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Strategies for the selection and characterization of aluminum-resistant variants from cell cultures of *Nicotiana plumbaginifolia*

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Abstract. The development of strategies for selecting and characterizing aluminum-resistant variants from *Nicotiana plumbaginifolia* Viv. cell cultures is described. Plated cells, smeared callus, in-vitro-grown shoots, and seedlings of wild-type *N. plumbaginifolia* all showed similar responses to Al, with total growth inhibition at or above 600 μM Al. The strict control of both cell density and aggregate size is important in selection experiments for total inhibition of the growth of wild-type cells. Two approaches for the selection of Al-resistant variants were used. In a direct method, cells were plated onto medium containing 600 μM Al which inhibited growth and chlorophyll synthesis in wild-type cells. A double selection strategy based on both cell growth and greening was used to isolate 29 Al-resistant variants. In the other approach, a rescue method, suspensions were cultured for 10 d in medium containing 600 μM Al, then plated onto standard medium for recovery of survivors. Using this strategy, 217 Al-resistant variants were selected. After six to twelve weeks of growth in the absence of Al, each variant was cloned and reselected from single cells. Al resistance was retained in 31% and 51% of the variants selected by the direct and rescue strategies, respectively. Seedling segregation data are presented for the progeny (selfed and backcrossed) of plants regenerated from one of the variants and are consistent with those expected for a single dominant mutation.

Key words: Aluminum resistant mutants – Cell culture (variant selection) – Cell selection strategies – Mutant (*Nicotiana*) – *Nicotiana* (Al resistant variants) – Variant selection.

Introduction

Aluminum toxicity is one of the most important environmental stresses limiting world food production (Anonymous 1977). It is a major growth-limiting factor for most crop plants in acid soils throughout the world, and is especially severe in developing countries of tropical regions (Sanchez et al. 1982; Foy 1983). Although selection and breeding for Al resistance is highly effective and well established (Foy 1983), a major limitation is the lack of sufficiently resistant germplasm in many crops. While screening large germplasm collections in the hope of finding Al-resistant accessions is one approach (Wright 1976), an alternative may involve the application of plant cell genetics.

The toxic effects of Al and most known mechanisms of resistance to Al have a fundamental cellular basis (Foy et al. 1978; Haug 1984). Consequently, Al resistance has been identified as an important agricultural trait that may be selectable in cell culture (Meredith and Conner 1985). For cell culture to contribute to the development of resistant germplasm, effective selection strategies must be designed. It is especially important to define conditions totally inhibitory to the growth of wild-type cells, so that resistant variants can be readily distinguished from a background of non-growing sensitive cells.

We have recently reported that several modifications to the inorganic composition of standard cell-culture medium are required to permit the expression of Al toxicity in plant cell culture (Conner and Meredith 1985). To maximize Al toxicity it is important to use unchelated iron and reduce the phosphate concentration, the calcium concentration and the pH to, in the order named, 10 μM,

0.1 mM and 4.0. Employing *Nicotiana plumbaginifolia* Viv. as a model system, we have carried out studies designed to define effective strategies for the selection and characterization of Al-resistant variants from cell culture.

Material and methods

Plant material and culture conditions

Homozygous diploid plants of *N. plumbaginifolia* (derived from a haploid plant obtained by anther culture) were maintained in vitro on MS salts (Murashige and Skoog 1962) plus 3% (w/v) sucrose and 0.7% (w/v) agar. Callus cultures were initiated from petioles of in-vitro-grown plants and grown on RMNO medium (Marton and Maliga 1975). Cell suspensions derived from the callus were maintained on a gyratory shaker at 140 rpm and subcultured weekly at a dilution of two parts suspension: three parts liquid RMNO medium. All media were autoclaved for 20 min at 103 kPa. Cultures were incubated at 25–26° C under light from cool-white fluorescent lamps (50–70 $\mu mol \cdot m^{-2} \cdot s^{-1}$; 16 h light:8 h darkness daily).

