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Selection of amitrole tolerant tobacco calli and the expression of this tolerance in regenerated plants and progeny

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Summary. Thirty-one clones capable of growth in the presence of 1.9×10^{-4} M amitrole (3-amino-1,2,4-triazole) were isolated from non-mutagenized cell suspensions of haploid *Nicotiana tabacum* cv. 'Wisconsin 38' plants at a frequency of 2.5×10^{-8}. Seven clones retained tolerance when grown on selective medium for three years. When clones were cultured in the absence of amitrole, tolerance persisted for 9 months in five clones. Some plants regenerated from three amitrole-tolerant clones were tolerant. Seven amitrole-tolerant clones were isolated from diploid *N. tabacum* cell suspensions and R plant tolerance was followed through two sexual generations. Simple Mendelian inheritance patterns were not observed.

Key words: Amitrole – 3-amino-1,2,4-triazole – Cell culture – Herbicide tolerance

Introduction

The development of herbicide-tolerant plants could enhance the benefits of herbicides. Selecting for tolerance to a biodegradable herbicide that leaves no active soil residue and is not toxic to wildlife or humans would minimize potential damage to the environment. In addition to the agronomic implications, selecting for herbicide tolerance may be valuable in elucidating the mechanism(s) of action which is not fully understood for many herbicides (Ashton and Crafts 1981).

Plant somatic cell genetics has tremendous potential to supplement conventional breeding programs by providing unique or new genotypes for utilization by plant breeders (Davies 1981; Thomas et al. 1979; Vasil et al. 1979). Cell culture is valuable in screening for variant types (Chaleff 1981; Handro 1981; Maliga 1980) and the applications of such methods in the search for herbicide tolerance have recently been reviewed (Hughes 1983; Meredith and Carlson 1982). Several successes indicate that cell culture selection schemes for herbicide-tolerant plants are viable (Chaleff and Parsons 1978; Miller and Hughes 1980; Radin and Carlson 1978; Thomas and Pratt 1982).

Amitrole (3-amino-1,2,4-triazole) is a broad-spectrum, biodegradable herbicide with a short soil half life (Campacci et al. 1977). It is toxic to non-photosynthetic cultured cells and is thus well suited for selection at the cellular level (Barg and Umiel 1977). Additionally, tolerance in plants can be scored by the extent of chlorosis in new growth. Amitrole has several proposed sites of action (Fedtke 1982), and natural resistance to a herbicide with multiple sites of action should arise more slowly than to single-site herbicides (LeBaron and Gressel 1982).

We report here the selection of amitrole-tolerant clones from haploid and diploid cell suspensions of *Nicotiana tabacum* cv. 'Wisconsin 38' and the expression of this tolerance in calli, regenerated plants and their progeny.

Materials and methods

Cell cultures

Callus cultures were obtained from pith of haploid and diploid *Nicotiana tabacum* cv. 'Wisconsin 38'. Haploid plants were derived from anthers of *N. tabacum* cv. 'Wisc. 38' and the two used in this study were designated H1 and H2. Callus cultures were maintained in the dark at 25 ± 1 °C on Linsmaier and Skoog (1965) medium supplemented with kinetin (0.1 mg/L), α-napthaleneacetic acid (2.0 mg/L), sucrose (4%), and agar (0.8%) (LS medium). Friable callus was subcultured in liquid LS medium (LS medium without agar) until a fine suspension of cells resulted. Suspension cultures were maintained on a gyratory shaker (140 rpm with a 2.5 cm stroke diameter) at a temperature of 26 ± 2 °C with 14 h of light per day. Shoot regeneration medium consisted of Linsmaier and Skoog (1965) medium supplemented with benzyl amino

purine (1 mg/L), sucrose (4%), and agar (0.8%). Shoots were regenerated at a temperature of 26±2 °C with 14 h of light per day.

