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Isolation and Characterization of Isonicotinic Acid Hydrazide-resistant Mutants of *Nicotiana tabacum*

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Summary. Twenty stable variant lines resistant to isonicotinic acid hydrazide (INH), an inhibitor of the conversion of glycine to serine in the glycolate pathway, were isolated in cell cultures initiated from allodihaploid *Nicotiana tabacum*. Plants were regenerated from 13 of these lines and explants were tested for resistance. For some lines virtually all of the regenerated plants scored as resistant; for others a mixed population of sensitive and resistant plants were obtained. One or more plants from 5 lines were fertile, presumably as a result of spontaneous diploidization of cells in the plant or culture. Callus initiated from the seed progeny of these plants was resistant to INH confirming the characteristic as a stable mutation. Seedlings from all INH-resistant plants were small and slow-growing, but the slow-growth trait could be separated from resistance in backcrosses of hybrids. In one case (line I21) crosses with sensitive lines show the resistant trait in that line to be dominant.

Key words: Isonicotinic acid hydrazide resistance – Glycolate pathway – *Nicotiana tabacum* – Somatic cell mutants

Introduction

Isonicotinic acid hydrazide (INH) inhibits the conversion of glycine to serine and CO_2 in the glycolate pathway in algae (Pritchard et al. 1963) and higher plants (Asada et al. 1965) and has been shown also to inhibit glycolate synthesis in tobacco leaf discs (Zelitch 1973). It is one of several inhibitors which (1) affect specific steps in this pathway and (2) are toxic to plant cells grown in culture. We are interested in isolating in cell culture and studying in culture and in regenerated plants mutations altering the enzymes in the glycolate pathway of photorespiration (Fig. 1) in order to understand and manipulate its regulation and to devise genetic blocks which will decrease photorespiration. It is important to determine whether permanent, genetic changes causing partial or complete blocking of photorespiration will lead to the levels of increased net photosynthesis observed temporarily after short-term chemical inhibition of the glycolate pathway reactions in leaf discs (Zelitch 1966, 1974). INH resistance may provide a class of mutants in which blockage of the glycine-serine conversion could be overcome by alteration of the enzyme complex catalyzing the conversion, or by increased use of alternate pathways of serine synthesis and glycine utilization. Such changes may affect glycolate synthesis or enhance viability of cells subsequently selected for total blocks in the glycolate pathway.

This report is concerned with isolation and genetic characterization of variant cell lines of tobacco which grow in the presence of INH and with the stability of the trait, regeneration of plants, and the occurrence of resistance in sexual progeny. The increasing number of mutants derived from plant somatic cell culture have been

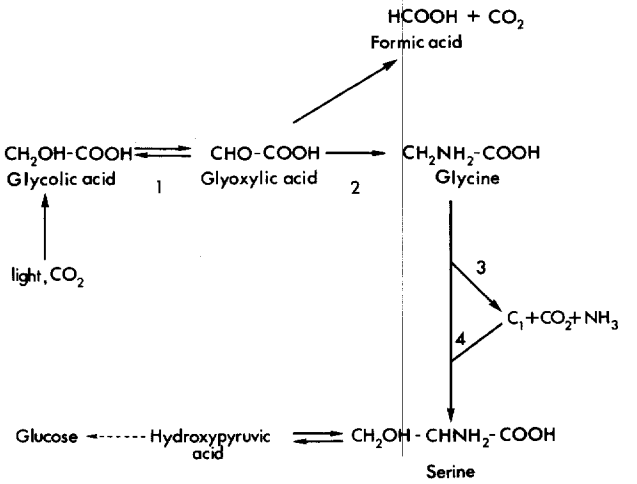

Fig. 1. The glycolate pathway. The enzymes shown are: *1* glycolate oxidase; *2* glutamate: glyoxylate aminotransferase; *3* glycine synthase; *4* serine hydroxymethyltransferase

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cited in many recent publications (e.g. Maliga 1978 and Widholm 1977). Only a few of these have been examined in subsequent seed generations (Bourgin 1978; Carlson 1973; Chaleff and Parsons 1978a, 1978b; Gengenbach et al. 1977; Maliga et al. 1975; Márton and Maliga 1975). We were able to examine a number of INH-resistant cell lines, plants regenerated from them, secondary callus cultures initiated from the regenerated plants, and progeny from those plants which were fertile. Results of our biochemical studies on these lines will be reported in a subsequent paper (Berlyn and Zelitch, in preparation).