Aluminum-toxic media

The MSMT-1 medium was used for Al toxicity experiments on cell suspensions, plated cells, and callus cultures. This medium (based on MS salts) was developed specifically for studying Al toxicity in plant cell cultures (Conner and Meredith 1985). A medium designated MSMT-2 (inorganic salts of MSMT-1 medium plus the organic components of RMOP medium; Sidorov et al. 1981), which induces greening during the first 10 d incubation in the light, was used in one dose-response experiment with plated cells. For Al toxicity experiments on in-vitro-grown shoots and seedlings, MSMT-3 medium was used (0.5-strength MS macronutrients with the additional modifications listed above for MSMT-1 medium, plus MS micronutrients and 3% (w/v) sucrose). Al was supplied as $Al_2(SO_4)_3 \cdot 18H_2O$. After autoclaving, the pH was aseptically re-adjusted to 4.0 with 1 M KOH.

Initiating experiments and monitoring cell growth

All experiments were initiated with cells, callus, or in-vitro-grown shoots 2 d after subculture (see Conner and Meredith 1985). Prior to the initiation of experiments with suspensions and plated cells, cells were filtered through a 500-μm teflon screen to eliminate large aggregates (leaving single cells and small aggregates of up to five to eight cells), allowed to settle, then rinsed with the control medium (no Al) to be used in each experiment. For experiments involving cell aggregates, the suspension fraction not passing through the 500-μm screen was resuspended in control medium. Two size classes of aggregates were separated by their rate of settling.

Cell suspensions. Experiments were established in 125-ml flasks with 50 ml of suspension (initial cell density of 10^5 plating units/ml). Every second day cells were allowed to settle out in 50-ml graduated centrifuge tubes. Settled cell volume was estimated and the supernatant exchanged for fresh MSMT-1 medium.

Plated cells. One-ml aliquots of cell suspension (10^5 plating units/ml, unless otherwise stated) were uniformly dispersed over the surface of 7.0-cm-diameter Whatman (Clifton, N.J., USA). No. 2 filter paper supported by polyurethane foam saturated with liquid MSMT-1 (or MSMT-2) medium (Conner and Meredith 1984). The filter paper with adhering cells was transferred to fresh medium every second day (see Conner and Meredith 1985). At the same time fresh cell weight was estimated as the difference between the weight of Petri dishes with and without filter paper plus adhering cells. Relative growth (RG) was calculated as:

$$RG = \frac{100(Wf - Wi)}{Wi},$$

where Wi and Wf are the initial and final fresh cell weights, respectively. Chlorophyll content was measured spectrophotometrically using the methods described by Harborne (1973, p. 205).

Callus cultures. Methods identical to those described above for plated cells were followed, except that callus was placed onto 4.5-cm-diameter Whatman No. 2 filter paper.

In-vitro shoots. Nodal segments (1.5–2.0 cm long) from in-vitro-grown plants were inserted into 4-mm-diameter holes through polyurethane foam supports saturated with MSMT-3 medium (five shoots per Petri dish). The length of the developed root system was measured after 14 d.

Seedlings. Seeds were soaked overnight in 10^{-3} M gibberellic acid, then sterilized in a bleach solution (1% NaClO) for 10 min and rinsed with sterile distilled water. About 60 seeds were evenly spaced over a 7.0-cm-diameter Whatman No. 2 filter paper supported by polyurethane foam saturated with liquid MSMT-3 medium. After 3–4 d ungerminated seeds were removed and the number of seedlings was reduced to 50 per Petri dish. The number of surviving seedlings as well as the number of leaves and length of root system for each seedling were recorded after 14 d.

Selection of aluminum-resistant variants

From the dose-response experiments described above, two potential approaches for selecting Al-resistant variants were envisioned:

1. Direct selection. Using a one-step approach, 10^5 cells were plated directly onto filter paper supported by polyurethane foam saturated with liquid MSMT-2 medium containing 600 μM Al (following the procedures outlined above). The filter paper with adhering cells was transferred to fresh medium every second day for 20 d, then every 6–8 d thereafter.