Selection scheme

Flasks containing 50 ml of liquid LS medium supplemented with 1.9×10^{-4} M amitrole were inoculated with 3.3 or 5.0 g fresh weight of H2 cells. A total of 73 g fresh weight was inoculated into 19 flasks and there were approximately 2.7×10^{6} cells per g fresh weight. After 5 weeks cells were suspended in LS medium at 40 °C containing 1.9×10^{-4} M amitrole and poured over solidified medium of the same composition. After 2 months, growing colonies were considered tolerant and transferred to fresh medium containing herbicide. Each colony was assumed to be a clone and was designated by a letter indicating the selection flask and a number, as the inoculum from some flasks gave rise to several colonies. Five flasks gave one or more clones and variant frequency was based on the assumption that all clones that arose in a herbicide-treated flask of cells originated from a single cell. Regenerated variants were referred to as R plants following the nomenclature of Chaleff (1981). A similar selection scheme was applied to diploid suspension cultures, but clones were identified by a number. Unless otherwise indicated, all results and discussion refer to the haploid selection.

Callus growth determination

Between 3 and 4 weeks after subculturing, 50 mg pieces of healthy callus were transferred to LS medium containing varying concentrations of amitrole (gift from Amchem or purchased from Sigma Chemical Co.). Each concentration had a minimum of three replicates. Fresh weight and, in some cases, dry weight (calli dried for 20 h at 110 °C) were recorded after a period of 4 weeks. Growth was defined as (W-Wo)/Wo where Wo equals initial fresh weight and W equals 4 week fresh weight. Percent relative growth, defined as the growth in the presence of herbicide divided by growth on medium without herbicide times 100%, was used as a measure of tolerance. A percent relative growth of 100% indicates equal growth on both media.

Maximum tolerance levels

Approximately 100 mg pieces of callus were grown on media containing amitrole concentrations higher than the selective concentration. The outline of each piece was traced on the bottom of the petri plate at 0 and 4 weeks. The maximum concentration permitting a visible increase in size was recorded.

Whole plant tolerance assay

Some regenerated plants from the haploid selection were vegetatively propagated to allow repeated testings and these groups were referred to as subclones. When plants were approximately 10 cm high and had four leaves between 10 and 15 cm long, 6×10^{-6} moles of amitrole in a 3% tween 20 solution were spotted on a single leaf. Treated plants were maintained in a growth chamber with 12 h of light daily. Expanding leaves of wild-type plants exhibited severe chlorosis after 2 weeks. Any plant with significantly reduced or no chlorosis after 2 weeks was considered tolerant. Tween 20 had no visible effect when spotted on wild-type leaves. For many R plants, leaf tissue was cultured on LS medium and resulting calli were tested for tolerance as described above. Amitrole tolerance was measured in regenerated plants and progeny from the diploid selection in a similar manner.

Cytological evaluation

Shoots from several plants regenerated from clones X2 and Y2 were rooted. These root tips and those from some R plant progeny were processed and evaluated according to the procedures of Collins (1979) to determine chromosome number.

Results

Variant frequency

Initially 31 amitrole-tolerant clones were isolated from 19 flasks of haploid cells with five flasks yielding one or more clones. The initial variant frequency for the haploid selection was estimated to be 2.5×10^{-8}.

Callus growth controls

Fresh weight growth of H2 callus was totally inhibited by 1.9×10^{-4} M amitrole, and dry weight followed the same trend (Fig. 1). Similar results were obtained with H1 and diploid callus cultures (data not presented). These data indicated that growth calculations based on fresh weight were justifiable. Tolerance of variant tissue was determined at 1.9×10^{-4} M amitrole and any callus clone yielding a percent relative growth greater than or equal to 20% was considered tolerant. Isolated variant

