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Variation in Nuclear DNA Content of Isonicotinic Acid Hydrazide-resistant Cell Lines and Mutant Plants of *Nicotiana tabacum*

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Summary. Isonicotinic acid hydrazide (INH)-resistant lines of *Nicotiana tabacum* have been maintained in callus culture for six years and mutant plants have been regenerated from a number of these lines. This study examines variations in DNA content in nuclei of several of these callus cultures, regenerated plants, and secondary callus from the regenerated plants. The lines selected for study include three easily regenerated lines (I 21, I 24, and I 9) and two lines of poor regenerating capacity (I 1 and I 18). Two of the regenerating lines eventually led to fertile plants and the third produced only sterile plants. In general, the range of total nuclear variability was not as high as anticipated from other studies of long-term tobacco callus cultures. The majority of nuclei in all the distributions were between 3 and 20 pg, and the most frequently encountered distributions concentrated in the 7–18 pg region corresponding to 2–5C by our estimate of the C value for tobacco. Distributions were not identical for plants regenerated from the same culture simultaneously, and the nuclear DNA content of secondary callus cultures from one of the plants examined did not reflect the quantitative DNA pattern of the plant from which it was derived. The greatest degree of variability and highest DNA content for individual nuclei were observed in the primary callus of the poorly- and non-regenerating lines. The variability in DNA content was not associated with the INH-resistant trait.

Key words: *Nicotiana tabacum* – Tissue culture – Microspectrophotometry – Isonicotinic acid hydrazide-resistant mutants – DNA content

Introduction

The isonicotinic acid hydrazide (INH)-resistant lines of tobacco previously described (Berlyn 1980) were isolated from anther culture-derived suspensions following UV irradiation and have been maintained as callus cultures in parallel for six years. They vary in their ability and propensity to regenerate adventitious shoots and roots and, in some cases, in the appearance of the callus and, for the regenerated plants, in the morphology, viability, and fertility of the plants. Our interest in these lines is based on the inhibitory effect of INH on the glycolate pathway of photorespiration and potential effects of such mutants on photosynthetic efficiency. Therefore, the capacity of these lines to regenerate normal plants is an important feature of the lines, and we are interested in examining possible reasons for failure of some lines to regenerate. Because of the well-known variability in the ploidy levels observed in cultured plant cells and tissues (recently reviewed by Bayliss 1980; D'Amato 1977), we examined DNA content in a number of the INH lines and plants to see if their regenerative and morphological characteristics were correlated with nuclear DNA content and whether the INH-resistant trait was correlated with ploidy changes in these lines.

Materials and Methods

Isolation and Growth of Cultures

The selection and growth of the INH-resistant lines of *Nicotiana tabacum* L., var. 'John Williams Broadleaf' have been described previously (Berlyn 1980). The stock cultures of these lines have been maintained on a Murashige-Skoog medium containing the additions of Linsmaier and Skoog (1965) (LS-1 medium, Berlyn 1980) and 0.3 mg naphthaleneacetic acid (NAA)/l and 0.3 mg isopentenyl-aminopurine (2 iP)/l at 27 C under continuous light with 4–6 week transfers and with periodic transfer to test medium containing 1 mM INH. Regeneration of plants has also been described previously (Berlyn 1980). Secondary callus cultures examined in this study were initiated from sterile stem explants of the regenerated plants grown initially on the LS-1 medium modified by increasing the naphthaleneacetic acid content to 3 mg/l and subsequently maintained on LS-1 medium.

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Microspectrophotometry

Axillary bud or apical region tissue was dissected from the plants, and undifferentiated callus was taken from the cultures. Callus and plant material was fixed in Carnoy's No. 2 (Berlyn and Miksche 1976) for 1 h, hydrated through an alcohol series to distilled water and treated with 1% pectinase in 0.01 M sodium citrate buffer pH 6.0 (Berlyn et al. 1979) at 35 °C until tissue fragmentation was evident (2–6 h for different samples). This macerated tissue was placed on slides coated with chrome alum adhesive (Berlyn and Miksche 1976) and containing an area of chicken erythrocytes which served as an internal standard and control for variation in staining efficiency (Dhillon et al. 1977; Rasch et al. 1971). The Feulgen staining procedure described by Berlyn and Miksche (1976) was used; prior to staining, cells were hydrolyzed for 20 min in 5 N HCl at 25 °C and hydrolysis was terminated by repeated transfers of the slides to distilled water at 4 °C. The microspectrophotometric apparatus and the two-wavelength procedure were those described by Dhillon et al. (1977) and Patel and Berlyn (1982). The coefficients of variation for the erythrocyte nuclei ranged between 2 and 8 percent, both for variation within slides and variation between slides stained simultaneously. The tobacco spectrophotometric readings were normalized against the erythrocyte readings and picograms of DNA were calculated, based on a value of 2.8 pg/-nucleus for the chicken erythrocytes (Dhillon et al. 1977).