2. Rescue selection. Using a two-step approach, cell suspensions were cultured in MSMT-1 medium containing 600 μM Al for 10 d (following the procedures outlined above), then 10^5 cells were plated onto each filter paper supported by polyurethane saturated with standard RMNO medium for recovery of survivors. In one experiment, the effect of incorporating feeder cells was examined by plating 10^5 cells from the standard RMNO medium over the filter paper. The cell population that had been exposed to the Al-toxic medium was then plated onto a second piece of filter paper positioned over the feeder cells.

Cloning and reselecting variants from single cells

Calli from the variants selected via the direct strategy were individually mashed, suspended in liquid RMNO medium and cultured as a suspension for 2 d. Each suspension was passed through a 300-μm teflon screen through which only small single cells and rare two- or three-cell aggregates passed (as determined by microscopic examination). The filtrates were plated at 200–400 cells/filter paper and the direct selection procedure described above was repeated. For the variants derived via

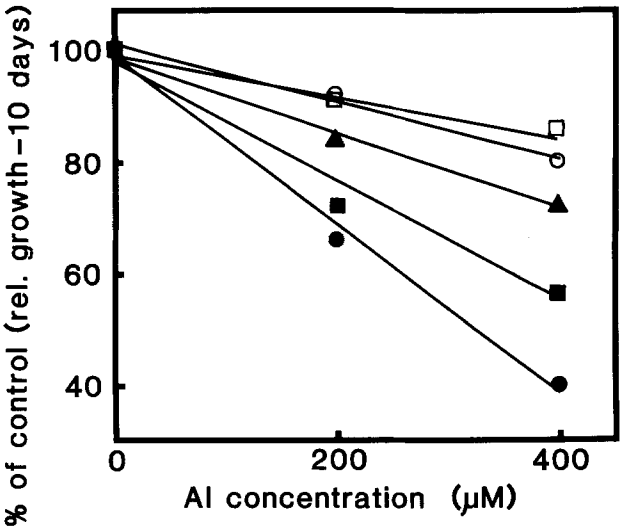

Fig. 1. Influence of cell density and aggregate size on toxicity of aluminum to plated cells of *Nicotiana plumbaginifolia*. Graphed points are the means of five replicates. Lines fitted by linear regression; slopes are highly significantly different from one another; $F_s = 22.96^{**}$. (Note: The initial cell weights of the aggregate treatments equalled that of $2 \cdot 10^5$ plating units/filter paper)
● $1 \cdot 10^5$ plating units/filter paper ($y = 99 - 0.15x$; $r^2 = 0.99$);
■ $2 \cdot 10^5$ plating units/filter paper ($y = 98 - 0.11x$; $r^2 = 0.98$);
▲ $4 \cdot 10^5$ plating units/filter paper ($y = 99 - 0.07x$; $r^2 = 0.99$);
○ small aggregates (0.4 ± 0.2 mm diameter) ($y = 101 - 0.05x$; $r^2 = 0.99$); □ large aggregates (0.7 ± 0.2 mm diameter) ($y = 99 - 0.04x$; $r^2 = 0.97$)

rescue selection, calli were mashed and suspended in MSMT-1 medium containing 600 μM Al. The rescue selection procedure described above was repeated, except that cells were passed through a 300-μm screen prior to plating at 200–400 cells/filter paper.

Transmission of aluminum resistance to sexual progeny

Shoots were regenerated from cloned variants and wild-type callus on RMOP medium, rooted on MS salts, then transferred to soil in a greenhouse. Controlled pollinations were made at flowering, and the resulting seeds sown onto MSMT-3 salt solution plus 600 μM Al (up to 50 seeds per Petri dish) following the procedures described above. Seedlings surviving after three weeks were considered Al-resistant.

Results

Influence of inoculum mass on aluminum toxicity. The toxicity of Al was greatly reduced as the density of plated cells from a fine suspension of *N. plumbaginifolia* was increased (Fig. 1). Reduced Al toxicity was also apparent in the presence of plated cell aggregates compared with a fine suspension (Fig. 1). Likewise, an increase in the inoculum size of callus cultures also decreased Al toxicity (Table 1).