Materials and Methods

Derivation of Cultures

The cultures used in this study were initiated from plants emerging from anther culture of the sulfur mutant of *Nicotiana tabacum* L. var. 'John Williams Broadleaf'. The sulfur mutant was characterized by Burk and Menser (1964) as heterozygous *Su/su*. Anther cultures give rise to white (*Su*) and green (*su*) haploid plants (Burk 1970). Callus from explants of the *su* plant was grown and used to make a cell suspension culture.

Culture Media

The media used were based on Linsmaier and Skoog (1965) medium (LS), which contains 0.55 mM inositol (100 mg/l) and 3 μM thiamin HCl (1 mg/l) in addition to the selected combination of hormones. For the major salts solution, Fe-EDTA solution of Kasperbauer and Collins (1972) was substituted in the LS medium or the Murashige-Skoog Plant Salt Mixture (Flow Laboratories) was used. Combinations of the hormones isopentenylaminopurine, naphthaleneacetic acid, and indoleacetic acid were added as indicated. LS medium containing 1.5 μM isopentenylaminopurine (0.3 mg/l) and 16 μM naphthaleneacetic acid (3 mg/l) will be referred to as LS-1 and that containing 1.5 μM isopentenylaminopurine and 1.6 μM naphthaleneacetic acid will be termed LS-2. Two percent sucrose and 1 percent agar were used in the media. The anther culture medium was that of Kasperbauer and Collins (1972). Cultures and regenerated plants on media were incubated at 28° under continuous light.

Irradiation and Incubation on Selective Medium

A suspension culture initiated from allodihaploid *Nicotiana tabacum* var. 'John Williams Broadleaf' was spread on petri plates of LS-1 medium at a density of approximately 1.5 g per plate and irradiated for 2 minutes with a Sylvania G8T5 germicidal lamp at a dosage of 3.3×10^5 ergs/cm^2/min. The plates were covered with aluminum foil for 12 hours, then incubated in the light for 16 days. Cells were transferred to LS-1 containing 10 mM isonicotinic acid hydrazide (INH; Eastman Organic Chem. Co.). After 1 week they were transferred to medium containing 1 mM INH or 2 mM INH or were further incubated on 10 mM INH-containing medium.

Quantitative Growth Tests

Quantitative comparisons of growth of selected and unselected lines on LS-2 medium with and without inhibitors were made by inoculating small pieces of callus (0.3-0.6 g divided into 7 or 8 pieces) onto plates of the test media, allowing growth for one 3 to 6 week passage, then re-inoculating and weighing samples of the callus (again, usually 7 or 8 pieces weighing 0.3-0.6 g per plate) for a second passage on the same medium. Weighed samples (0.3-1.0 g) of each culture used for inoculation and of each culture at the end of the passage were dried in a 110°C oven for 24 hours or more and the dry weight: fresh weight ratios were used to calculate increases on a dry weight basis. Weighed passages of 6-week duration or repeated 3-week weighed passages were routinely used.

Regeneration of Plants

Shoots appearing in cultures on LS-1 or LS-2 medium were transferred to jars of LS medium without hormones with 0.8% agar. Subsequent transfers were made as necessary to allow continued growth. If roots developed on this medium, the small plants were transferred to small pots of vermiculite, covered with plastic wrap, and grown under lights for 1 week or more. Plants were subsequently transferred either to a sand bench or to pots of peat moss: sand: vermiculite mixture. If roots did not develop on the hormoneless medium, the shoots were transferred to medium containing 0.57 μM indoleacetic acid (0.1 mg/l) and plants that developed roots on this medium were then transferred to pots. If shoots did not form on callus cultures on either of the standard media, large pieces of callus were inoculated onto plates of LS medium containing either 4.5 or 15 μM isopentenylaminopurine (0.9 or 3 mg/l) or 1.7 μM indoleacetic acid (0.3 mg/l) and 50 μM isopentenylaminopurine (10 mg/l). Shoots developing on these plates were subsequently transferred as described above.