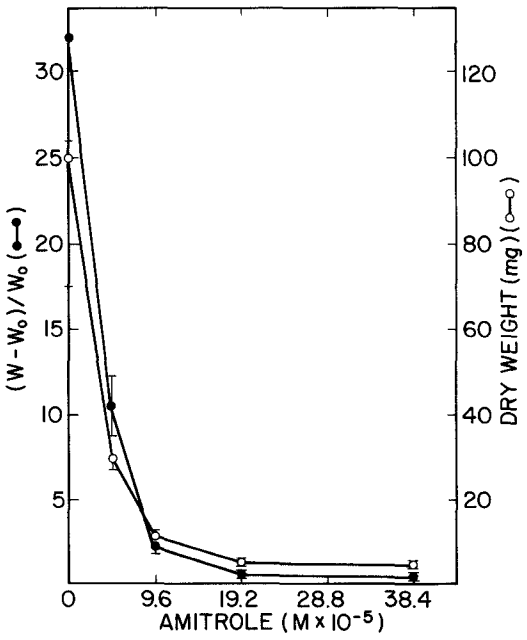

Fig. 1. Effect of amitrole on H2 callus growth. Each point represents the mean of 12 replicates ± SE

Table 1. Differences in growth rates among tolerant clones

Clone	Growth	Doubling time (days)
U1	25±3	6
U12	7±2	9
U16	8±3	9
X2	14±2	7
Y2	7±1	9
Y3	7±2	9
Y4	14±4	7
Control (H1)	65±6	5

Callus was tested on LS medium. Each growth value represents the mean of 3 replicates ± SE

Table 2. Stability of amitrole tolerance in culture

Clone	Months cultured on nonselective medium	% Relative growth ± SE (n=3 or 4)
X2	0	70±10
X2	9	74±12
X2	16	5± 0
U12	0	100±15
U12	9	22± 2
U12	16	1± 0
Control (H2)	18	2± 0

Test medium contained 1.9×10^{-4} M amitrole

clones and control cultures grew at different rates (Table 1). For this reason percent relative growth was used to quantify tolerance, since direct comparisons among clones which varied in growth rate could lead to inaccurate conclusions.

Stability of tolerance in callus cultures

Only seven of the 31 initially tolerant clones retained tolerance when grown on selective medium for three years (Fig. 2). Growth of these seven amitrole-tolerant clones cultured on nonselective medium for 9 and 16 months was compared with growth of tissue continuously maintained on amitrole containing medium. Clones fell into two categories as illustrated by representative data in Table 2. Tolerance was unaffected (X2 and Y2) or tolerance decreased (U1, U12, U16, Y3 and Y4) after a 9 month absence of selective pressure. All clones except U16 and Y4 were tolerant after 9 months, but none were tolerant after 16 months of subculturing away from amitrole. Tolerance of all clones from the diploid selection was significantly reduced or eliminated after lengthy culture passages away from amitrole.

Maximum levels of tolerance

Clone X2 grew at a maximum concentration of 2.8×10^{-4} M amitrole, while Y2 grew at 5.6×10^{-4} M amitrole. U1, U12, U16 and Y4 tolerated 9.5×10^{-4} M amitrole. Y3 grew at an amitrole concentration ten-fold higher than the selective concentration.

Whole plant tolerance

Plantlets were regenerated from six amitrole-tolerant clones. Healthy plants were obtained from five of these