Table 1. Influence of inoculum mass on aluminum toxicity in callus cultures of *Nicotiana plumbaginifolia*. Mean relative growth ± SE after 10 d ($n = 5$)

Inoculum	Aluminum concentration (μM)		Significance[a]
	0	600	
Smeared callus (100 mg)	312 ± 18	132 ± 3	$t'_s = 9.68^{***}$
Callus pieces (150 mg)	159 ± 4	110 ± 17	$t'_s = 2.82^{*}$
Callus pieces (300 mg)	168 ± 16	134 ± 12	$t_s = 1.69$ ns

[a] ***, * and ns represent significant differences between means at the 0.1% and 5% probability levels and non-significance, respectively, as determined by a t-test or t′-test (for means with unequal variances) (Steel and Torrie 1960)

Table 2. Response of *Nicotiana plumbaginifolia* cells plated directly on aluminum-containing medium. Initial inoculum of 10^5 plating units/filter paper was plated onto MSMT-2 medium. Mean relative growth ± SE after 10 d ($n = 5$)

Aluminum conc. (μM)	Relative growth	Chlorophyll content (μg g^{-1})[a]	pH of spent medium
0	371 ± 19	33.8 ± 3.7	5.45 ± 0.18
200	242 ± 29	37.4 ± 4.3	5.01 ± 0.13
400	160 ± 25	12.6 ± 2.4	4.71 ± 0.05
600	20 ± 9	–	4.47 ± 0.01
800	8 ± 9	–	4.38 ± 0.02
1000	−2 ± 8	–	4.32 ± 0.01
	$F_s = 70.07^{***}$	$F_s = 14.23^{***}$	$F'_s = 31.74^{***}$

[a] On FW basis; – indicates insufficient tissue to analyze; *** represents significant differences among means at the 0.1% probability level as determined by analysis of variance

Aluminum dose responses. Plating *N. plumbaginifolia* cells directly on an Al-containing medium resulted in decreases in both cell growth and chlorophyll production as the Al concentration increased, with total inhibition occurring at 600 μM Al (Table 2). When suspension cells were cultured in the presence of Al and then plated onto a standard medium, a longer lag phase occurred before recovery as both the Al concentration and the length of exposure to Al increased (Fig. 2). To prevent the recovery of wild-type cells completely it was necessary to culture the suspensions for 10 d in at least 600 μM Al.

Aluminum toxicity in various developmental states. Cell suspensions were very sensitive to Al, with growth being totally inhibited at even the lowest concentration (200 μM) (Table 3). Plated cells, cal-

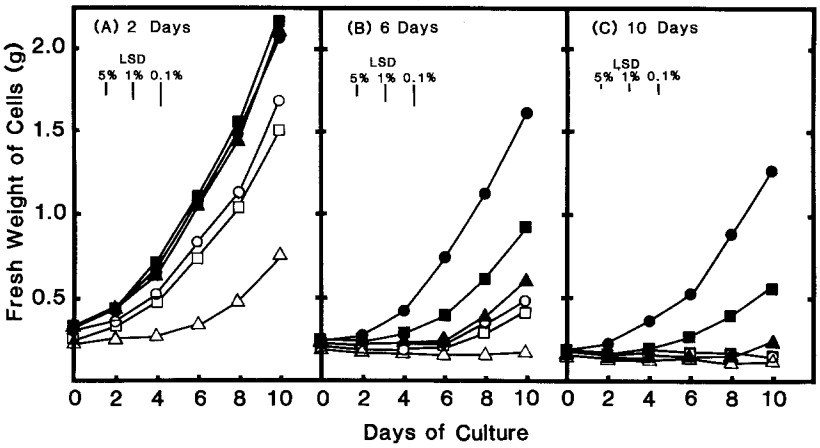

Fig. 2A–C. Recovery of *N. plumbaginifolia* cells after exposure to aluminum in suspension culture. Cell suspensions were cultured in MSMT-1 medium with 0–1000 μM Al for (**A**) 2 d, (**B**) 6 d, and (**C**) 10 d; then plated onto the standard medium (RMNO). Graphed points are the means of five replicates. LSD = least significant difference at the 5%, 1%, and 0.1% significance levels. ● 0 μM Al pre-treatment; ■ 200 μM Al pre-treatment; ▲ 400 μM Al pre-treatment; ○ 600 μM Al pre-treatment; □ 800 μM Al pre-treatment; △ 1000 μM Al pre-treatment