Tests of Explanted Tissue from Regenerated Plant

Stem sections or leaf midrib sections were washed, cut into pieces of 1 inch or less, and surface sterilized by successive (1-3 min.) baths in 1 or 3% sodium hypochlorite, sterile water, 70% ethyl alcohol, and sterile water, 70% alcohol, and sterile water. Cross sections of about 1 mm thickness were cut and placed on LS-1 medium and incubated at room temperature. Callus which grew from these cut sections was transferred to the same medium for one passage, then to LS-2 medium and incubated in the light. After this passage, growth tests were made as described above.

Results

Isolation of Variant Cell Lines

After 6 weeks, small green outgrowths from the irradiated cultures on the 1 mM INH plates were observed. The remaining cells of the culture were colorless and showed no apparent growth. No growth was observed from the irradiated cells incubated on 2 or 10 mM INH; cells from these plates were transferred to medium lacking INH, incubated for 8 weeks, then transferred to medium containing 2 mM INH. After 3 months on INH-containing medium, small green outgrowths from these cultures were observed and transferred. Plates which were further incubated and inspected subsequently for new areas of growth did not yield any new isolates. Attempts to select cells on 6 or 25 mM INH were not successful.

A total of 58 isolates were obtained after incubation on the 1 and 2 mM INH-containing medium. Following growth on LS-2 medium, 41 of these isolates survived a second screening on INH. After several passages (at least 2 months) of growth in the absence of INH, these lines as well as several unselected (WT) lines serving as controls were again tested for vigorous growth in the presence of 1 mM INH. Twenty lines survived on INH-containing medium in these retests and were judged stably resistant and vigorous enough to allow further study. The WT lines turned brown or tan and appeared to grow slowly or not at all.

Test of Resistance of Regenerated Plants

Plants were obtained from 13 of the INH cell lines, but for 3 of these lines the plants did not grow well or did not survive greenhouse transplantation (Table 1). Explants from 51 of these regenerated plants and 6 control plants derived from unselected (WT) cultures were induced to form callus and the callus was tested for ability to grow on 1 mM INH (Table 1). Table 2 shows the quantitative results from plants from 2 of the INH-resistant lines compared with control plants. In the cases of I24 and I29, callus from all of the plants tested grew on INH and were scored resistant. None of the 6 control plants were scored as resistant, although one which originally showed sensitivity to INH became resistant after prolonged culture. For the lines I5, I9, and I16 a large proportion (40-60%) of the plants were scored as sensitive (Table 1).

Inheritance of INH Resistance

Although the original tissue was derived from anther culture, seed production occurred in plants regenerated from some of the lines, as indicated in Table 1. (Spontaneous diploidization may have occurred in cells of the original anther-derived plant, the suspension or callus cultures, or the regenerated plants.) Even for lines in which most of the plants produced seed, the seed set was generally poorer than in plants from the control lines. Seed germination tests on blotters with water containing 0, 1, 2, or 5 mM INH did not discriminate between plants that were sensitive or resistant in terms of callus growth. Therefore it was necessary to make tissue explants to test for resistance, and this procedure limited the number of plants which could be examined. Figure 2 and Table 2 show growth of callus from a seedling of I24 and of WT on media with and without INH, as well as similar data for regenerated plants. Germination and growth characteristics of seedlings originating from selfings and crosses involving these and other fertile culture-derived plants are shown in Table 3. INH resistance was observed in tested progeny of selfings of all five types of fertile INH-resistant plants and also in crosses between two INH-resistant plants. Crosses between WT and I21 result in resistant progeny, indicating that the resistant trait carried by I21 is dominant. The results for the crosses between I24 and WT indicated sensitivity in the passage shown in Table 3, but in the subsequent passage resistant growth was observed. Such a response was not observed in the sensitive WT line, and the