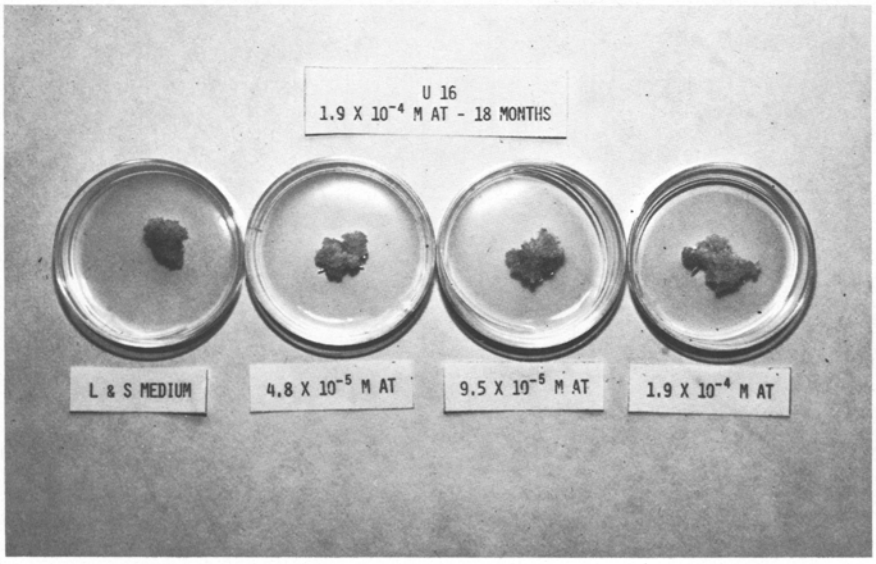

Fig. 2. Growth of amitrole tolerant variant U16 in the presence or absence of amitrole after growing in the presence of 1.9×10^{-4} M amitrole for 18 months. Each callus initially weighed 50 mg. Picture taken after four weeks

Table 3. Whole plant tolerance

Clone	No. subclones tested	No. tolerant subclones
U12	1	0
U16	5	3
X2	9	1
Y2	6	1
Y4	3	0
Regenerated controls[a]	5	0
Wild-type controls	72	3

[a] Plants were regenerated from H1 and H2 cells which had not been exposed to amitrole

clones and tolerance was expressed in some plants from clones U16, X2, and Y2 (Table 3). Since chlorosis appears only in expanding leaves, a plant that fails to grow would appear tolerant. For this reason several plants from a subclone were screened when possible. All plants had to pass the amitrole screen for the subclone to be considered tolerant. Three wild-type plants or 4% of the plants tested showed reduced chlorosis and were considered tolerant. Thus, the whole plant tolerance assay may have as much as a 4% internal error in identifying tolerant plants.

Tolerance in tissue from R plants

Tissue capable of growth in the presence of 1.9×10^{-4} M amitrole was isolated from some sensitive plants (Table 4). Table 5 summarizes the relationships observed among stability of tolerance in culture, tolerance in R plants and tolerance in tissue from the R plants. The observation that tissue from tolerant plants was sensitive was unexpected. Similar results were observed when tissue was isolated from plants regenerated from six diploid clones that were amitrole tolerant. Tissue from one tolerant plant (9) yielded tolerant tissue, but all other plants yielded sensitive tissue.

Heritabiliy of tolerance

Amitrole-tolerant plants and plants yielding tolerant tissue flowered but were not fertile. Ploidy levels were determined for plants regenerated from clones X2 and Y2 because tolerance was stable in culture and tolerant plants or plants with tolerant tissue had been obtained. Most plants regenerated from clone X2 appeared to be haploid since their cells contained approximately 24 chromosomes; however, cells from plant X2-8 contained about 34 chromosomes. Y2-1 may be chimeral since some cells contained about 24 chromosomes and others contained about 37. Attempts are now being made to produce fertile plants. R plants from the diploid selection were followed through two sexual generations, but simple Mendelian inheritance patterns were not observed (Table 6). Although half of the progeny of selfed variant 1 plants and many of the progeny of backcrossed variant 1 plants were tolerant (Fig. 3), tolerance vanished when tolerant R1 and F1 plants were selfed. Cells of one R1 plant from variant 1 contained approximately 40 chromosomes, while cells from others contained up to 73 chromosomes. Chromosome numbers in R2 plants of variant 1 ranged between 30 and 70. Variability in the chromosome number of plants regenerated from tissue culture is well-known (Bayliss 1980; D'Amato 1978).