Table 3. Influence of aluminium on *Nicotiana plumbaginifolia* in various developmental states

Developmental state	Length of incubation (d)	Growth parameter	*n*	Aluminium concentration (μM)						F_s
				0	200	400	600	800	1000	
Cell suspension	10	Relative growth	5	432	0	0	0	0	0	–
Plated cells	10	Relative growth	5	371	242	160	20	8	−2	70.1***[a]
Smeared callus	10	Relative growth	5	312	194	178	132	126	130	7.0***
In vitro shoots	14	% rooting	10	80	60	60	40	30	40	–
		Root length (mm)	10	7.8	2.7	2.7	2.2	1.3	1.8	3.5**
Seedlings	14	% survival	100	97	89	65	2	1	0	–
		Root length (mm)	100	8.5	3.7	0.7	0.4	0.1	0.1	662.9***
		No. of green leaves	100	3.5	2.9	1.8	0.9	0.5	0.3	93.6***

[a] *** and ** represent significant differences at the 0.1% and 1% probability levels as determined by analysis of variance. For seedling root length, data was transformed to logarithms (X + 1)

lus cultures, in-vitro-grown shoots, and seedlings all showed similar responses to Al, with total growth inhibition at or above 600 μM Al. Aluminum had no effect on seed germination, but in the presence of Al, seedlings gradually deteriorated during the two-week incubation period. Transferring seedlings to 0.5-strength MS salts 2 d after germination allowed full recovery of all seedlings, even those from the 1000-μM Al treatment (data not shown).

Selection of aluminum-resistant variants. With the direct selection strategy, Al-resistant variants were very slow to appear. On some selection plates no cell growth was apparent, whereas on other plates numerous brown, senescent colonies appeared within 28 d (Fig. 3a). Eleven viable colonies (eight of which were green) slowly appeared 35–70 d after plating (Table 4). These Al-resistant variants were very slow growing on the selection medium and remained under 1 mm diameter after 102 d. The filter paper with adhering cells under selection was therefore transferred to standard medium without

Table 4. Influence of selection strategy on the recovery of aluminum-resistant variants. Each plate was inoculated with 10^5 plating units/filter paper. See text for other details

Selection strategy	No. of plates	Days of culture	No. of variants
Expt. 1:			
Direct (no feeder cells)[a]	100	35	0
		70	11[b]
		102	11[b]
		123	29[b]
Expt. 2:			
Rescue (no feeder cells)[c]	15	10	0
		21	32
Expt. 3:			
Rescue (no feeder cells)[c]	50	10	9
		21	98
Rescue (feeder cells)[c]	50	10	62[d]
		21	87

[a] Filter paper with adhering cells under selection was transferred to standard medium (RMOP) with no Al after 102 d; no further variants appeared after 123 d
[b] Eight of these variants were green
[c] No further variants appeared after 21 d
[d] Colonies appeared after 5–6 d