Table 1. Characteristics of plants regenerated fron INH resistant cell lines

Cell line designation	Number of plants regenerated	Survival to maturity	Number of plants tested for resistance	Number of resistant plants[a]	Number of seed-producing plants
I21	6	+	6	5	4
I24	15	+	8	8	11
I28	11	+	3	2	2
I29	13	+	3	3	0
I32	1	–	0	0	0
I42	4	–	0	0	0
I4	4	–	1	1	0
I5	8	+	3	1	0
I6	7	+	7	4	5
I9	10	+	10	5	1
I15	5	+	4	3	0
I16	19	+	11	6	9
I19	3	–	0	0	0
WT	3	+	3	0	2
WT-S[b]	(12)[b]	+	3	0	3

[a] Plants were scored as resistant if callus from explants showed growth on INH-containing medium which was 40% or more of the growth during the same passage on the standard medium lacking INH. Quantitative growth tests are described in Materials and Methods

[b] Plants from seeds of regenerated WT plants were also used as controls in these explant studies and these are designated WT-S and the number in this case represents seedlings

resistant lines were also consistent in both passages. This anomalous result for these hybrids was observed in four groups of plants tested at two different times. Further study is necessary on plants and callus of this type.

Morphological Abnormalities

It has often been observed that many of the plants derived from tissue culture have unusual morphological characteristics and that has been the case in this study. The plants regenerated from INH-resistant lines as well as seedlings from these regenerated plants showed slower growth and poorer root development than WT-derived plants and seedlings. Abnormalities in leaf morphology and growth habit were also observed in plants of the INH-resistant lines, but these traits were more pronounced in the regenerated plants than in subsequent generations of seed-derived plants. In preliminary cytological analysis, varying degrees of hypoploidy and hyperploidy were implicated for cells of some regenerated plants and callus cultures. However, no obvious correlation between the degree of ploidy abnormality and morphological traits of the plants is apparent. The occurrence of unusually narrow leaves and multiple adventitious shoots (characteristics which typify the plants regenerated from INH-resistant lines) in some of the plants of back-cross progeny may allow further investigation into possible relationships between chromosomal and morphological abnormalities.

The most striking morphological differences observed among seedlings of selfed and backcrossed plants is the seedling size difference indicated in Table 3 and illustrated in Figure 3. Seedlings from selfed plants of five different INH-resistant lines were all very small. When I24 and I21 plants were crossed with WT plants the seedlings were comparable to those of WT when germination and growth occurred on sterile media, but smaller following germination in soil. Size differences between WT/WT and WT/I24 or WT/I21 plants could also be observed in older seedlings after transplantation from sterile medium to soil.

In progeny of crosses between I21 and I24, complementation was not observed; only slow-growing plants were obtained (Fig. 3b). Backcrosses and selfings of the heterozygous traits resulted in segregation of the size traits. Crosses of I24/WT × WT or I21/WT × WT showed size segregations of 22 Large: 20 Medium: 0 Small for I24 and 13:16:0 for I21. Selfed hybrids segregated 5 Large: 13 Medium: 6 Small for I24/WT and 2:12:1 for I21/WT. These values do not deviate significantly from 1:1 and

Table 2. Explant growth data from plants of two of the INH-resistant lines and of unselected lines

Cell line designation	Plant designation	0 mM INH Factor of increase per passage[d]	1mM INH Percent of growth on standard medium
I24	P39	11	86
	P40	26	55
	P41	18	63
	P78	7	60
	P83	10	47
	P151	19	79
	P152	14	82
	P153	15	49
	P69	+[c]	+[c]
I24-S[a]	P165	14	63
I29	P76	17	80
	P77	8	65
	P185	15	35
WT	P4	30	22
	P18	11	11
	P154	17	20
WT-S[b]	P158	11	18
	P161	15	20
	P163	15	27

[a] Callus from a seedling from an I24 plant
[b] WT-S designation, as in Table 1
[c] Only qualitative observations were made in this case
[d] Calculated as ratio of harvest weight at the end of the passage/inoculum weight, calculated on a dry weight basis. The passages were of 6-week duration, and in most cases, the average for 2 determinations is given

1:2:1 ratios, respectively, in the χ^2 test. Callus tests of backcross progeny indicate that the resistant trait is segregating in plants of normal as well as small size.