Table 4. Tolerance of R plant tissue

Subclone	% relative growth ± SE
U12-1	0±0
U16-1	2±1
X2-2	42±5
X2-3	6±1
X2-7	75±8
Control[a]	1±0

Callus was tested on medium containing 1.9×10^{-4} M amitrole. Tolerance was tested several times between 2 and 9 months after tissue isolation, but the level of tolerance did not change

[a] Tissue from a plant that was regenerated from H1 cells which had not been exposed to amitrole. Similar results were obtained with tissue from 9 such plants regenerated from H1 and H2 cells

Table 5. Summary of amitrole tolerance

Subclone	Stable tolerance in culture	R plant tolerance	R plant tissue tolerance
U12-1	Yes	No	No
U16-1	No	Yes	No
U16-2	No	Yes	No
U16-6	No	No	No
U16-7	No	Yes	No
U16-8	No	No	No
X2-1	Yes	No	Yes
X2-2	Yes	No	Yes
X2-3	Yes	No	No
X2-4	Yes	No	Yes
X2-5	Yes	Yes	No
X2-6	Yes	No	Yes
X2-7	Yes	No	Yes
X2-8	Yes	No	Yes
Y2-1	Yes	Yes	No
Y4-1	No	No	No
9[a]	Yes	Yes	Yes
H1-1 Control	No	No	No
H1-2 Control	No	No	No

Stability of tolerance in culture was based on a 9 month subculturing on nonselective medium

[a] From the diploid selection

Table 6. Whole plant tolerance in progeny from diploid selection

Variant	Selfed		♀ Variant × ♂ wild-type		♀ Wild-type × ♂ variant	
	Tol R1(*n*)	Tol R2(*n*)	Tol F1(*n*)	Tol F2(*n*)	Tol F1(*n*)	Tol F2(*n*)
1	13 (24)	1 (36)	11 (54)	1 (76)	8 (96)	0 (76)
6	4 (67)	4 (80)	5 (46)	3 (59)	0 (4)	–

R plants were selfed or backcrossed. Tolerant R1 and F1 plants were selfed. *n* = the number of plants tested

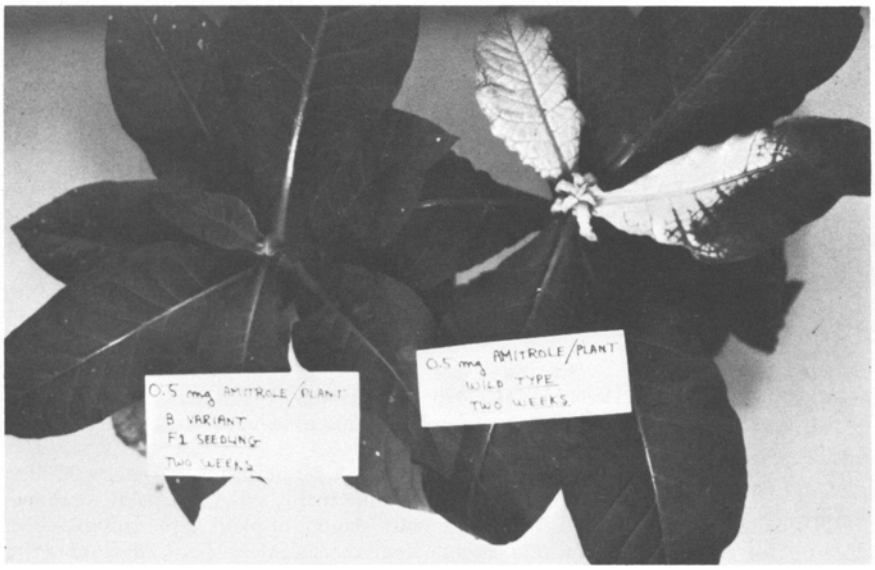

Fig. 3. Amitrole tolerant F1 plant. Plant on the *left* is a variant 1 (initially called variant B) F1 seedling which showed no chlorosis two weeks after being exposed to 6×10^{-6} moles of amitrole. Plant on the *right* is a wild-type plant showing extensive chlorosis after two weeks

Discussion

Tolerance with varying degrees of stability was obtained in both selections. Of 31 tolerant clones from the haploid selection, seven still retain the trait after three years on selective medium. Tolerance persisted in five of these clones after a 9 month absence of selective pressure, but none of the clones tested after 16 months were tolerant. Individual clones gave rise to both tolerant and sensitive plants as well as plants with sensitive tissue and plants with tolerant tissue.