The non-parametric Wilcoxon Rank Sum Test was used to evaluate differences between distributions (Snedecor and Cochran 1967) because of the non-normal nature of some of the distributions.

Results

Examination of 69 nuclei of shoot apices of anther-derived control plants revealed a distribution (Fig. 1 g) which suggests a C value corresponding to a mean of 4.5 pg (±0.2)[1] for 51 nuclei and an apparent 2C value of 8.4 pg (±0.7) for 18 nuclei. For 55 nuclei of the shoot apex of a *Nicotiana tabacum* diploid (albeit of allo-tetraploid hybrid origin) control plant, the mean of the 49 apparently 2C nuclei was 7.8 (±0.3, Fig. 1a). A true diploid species, *Nicotiana sylvestris,* was also examined, and the mean DNA content for 30 nuclei was 4.4 pg (±0.6). The frequency distribution of DNA content of 69 nuclei of an early and sterile regenerated plant of the INH-resistant line I 21 is shown in Fig. 1 h. The largest frequencies occur between 2 and 4 pg and between 6 and 7 pg. The mean and SD for the entire distribution are 4.6 and 2.0 and the total range is 2.0–13.6 pg. A number of division figures were observed and their measurements are indicated in the Figure. Anaphases and telophases (1C division figures) were in the 2–5 pg range and prophases and metaphases (2C figures) were between 5 and 12 pg suggesting a heterogeneous population of hypodiploid nuclei, predominantly haploid and hypohaploid. A plant regenerated from callus of the same line a year later was weakly fertile and bore viable seeds which did not give rise to fertile plants. Although microspectrophotometric data are not available for this plant, its fertility indicates that at least some cells of the plant were diploid or nearly diploid.

Regenerated plants from the most extensively studied line, I 24, (Berlyn 1980; Zelitch and Berlyn 1982), like subsequent I 21 plants, set a modest number of viable and apparently diploid seeds. Examination of axillary buds from two of these plants which had developed in culture simultaneously and were transferred and transplanted in parallel indicated that in neither plant were all nuclei diploid. The two plants did not exhibit the same pattern of nuclear DNA content. For the plant designated P 39 (Fig. 1 k) 60% of the nuclei had values between 2 and 6 pg (predominantly haploid and hypodiploid values) and 27% measured 6–15 pg. For plant P 41 (Fig. 1 n), 55% of the nuclear values were between 9 and 13 pg and 31% were greater than 16 (the approximate 4C value for the diploid control).

Secondary callus cultures were obtained from these plants and another plant with the same development and maintenance history, and these secondary cultures were again maintained in parallel. After 3 years in culture, a comparison of the range of DNA content of nuclei of these cultures was made. As shown in Table 1 and Fig. 1 k–o, the plant P 41 in which most nuclei contained between 8 and 13 pg (2C–3C, in comparison with our controls) yielded a culture with nuclear DNA distribution similar to the parent plant (P 41) distribution; the mode of the callus culture was 9–10 and that of the plant 11–12. However, the predominantly haploid-hypodiploid plant, P 39 (Fig. 1 k, l) showed a large shift in nuclear DNA content in culture, with a distribution centered around 15–18 pg, approximately the 4C level, compared with the plant mode of 3–6 pg. Thus two plants which developed simultaneously in

Table 1. Comparison of sample nuclei from similar plants (see Fig. 1)

Plant	Sample size (n)	DNA content (pg)		
		Mean	Mode	SD
I 24 – P 39	84	7.1[a]	4.5	5.0
I 24 – P 41	67	14.9[a]	11.5	6.9
Haploid control	70	5.5[b,d]	4.5	2.0
I 21 – P 1	69	4.6[b]	3.5	2.0
I 9 – P 199	87	8.3[c]	5.5	3.3
I 9 – P 200	74	12.6[c]	12.5	5.3