Discussion

The ability to grow in the presence of 1 mM INH discriminated between 20 selected variant lines and several unselected lines in the original callus cultures and in similar cultures derived from plants regenerated from 10 of these lines. Fertile plants were obtained from 5 variant lines, and growth in the presence of INH was observed in callus from the first seed generation resulting from selfing of these plants (but not from seedlings similarly derived from control lines). This evidence implicates a stable genetic change as the effector of INH resistance in these lines. Plants from two lines were successfully crossed with the WT control plants as well as selfed. For the line I21 resistance appears to be a dominant trait. Cultures initiated from hybrids involving the I24 line were severely inhibited in growth during 2 passages on INH, but showed considerable growth during the third such passage. Whether this unusual result, not encountered in WT or other resistant lines, is due to an error in the method of testing or scoring (despite repeated tests on a number of different plants) or to a kind of adaptation characteristic of this line remains to be determined. Further clarification is necessary to establish whether resistance in this line is in fact recessive. An effect on growth rate, semidominant for seedlings germinated in soil, is also segregating. Preliminary analysis of backcrosses between hybrids and WT suggests that this mutant trait is segregating independently from the INH-resistant trait. Study of these crosses is continuing.

Several characteristics of the isolates as now observed, limit the attractiveness of these mutations for extensive genetic and physiological study. The level of resistance is low; thus the range of inhibitor concentration that distinguishes between WT and mutant phenotypes is very restricted. Lack of seed germination differences between WT and mutant on INH precludes easy classification of the germinating mutant plant; tests must be made on tissue cultures of explants of the mutant and control plants. Quantitative growth tests were helpful in ensuring that qualitative observations were not misleading, but such tests are cumbersome. These factors impose a limit on the size of the progeny which can be tested. Abnormalities in leaf morphology and plant size of the regenerated mutant plants and first seed generation did not allow straightforward comparisons of photosynthesis and photorespiration in these plants.

Several possibilities exist for overcoming these limita-

Table 3. Callus resistance tests and seedling characteristics of progeny from crosses involving INH-resistant plants

Cross	Seedling characteristics		Selective growth ratio[a] in callus resistance tests			Score[c]
	In soil	On media	Mean	(S.E.)	Number of plants[b]	
WT selfed	Large	Large	0.16	(0.03)	3	S
I21 (P62) selfed	Very small	Very small Poor roots	0.63	–	1	R
I24 (P78, P40, P83, P153) selfed	Small	Small	1.2	(0.6)	3	R
I28 (P75, P80) selfed	Very small	Small	0.57	–	2	R
I16 (P43, P67) selfed	Very small	Small Poor roots	0.61	(0.20)	2(4)[b]	R
I6 (P81, P50, P28) selfed	Very small	Small Poor roots	0.50	(0.04)	3(5)[b]	R
WT × I21 (P62)	Small	Large	0.44	(0.08)	4	R
I21 (P62) × WT	Small	Large	0.58	(0.13)	5(6)[b]	R
I21 (P62) × I24 (P78)	Very small	Very small	0.68	–	1(2)[b]	R
I24 (P78) × I21 (P62)	Very small	Small Poor roots	0.89	(0.27)	2(4)[b]	R
WT × I24 (P78)	Small and very small	Large	0.18	(0.02)	10(11)[b]	S[d]
I24 (P83) × WT	Small	Large	0.28	(0.03)	4	S[d]

[a] The ratio of growth on medium with 1 mM INH/growth on standard medium. Growth ratios calculated on a dry-weight basis (Materials and Methods)

[b] The numbers in parentheses indicate the number of growth tests made in cases in which more than one independent test was made on callus from the individual plants indicated

[c] S = sensitive; R = resistant

[d] The result in this passage is scored as sensitive; however, in subsequent passages these cultures gave a uniform resistant response; this contrasts with the consistency in scoring observed for subsequent passages of the other R and S cultures