In other reported selections for herbicide tolerance via cell culture, tolerance with varying degrees of stability has been found (Aviv and Galun 1977; Chaleff and Parsons 1978; Miller and Hughes 1980; Radin and Carlson 1978; Thomas and Pratt 1982). After a minimum of 4 weeks of growth on herbicide-free medium, 87 of 140 paraquat-tolerant tobacco clones isolated by Miller and Highes (1980) retained tolerance to paraquat. Only 15 of 43 plants regenerated from paraquat-tolerant clones were tolerant; however, tissue isolated from all but a chimeral R plant exhibited some level of tolerance. Simple Mendelian inheritance was not observed (Hughes, personal communication). Thomas and Pratt (1982) isolated 19 paraquat-tolerant clones from tomato cultures. There was evidence of a stable genetic basis for tolerance for three of the clones. Paraquat tolerance was observed in callus of plants regenerated from six of nine tolerant clones, but whole plant tolerance was evident for only one clone. In both paraquat selections, with minor exceptions, all R plants from a single clone were either tolerant or sensitive and isolated tissue from each clone was either sensitive or tolerant. This was not true for the amitrole-tolerant clones where significant variability was evident among R plants from single clones. Of 144 putative herbicide-tolerant mutants isolated by Radin and Carlson (1978) only ten had a stable genetic basis. Chaleff and Parsons (1978) isolated seven picloram-tolerant tobacco clones and simple Mendelian inheritance was reported for four of these. These studies indicate that only a small proportion of the initially tolerant clones will retain tolerance and have a stable genetic basis.

There is more than one possible explanation for the loss of tolerance in culture. A plausible explanation is that plant cells grow in aggregates and a wild-type cell could be protected by nearby tolerant cells. In the absence of selective pressure wild-type cells would outgrow variant cells (Table 1). Alternatively, tolerance may arise from an epigenetic rather than a genetic change (Chaleff 1981). These and other possibilities for tolerance expression and instability have recently been discussed (Chaleff 1981, 1983; Larkin and Scowcroft 1981).

It is not surprising that tissue from some regenerated plants was tolerant while the plant was sensitive since cultured cells and whole plants are at very different developmental stages. However, when tolerance is selected for at the tissue level, it seems unlikely that tolerance should be lost at this level and yet evident in the whole plant. All but one of the amitrole-tolerant plants tested yielded sensitive tissue. This whole plant tolerance did exhibit some stability since plants vegetatively propagated from tolerant plants expressed tolerance.

In the diploid selection tolerance is a dominant trait since it is expressed in heterozygotes. Simple Mendelian inheritance patterns in crosses, however, were not observed. Non-Mendelian segregation ratios, and the loss of tolerance in R2 and F2 plants may be explained by aneuploidy and irregular chromosome transmission through meiosis.

Our study has shown that the level of whole plant tolerance to amitrole can be increased by employing tissue culture procedures. Only a slight increase in tolerance to amitrole has been reported for ecotypes of *Agropyron repens* (Haddad and Sagar 1968) and *Cirsium arvense* (Hodgson 1970; Smith et al. 1968). High levels of stable tolerance to amitrole appear to be difficult to attain both in the field and through cell culture. This may be due to the multiple sites of action for amitrole. While herbicides with multiple sites of action exacerbate the problems of selecting for tolerance, they are valuable agronomically because of the expected low frequency of occurrence of natural resistance.

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