[a,b,c] The distributions for these pairs differ at the < 1% level of significance by the Wilcoxon Rank Sum Test.
[d] The overall mean for the bimodal population

1 Values in parentheses represent the 95% confidence interval for the mean, unless otherwise indicated

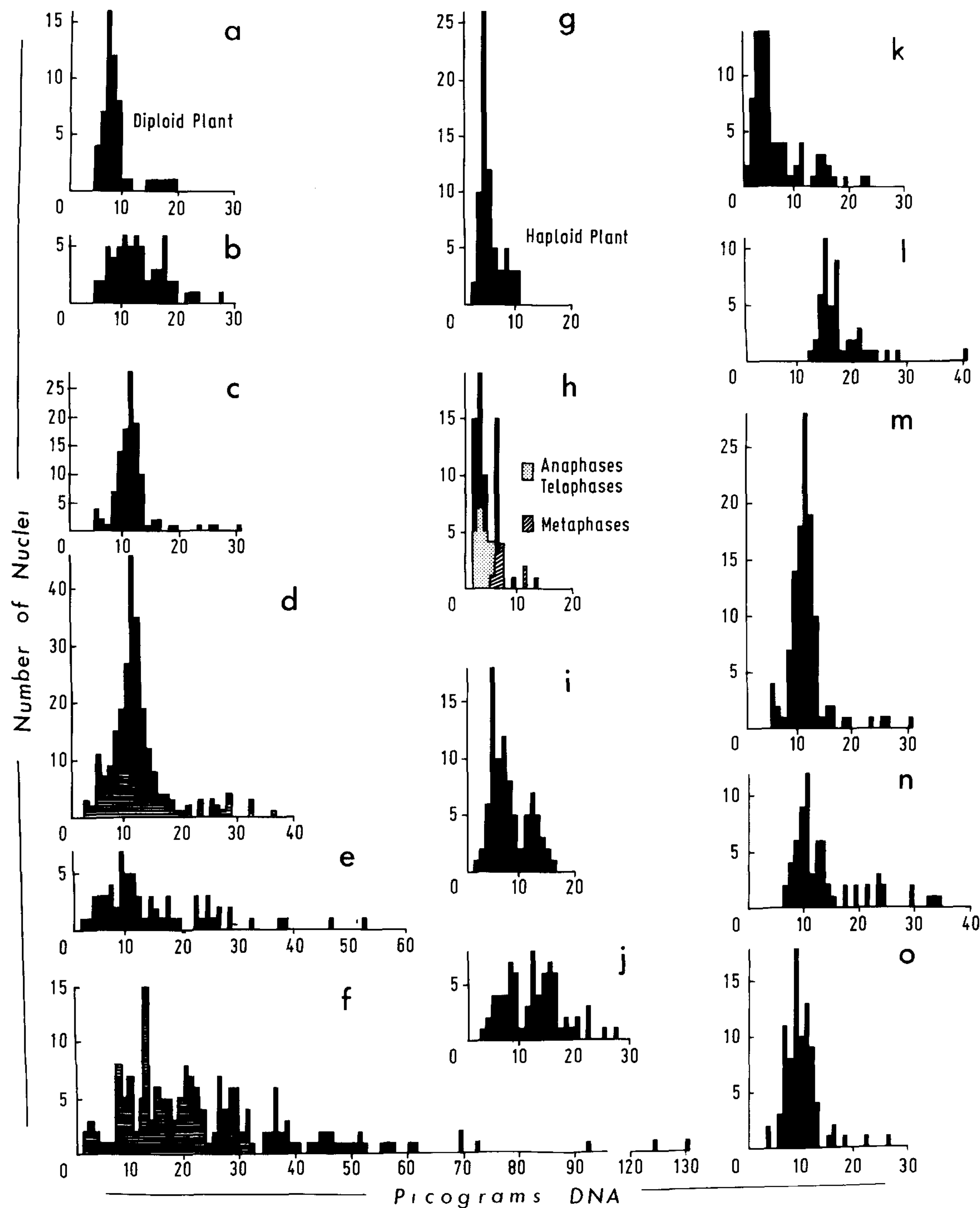

Fig. 1a–o. Frequency distribution of DNA content/nucleus, based on internal standards of chicken erythrocytes, 2.8 pg. **a–f** compares the distribution of a wild-type diploid plant with that of callus of wild-type and 4 different INH-resistant lines, with **e** and **f** representing poorly regeneration lines: **a** diploid control plant; **b** wild-type primary callus culture, approximate age 6 years; **c** secondary callus from a plant regenerated from the INH-resistant line I 24, approximate age 3 yrs; **d** primary callus from the resistant line I 9; stippled grey area represents callus of approximate age 4 yrs and black area represents callus of approximate age 6 yrs; **e** primary callus from line I 1, approximate age 4 yrs; **f** primary callus from line I 18, ages as indicated in **d**. **g–j** compares nuclei from the apical region of a haploid plant with those of plants regenerated 2–3 yrs after mutagenesis from 2 INH-resistant lines: **g** haploid control plant; **h** plant I 21–P 1, regenerated from INH-resistant line I 21; DNA content of division figures are shown; **i** plant I 9–P 199, regenerated from line I 9; **j** plant I 9–P 200, regenerated from I 9. **k–l** shows DNA distributions for nuclei of the apical region and axillary buds of plants and of secondary callus from line I 24, approximate age 3 yrs: **k** plant I 24–P 39, regenerated from line I 24; **l** secondary callus from plant I 24–P 39; **m** secondary callus from plant I 24–P 40; **n** plant I 24–P 41; **o** secondary callus from plant I 24–P 41

culture and were maintained in parallel had different patterns of nuclear DNA content and gave rise to secondary callus cultures which differed in the degree to which they reflected the DNA distribution observed in the original plant. The callus culture from a third plant in this group was very similar to the apparently more stable hyperdiploid explant (Fig. 1 m, o).

Many of the INH-resistant lines formed numerous small narrow-leaved shoots on culture media used for maintaining undifferentiated callus in wild-type lines, and they regenerated into plants with narrow leaves and multiple branching. The viable regenerated plants from such lines were usually sterile. The line I 9 showed these characteristics to a marked degree. Plants and callus cultures of this line were examined. Two I 9 plants