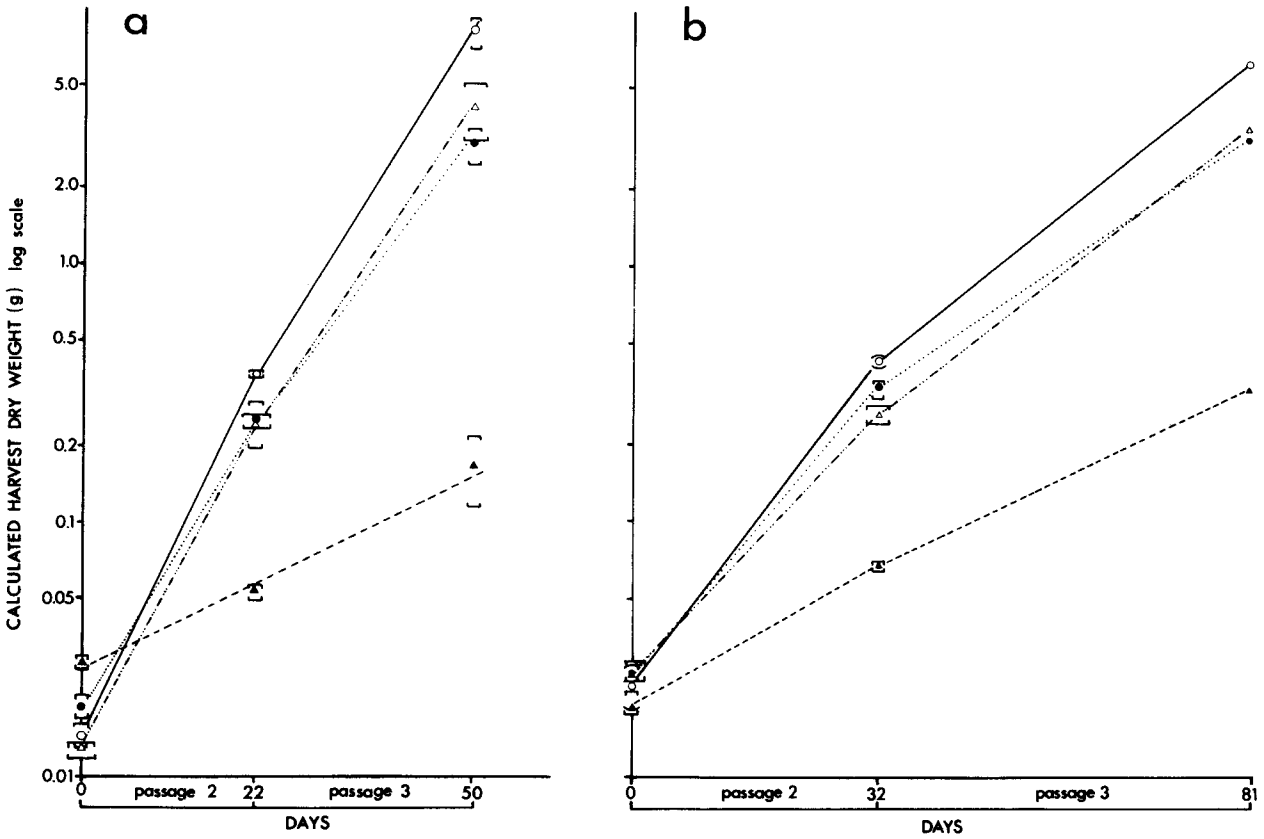

Fig. 2a and b. Graphic illustration of calculated growth rate curves during 2 sequential passages of callus from **a** seedlings and **b** regenerated plants, derived from WT lines on media with (▲) and without (●) INH (1 mM) and from the I24 line on media with (△) and without (○) INH (1 mM). These do not represent growth rate curves since long passages were used and determinations were limited to 2-3 passages. Growth rate experiments indicate that a lag in growth rate occurs on the standard medium after 3-4 weeks; thus the slope of the total growth line in (**b**) and in passage 3 of (**a**) reflects the length of the passage rather than the maximum growth rate. Brackets indicate standard errors of the mean of triplicate determinations. After the final passage in (b) the 3 plates were combined prior to sampling and weighing, so that standard errors could not be calculated

tions. It may be possible to obtain from a second cycle of selection mutants showing higher levels of resistance. In lines in which enzymatic differences can be demonstrated in both callus cultures and leaves, direct classification of the mutant type may be made in the intact plants (Berlyn and Zelitch, manuscript in preparation). The morphological traits appear to segregate independently from the resistant trait, so that comparative photosynthetic and photorespiratory measurements of selected progeny in subsequent generations will be possible. Because of the small size of 5 different INH-resistant lines, it appears that the plant size characteristic represents an independent mutation that occurred very early in the culture, prior to mutagenesis and selection.