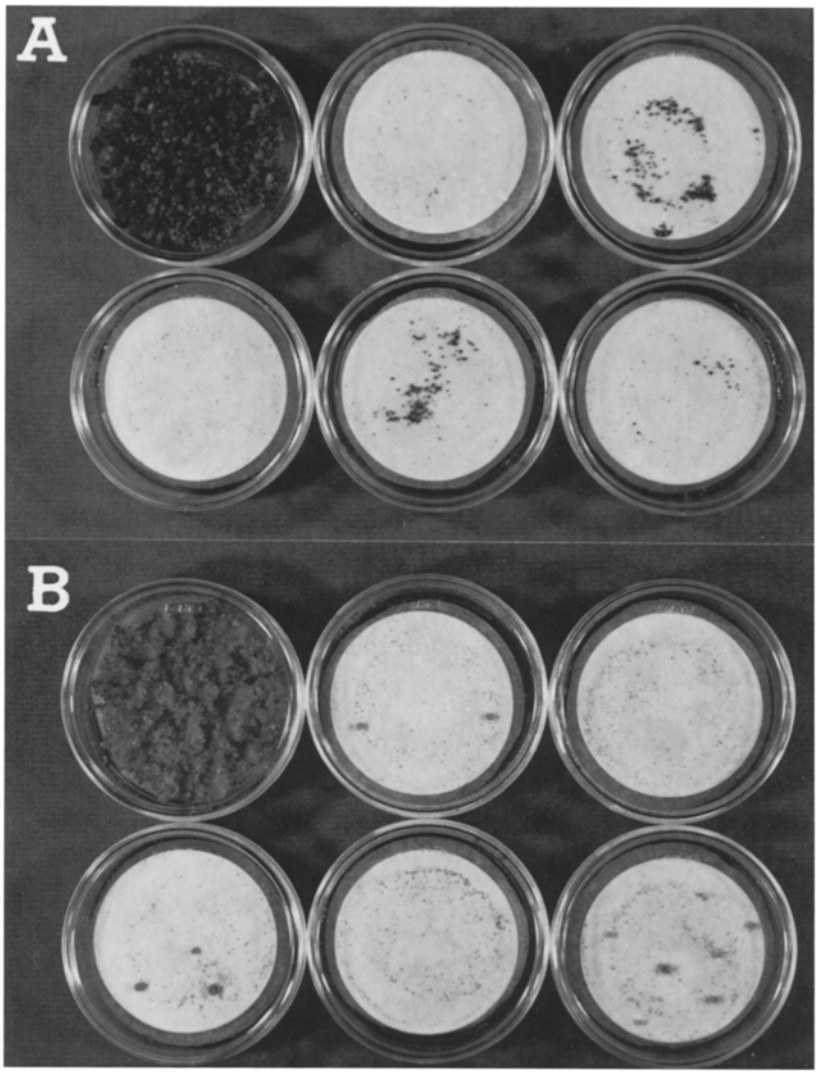

Fig. 3A, B. Comparison of the direct and rescue selection strategies four weeks after inoculation with 10^5 plating units/filter paper. A control plate (no Al, upper left) and five selection plates under direct selection (**A**) and rescue selection (**B**). Note the difference in background growth of wild-type cells on the selection plates and the appearance of discrete variant colonies with the rescue selection method

Table 5. Stability of aluminum resistance after cloning and reselection from single cells. See text for details

Selection strategy	No. selected	No. stable[a]	% stable
Expt. 1:			
Direct – green variants	8	5	63
Direct – non-green variants	21	4	19
Expt. 2:			
Rescue (no feeder cells)	32	15	47
Expt. 3:			
Rescue (no feeder cells)	98	52	53
Rescue (feeder cells)	87	43	49
Total	246	119	48

[a] A stable variant retained Al resistance when cloned and reselected from single cells after 6–12 weeks growth in the absence of Al

Al. In addition to the rapid growth of these variants, 18 new cell colonies appeared within 21 d (Table 4).

With the rescue selection strategy, all the Al-resistant variants appeared within 21 d of plating (Table 4). When feeder cells were incorporated, rescued colonies were apparent after only 5–6 d incubation and had reached 1–2 mm diameter by 10 d. By comparison, in the absence of feeder cells, rescued colonies were not usually evident until two weeks after plating (Table 4). By 28 d, large cell colonies (4–7 mm diameter) were actively growing against a background of totally inhibited wild-type cells (Fig. 3b).