were transplanted from callus culture approximately 28 and 30 months after the original mutagenized suspension cultures were transferred to solid medium. They were transplanted to soil at dates less than a month apart and were maintained in the greenhouse for 12 months prior to sampling of axillary buds for this study. In the younger plant, P 199, 68% of the nuclei were between 4–10 pg and 23% between 1 and the highest value of 16 pg (Fig. 1 i). For the other I 9 plant, P 200 (Fig. 1 j), a broader distribution, between 3 and 28 pg, was observed, with 61% of the nuclei having values above 11 pg, including 17 values above 16 pg. Table 1 indicates that these distributions are significantly different, as are the differences between plants P 39 and P 40. Callus from the original I 9 culture was sampled 4 and 6 years after the first transfer of the mutagenized suspension to solid medium. Figure 1 d contrasts the distributions for these two samples. The mean for 85 nuclei of the earlier sample is 12.7 pg (SD 7.3) and the mean for 161 nuclei of the second sample was 12.3, with a smaller SD, 3.4. Samples taken only 2 months apart did not appear to differ in their distribution (data not shown).

Two other callus lines were selected for study. The line I 18 has never regenerated shoots during maintenance for 6 years or on any of a number of media that have resulted in shoot formation for other lines (Berlyn 1980). Line I 1 very rarely formed shoots despite numerous attempts on regeneration media, and these shoots did not lead to a viable plant. The distributions of the nuclear values for these lines are compared with those of I 9, secondary callus of I 24, the diploid *N. tabacum* plant, and the unmutagenized parent culture in Fig. 1 a–f. Callus from I 18 was sampled after 4 and 6 years of maintenance on solid medium, as described for I 9, and I 1 was sampled only at the earlier time. For I 18 results for the two sampling times are indicated in the Figure and it can be seen that a wide range is observed for both samples; however, the earlier sample range was 2.6 to 42 pg and the later range was higher, 12 to 130 pg. Although in both I 1 and I 18 there are nuclei with approximately C and 2C values and also nuclei with DNA content in the 2C–4C range that predominated in the examined cultures capable of regeneration, the overall distributions for these lines are broader and are characterized by the occurrence of more highly hyperploid nuclei. The more highly disperse distributions for nuclei of the poorly regenerating and non-regenerating lines are obvious in Fig. 1 and are indicated by calculation of variances, recorded in Table 2.