The regeneration of resistant and non-resistant plants from I6, I9, and I16 (Table 1) suggests heterogeneity in the callus cultures of these lines. Since they were not cloned by protoplast isolation and selection, such heterogeneity may have been present in the early isolates.

The well-known observations of high ploidy levels and frequent aneuploidy occurring in cell and tissue cultures (d'Amato 1975; Sunderland 1977) as well as recent results on habituation in plant tissue cultures (Meins and Binns 1977) raise questions about the nature of phenotypic changes observed. Although gross chromosomal (rather than specific gene) changes cannot be ruled out as the cause of the observed phenotype of INH resistant lines, the changes responsible for the observed characteristics appear not to be random changes arising from the culturing process and unrelated to the selections imposed, since unselected cultures do not exhibit these phenotypes. Our preliminary cytological studies on the callus cultures and regenerated plants have revealed variation in ploidy levels but no obvious correlations between ploidy variation and observed phenotypic characteristics. There is nothing in our evidence to date that would negate the assumption of a stable gene mutation, suggested by the regular transmission of the trait through seed. It is hoped that in at least

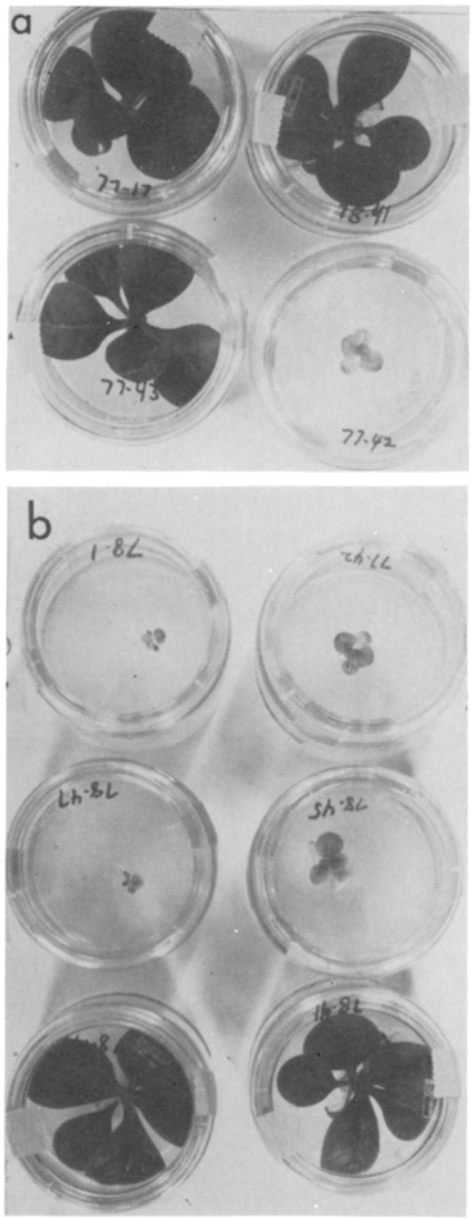

Fig. 3a and b. Size differences observed in seedlings from selfings and crosses. a Seedlings growing on sterile media. Clockwise from upper left: WT/WT; WT/I24; I24/I24; I24/WT. b Seedlings growing on sterile media: left column I21/I21; I21/I24; I21/WT. Right column: I24/I24; I24/I21; WT/I24

some of the mutants, ongoing enzymological studies may provide evidence for direct effects on primary gene products.

The relevance of INH-resistant mutants to glycolate pathway regulation depends on the mechanism of resistance. Cells that are resistant because of changes in glycine or serine synthesis, accumulation, or metabolism are of most interest; those resistant because of altered INH uptake or metabolism are less interesting. Evidence for similarity of uptake and metabolism of INH in resistant and sensitive lines and evidence for a higher level of resistance of glycine synthase to INH inhibition in mitochondrial preparations from some of the lines (e.g., I24) will be presented in a subsequent paper (Berlyn and Zelitch, in preparation).

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