Stability of aluminum-resistant variants. After 6–12 weeks of growth as callus on standard medium (RMNO) in the absence of Al (at least two subcul-

Table 6. Segregation of aluminum resistance in seedling progeny of *Nicotiana plumbaginifolia* plants regenerated from variant Alr 105

Cross	Observed No. of seedlings		Expected No. of seedlings		χ^2	Probability
	Al-resistant	Al-sensitive	Al-resistant	Al-sensitive		
Wild-type (selfed)	0	154	–	–	–	–
	0	138	–	–	–	–
Alr 105 (selfed)	69	31	75	25	1.92	0.1–0.2
	105	45	112.5	37.5	2.00	0.1–0.2
Wild-type × Alr 105	25	29	27	27	0.30	0.5–0.7
	19	13	16	16	1.13	0.2–0.3

tures), each variant was individually cloned and reselected from single cells. Of the 29 Al-resistant variants selected via the direct strategy, only nine retained their Al resistance (Table 5). However when considering only the eight initially green variants, five were stable in their Al resistance. Approximately half of the Al-resistant variants selected via the rescue strategy retained their Al resistance, with no difference between those arising with or without the aid of feeder cells (Table 5). Of the 135 variants failing a first attempt at cloning and reselection, only eight showed Al resistance when retested (the initial failure to clone and reselect these eight variants was probably a consequence of the very low plating density).

Inheritance of aluminum resistance. Seedlings from selfed wild-type plants were sensitive to 600 μM Al, whereas those from variant Alr 105 segregated 3 resistant:1 sensitive (Table 6), indicating that the originally selected cell line was heterozygous for a single dominant mutation. Seedlings from backcrosses of Alr 105 to wild-type plants confirmed this hypothesis with a 1 resistant:1 sensitive segregation ratio (Table 6).

Discussion

The expression of Al toxicity in cell cultures of *N. plumbaginifolia* was highly dependent on cell density and aggregate size in plated cells (Fig. 1) and inoculum size in callus cultures (Table 1). In selection experiments both cell density and aggregate size must therefore be strictly controlled to permit a repeatable, uniform exposure of cells to Al. Such stringent selection conditions are important so that Al-resistant variants can be readily distinguished from the background of non-growing Al-sensitive cells. The influence of inoculum mass on the response to toxicants also has important implications for the characterization of resistant variants (and comparisons of different genotypes in cell culture). Comparisons between different cell lines must involve equating their cell density and aggregate size when using suspensions or plated cells, or inoculum size and friability when using callus cultures.

One advantage of using polyurethane supports for studying Al toxicity in cell culture is that spent medium can be easily squeezed out after the incubation period to monitor changes in the medium pH (Conner and Meredith 1984). The pH of the spent medium was more acidic at higher Al concentrations (Table 2). These values were typical for all experiments reported in this paper (data not shown). The possibility that low pH rather than Al may be inhibiting cell growth has been eliminated (Conner and Meredith 1985). The drift from the initial pH of 4.0 (Table 2) was insufficient to cause more than negligible precipitation of Al (Conner and Meredith 1985).

For the selection and full characterization of Al-resistant variants isolated in cell culture, a clear understanding of the degree of Al toxicity in various developmental states is important. We attribute the higher sensitivity of cell suspensions to Al (Table 3) to the intimate contact between the cells and the toxic medium. This reduces the possibility for establishing micro-environments in the immediate vicinity of the cells. The similar response to Al of plated cells, smeared callus, in-vitro-grown shoots, and seedlings (Table 3) reflects the fundamental cellular basis at which the toxic effects of Al are expressed (Haug 1984). Provided the initial inoculum mass is carefully controlled, 600 μM Al can be used for both the selection of Al-resistant variants via both the direct and rescue strategies, and their subsequent characterization at various levels of development.

Aluminum-resistant variants of *N. plumbaginifolia* were successfully selected using both the direct and rescue selection strategies (Table 4). An

advantage of rescue selection is that feeder cells can be incorporated directly beneath the filter paper on which the cells under selection are plated. This is known to facilitate greatly the recovery of viable cells plated at very low densities (Horsch and Jones 1980; Conner and Meredith 1984). (Note: With direct selection, feeder cells in this position decrease the effectiveness of selection because of cell-density effects.) However, the use of feeder cells in the rescue selection approach had no influence on the total number of variants selected; it only shortened the time required for the variants to appear (Table 4).