Table 2. Comparison of nuclear DNA content of primary callus lines I 9, I 1 and I 18 with that of a wild-type plant, primary callus of the anther-derived wild-type culture, and secondary callus from the INH-resistant plant I 21 – P 40 (see Fig. 1 a – f)

Cell line (or plant)	Sample size (n)	DNA content		
		Mean	Range	Variance
(Wild-type plant)	55	8.8	5.8– 19.4	9.6
Wild-type callus (anther-derived)	87	13.5	5.4– 29.4	23.2
I 24 – P 40 secondary callus	114	11.7	5 – 30.8	13.7
I 9: 4 yr sample	85	12.7	3 – 36	53.3
6 yr sample	161	12.3	4.9– 32	14.1
combined samples	246	12.5	3 – 36	27.6
I 1	43	16.6	2.9– 57.9	174.2
I 18: 4 yr sample	100	16.4	2.6– 48.8	76.8
6 yr sample	65	36.5	12.1–130.5	637.8
combined samples	165	24.3	2.6–130.5	392.1

Discussion

Although the lines in this study have been in culture for 6 years, the ploidy levels were not as high as those reported in a number of other studies of long-term (3–17 yr) tobacco cultures (Fox 1963; Murashige and Nakano 1967; Traynor and Flashman 1981) and are more in line with the modest (aneuploid) increases in chromosome number observed in the 8-yr-old cultures of Sacristan and Melchers (1969) and the 1½-yr-old cultures of Shimada and Tabata (1967). The modes of the distributions in the INH-resistant lines examined were between 2C and 4C.

Our estimate of the C value for *N. tabacum* in this study is approximately 3.5–4.5 pg. A similar earlier microspectrophotometric examination of division figures led to an estimate of 3.5 pg (unpublished data). These values are somewhat lower but comparable to the recent 6 pg-equivalent estimate of Traynor and Flashman (1981), also based upon comparison with chicken erythrocytes. Using the diphenylamine method,

Siegel et al. (1973) found 10 pg DNA/(diploid) leaf cell. The value of 1.65 pg for the 1× level (single genome of the 4× allotetraploid) obtained from kinetic data by Zimmerman and Goldberg (1977) is also quite comparable to our estimated value. A more precise estimate could be obtained by interference microscopy but is not necessary for the comparisons which we are examining.

Variation in ploidy level in cultured cells has been examined from several standpoints. In a number of different systems it has been shown that hormone levels affect the degree of polyploidy of a culture (e.g., Torrey 1961; Bennici et al. 1971); stimulation of polyploid divisions was found with relatively high levels of kinetin, ca. 1 mg/l (Torrey 1961; van't Hof and MacMillan 1969) and also with 0.1 mg/l (Gosh and Gadgil 1979). However, in *Allium cepa* callus and regenerated roots, 2iP (0.04 to 40 mg/l) stimulated diploid divisions from mixoploid populations (Nandi et al. 1977), and 1 mg kinetin/l stimulated formation of aneuploid buds from tobacco callus (Brossard 1975). A study of regenerated tobacco plants by Ogura (1976) showed variation in chromosome number unrelated to growth regulator regimes, and Binns and Meins (1980) found no correlation between chromosome number and cytokinin habituation. Bayliss (1980) suggests that in carrot it is the degree of organization rather than the presence of 2,4-D that results in mitotic irregularities. Some studies have attributed changes in ploidy patterns to other cell conditions, e.g., callus vs. suspension growth (Singh and Harvey 1975; Bayliss 1977).

The INH-resistant variant lines are of common origin, were grown in suspension for only a brief period prior to mutagenesis, and have been maintained in parallel on agar medium with the same hormone supplement regime, namely 0.3 mg 2 iP/l and 0.3 mg NAA/l. Thus the variations in ploidy level in the different lines cannot be ascribed to differences in culture media or culture conditions.

