}-4, Ns^{VR}-5, Ns^{VR}-9 and Ns^{VR}-13) produced seeds which germinated in the presence of 0.5 mM valine while plants from other 11 callus lines produced valine sensitive seeds. It should be noted that 0.25 mM valine is already toxic to germinating seeds of normal *N. sylvestris* plants. An additional experiment starting with the same number of U.V. treated Ns(n) protoplasts did not result in valine resistant plants.

Finally, diploid *N. sylvestris* protoplasts were tested as a source for valine resistant plants. Thus 4.16×10^6 Ns(2n) protoplasts were exposed to an U.V. dose of 625 erg · mm^{-2} and 1.53×10^6 out of 1.84×10^6 surviving protoplasts were further cultured and processed through the double selection system (4 mM and 8 mM valine). Only one callus was recovered which grew normally in the presence of 8 mM valine. This cell line retained its valine resistance and was regenerated to plants in the presence of 4 mM valine. The regenerated plants had 48 chromosomes and a tetraploid morphology. Seeds of these plants germinated normally at 0.25–0.5 mM valine but not at higher valine concentrations, hence were only slightly more resistant than normal *N. sylvestris* seeds.

Discussion

The results presented in this study contribute towards a better understanding of two basic aspects of angiosperm cell genetics, namely, the possible correlation between ploidy levels and U.V. sensitivity and the possible correlation between ploidy levels and mutagenesis. The ploidy levels dealt with in this study were haploid [Ns(n)] and diploid [Ns(2n)] *N. sylvestris* derived protoplasts and haploid [Nt(n)] and diploid [Nt(2n)] *N. tabacum* derived protoplasts respectively. The different genomes can also be presented as a aa ab and aabb respectively, where a and b represent different sets of 12 chromosomes. Several studies on the effect of X-rays and gamma rays on Solanaceae protoplasts were conducted (e.g. Galun and Raveh, 1975; Krumbiegel 1979; Magnien and Devreux 1980) but we lack information

on U.V. sensitivity in angiosperm protoplasts. Although in a few reports on isolation of metabolic mutants in plants U.V. irradiation was employed as mutagenic agent (Bourgin 1978; Christianson and Chiscon 1978; Berlyn 1980; Horsch and Jones 1980; Weber and Lark 1980; Negrutiu 1981) there are either no or only limited data on U.V. sensitivity per se. Moreover in all but two (Bourgin 1978; Negrutiu 1981) of these studies, cell suspensions with probably variable chromosome numbers, rather than mesophyll protoplasts, were used. Eapen (1976) reported a survival curve after U.V. irradiation of cell suspensions derived from dihaploid *N. tabacum* plants. The DL_{50} of the latter cells was about 4700 erg · mm^{-2}; the chromosome number of the cells deviated considerably from the dihaploid level. Bourgin (1978) found that about 0.5 survival of dihaploid *N. tabacum* protoplasts resulted from exposure to 1000 erg · mm^{-2} U.V. indicating a considerably higher U.V. sensitivity of protoplasts as compared to cell suspensions. Nugrutiu (1981) reported a similar U.V. sensitivity ($LD_{50} = 750$ erg · mm^{-2}) for haploid *N. plumbaginifolia* protoplasts. In this study we found that 0.5 survival of Ns(n), Ns(2n), Nt(n) and Nt(2n) protoplasts resulted from about 400, 600, 1000 and 3000 erg · mm^{-2}, respectively, thus confirming and extending Bourgin's findings (loc. cit.). Studies of Bourgin (1978), Christianson and Chiscon (1978) and Weber and Lark (1980) indicated an increase in mutation rate following exposure of the cells to U.V. irradiation, relative to control cells. A total of 8 distinct and verified valine resistant mutants were obtained in this study (4, 1 and 5 from Ns(n), Ns(2n) and Nt(n) protoplasts, respectively). No truly resistant mutant was derived from 1.8×10^7 U.V. irradiated Nt(2n) protoplasts.

The total number of protoplasts and derived verified mutants do not allow a statistical evaluation of mutation rates in the 4 *Nicotiana* protoplast systems, but the results are in conformation with two important assumptions; U.V. irradiation of angiosperm protoplasts increases mutation rates and mutagenesis is more efficient when haploid rather than diploid protoplasts are employed.

When U.V. irradiated Nt(n) protoplasts were exposed to valine selection only 5 out of 29 apparently resistant cell lines were finally verified as true valine resistant mutants. Similar results were obtained with the two other protoplasts systems. Thus, our findings re-emphasize the need for multiple stage selection to obtain metabolic mutants in angiosperm (e.g. Sung 1976; Bourgin 1978; Lawyer et al. 1980). It should be noted that some of our cell lines, which were secured after repeated screening for valine resistance but were not capable of differentiating into plants may be true mutants which lost the ability to regenerate functional plants (Vunsh 1981).

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