With the direct selection approach, increasing the Al concentration reduced both cell growth and chlorophyll levels (Table 2). This suggested a double selection scheme for isolating Al-resistant variants from cell culture. Cells could be selected not only for growth, but also screened on the basis of greening. The direct selection method is very time consuming. The filter paper with adhering cells under selection needs to be repeatedly transferred to fresh medium to restore the initial pH of 4.0 and to replenish the low phosphate supply (Conner and Meredith 1985). Furthermore, resistant variants do not appear until at least five weeks after plating on the selection medium and remain under 1 mm diameter even after three months (Table 4). The rescue selection strategy overcomes both of these disadvantages. Once the 10-d incubation in suspension culture has been completed, transfer to fresh medium is not required, and the incorporation of feeder cells permits resistant variants to appear after 5–6 d (Table 4). Even without feeder cells Al-resistant variants are evident within three weeks. A further advantage of rescue selection is the complete inhibition in the background growth of wild-type cells. The only growth on selection plates is isolated colonies of Al-resistant variants, which are easily identified (Fig. 3b). In contrast, with the direct selection strategy, background growth of wild-type cells persists on some selection plates (Fig. 3a), even with increases in the Al concentration. This appears as small brown senescent cell colonies, making it difficult to discriminate between escapes and Al-resistant variants, although double selection for green colonies improves the effectiveness of selection (Table 5). A disadvantage of the rescue selection strategy is the high probability of independently selecting the same variant several times. Although the same problem exists with direct selection, it is exaggerated as a consequence of the methodology used in rescue selection. Any Al-resistant cells present at the initiation of selection may undergo up to two or three mitotic divisions during the culture in suspension. If the resulting cells dissociate prior to plating, they may give rise to separate cell colonies and be identified as distinct variants.

A total of 246 Al-resistant variants were selected from cell cultures of *N. plumbaginifolia* (Table 4). Each variant was individually cloned and reselected from single cells by the same strategy used in the initial selection. It was important to do this prior to characterizing the variants in order to eliminate the possibility that the initially selected variants were chimeras of Al-resistant and wild-type cells. Some Al-sensitive cells may survive within resistant colonies via cross feeding (Meredith 1983, 1984). Although the cloning procedure did not eliminate rare aggregates of two or three very small cells, such aggregates are very unlikely to be chimeras and the component cells are most probably of very recent clonal origin (certainly since the original selection). Reselection by plating at low cell density prevents cell colonies from coalescing and allows discrete colonies (from presumptive single cells) to be isolated. For each cloned and reselected variant, a single small colony (larger colonies were more likely to have originated from the rare aggregates of two or three cells) was chosen for further characterization.

After 6–12 weeks of growth in the absence of Al, 48% of the 246 Al-resistant variants initially selected retained their Al resistance (Table 5). There are two possible explanations for the failure to reselect the other variants: (1) They may have been escapes from the original selection pressure. Considering the influence of cell density and aggregate size on the growth responses of cells to Al stress (Fig. 1), it is not difficult to envision some escapes arising. (2) They may have represented a transient physiological adaptation to Al stress which was lost during the culture period in the absence of selection pressure, similar to that reported for unstable cycloheximide resistance (Maliga et al. 1976).

A large number of *Lycopersicon esculentum* cell lines with stable resistance to Al-EDTA have been previously selected (Meredith 1978). The possibility of a genetic basis for the resistance could not be confirmed since plants could not be regenerated. More recently, a cell line of *Daucus carota* with increased resistance to $AlCl_3$ has been isolated (Ojima and Ohira 1983). Preliminary results indicate that the resistance is retained in the seedlings progeny of regenerated plants (Ojima and Ohira 1982), but unequivocal evidence for a mutation has not been reported. The genetic and physiological basis of Al resistance in all the variants reported

in this paper is now under investigation. One of the variants has been confirmed as a true mutant with Al resistance being expressed in seedling progeny in segregation ratios expected for a single dominant mutation (Table 6).

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