Two of the anther-derived mutant plants regenerated early in the study, I 24–P 39 and I 21–P 1, had nuclei predominantly in the haploid region but showed enough variability in this region to suggest the presence of aneuploid (hypohaploid and hypodiploid) nuclei as well. In contrast to a simultaneously regenerated plant with predominantly 8–14 pg nuclei, secondary callus from the P 39 plant differed greatly from the original plant. The callus had a mode of 14–15 pg, approximately 4C, compared with the plant mode of approximately 1C.

A correlation between degree of aneuploidy and polyploidy and ability to regenerate plants has been reported for tobacco (Murashige and Nakano 1968; Zagorska et al. 1974) and for other plants (Muir 1965; Torrey 1967). Based on early studies of regeneration from mixed cell populations, it has been suggested that the regeneration process in some systems selects for diploid, or at least euploid, nuclei (Cf. Partanen 1965). The appearance of nuclei with much higher DNA levels in our poorly and non-regenerating lines is consistent with the idea that highly aneuploid lines have lost regeneration capacity. However, 2C nuclei are present in these populations and would presumably be available for selection in the regeneration process. Furthermore, axillary buds of the regenerated plants of other INH-resistant lines did not contain strictly euploid nuclei and in fact appear to be mixoploid. There have been other studies demonstrating aneuploid and mixoploid differentiation from tobacco (Sacristan and Melchers 1969; Sacristan and Lutz 1970; Ogura 1976) and other (e.g., Orton 1980) cultures. It appears that in our lines consistent selection has not occurred.

We are looking further at DNA content in developing shoots from some of these callus cultures. Our preliminary results indicate that in some instances a restriction in range of DNA content may occur in the developing shoot, but is not consistently maintained and that the pattern is not the same in all lines. Traynor and Flashman (1981) found a definite pattern of limitation in DNA levels during differentiation in 3 lines that they examined, but not in a fourth line. Similarly, dramatic differences were also reported between *Datura innoxia* suspension cultures and meristematic clusters within the cultures (Kibler and Neuman 1980). These studies, however, were dealing with callus and suspension cultures that had a much higher level of ploidy than the regenerating INH-resistant lines. Nuclei in meristematic cells at interphase have been shown to have the 2C DNA content (van't Hof 1974; D'Amato 1972, 1978). If this is the case in callus culture also, then in our lines there has been an increase above the normal 2C value (and certainly above the C value of the starting haploid line). However, it does not represent a large increase; most frequently observed classes are in most cases between 2 and 4C. This is true even for the non-regenerating line I 18. Nonetheless, I 18 does contain many nuclei with much higher DNA levels than those of most other cultures that we have examined, and an increase in the amount of DNA/nucleus was seen after an additional 2 years in culture. Murashige and Nakano (1967) reported a similar increase in chromosome number with time in culture, in variably aneuploid cultures of tobacco that they characterized as low morphogenetic potential lines.

There has been an increase not only in the mean amount of DNA/nucleus, but also in the variation in DNA content/cell in the populations of nuclei from the callus cultures compared with plant nuclei. This is most striking in the poorly regenerating lines. In fact, the distinguishing feature of both the non-regenerating line I 18 and the poorly regenerating line I 1 is a highly heterogeneous mixoploid population including nuclei with very high levels of DNA (Fig. 1 a–f, Table 2). It is possible that the variability per se rather than the actual DNA level may be an indicator of poor regeneration capacity. An assessment of uniformity of nuclei in the culture may predict whether a variant line has potential for regeneration into a mutant plant.

Because of all the genetic changes which occur in culture, some altered phenotypes might be expected to be due to karyotypic instability, and it is important to

show that genetic changes attributed to classical mutations are not in fact due to karyotypic changes. The differences in DNA content of cultures and plants in this study are not correlated with the INH-resistant properties of the cells (described by Berlyn 1980; Zelitch and Berlyn 1982). Although in tests of INH inhibition of mitochondrial extracts from callus of the I 24 plants P 39, P 40, and P 41 (Zelitch and Berlyn 1982), the lowest mean inhibition was observed for P 39 and the highest for P 41 (DNA profiles shown in Fig. 1 l, m, o), the difference between these mean values was not significant. Furthermore, in fertile seedlings of P 40 and among resistant segregants of backcross and F_2 progeny of another I 24 plant, all presumably diploid, the INH-resistant phenotype was detected in mitochondrial extracts. Therefore, the observed aneuploidy in culture is not responsible for the INH-resistant phenotype.

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