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Clonal Variation for Tolerance to Polyethylene Glycol-Induced Water Stress in Cultured Tomato Cells[1]

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ABSTRACT

Cell clones were isolated from a population of cultured tomato (*Lycopersicon esculentum* Mill cv VFNT-cherry) cells and their tolerance to polyethylene glycol (PEG)-induced water stress was measured. Considerable variation for tolerance among the clones was found. Tolerance differences between clones appeared to be spontaneous and were different from tolerance differences between adapted and unadapted cells. Unlike adapted (selected by exposure to PEG) cells, cell clones retained their relative tolerance for many generations in the absence of selection pressure, and tolerance of both relatively tolerant and intolerant clones was very dependent on growth cycle stage and inoculum density. Analysis of subclones isolated from relatively tolerant and intolerant parent clones revealed that each parent clone gives rise to progeny with tolerances near the mean tolerance of both parents. However, progeny populations of both tolerant and intolerant parents are enriched with individuals with phenotypes nearer the mean response of their respective parent populations. When exposed to PEG, relatively tolerant and intolerant clones alike become adapted to the level of PEG to which they are exposed, and have the same phenotypic level of tolerance. Thus, selection by exposure to stress is unable to discriminate (on the basis of growth) between the innately tolerant and intolerant cell types within the population. This is indicated also by the fact that clones isolated from a population of cells adjusted to growth on 25% PEG do not show an enriched frequency of tolerant phenotypes when grown in the absence of PEG compared to the nonselected normal cell population which has never been adjusted to growth on PEG.

The use of plant cell cultures to obtain variants much akin to the selection of variants in bacterial cultures has been proposed and discussed in the literature for some time. In fact, many plant cell variants have been selected and these efforts have been reviewed (6, 12). The possibility of using plant cell cultures to obtain variants which would have practical application to agriculture is receiving increased attention. Among such agriculturally useful variants are stress-tolerant phenotypes which might be selected directly from cultured cell populations exhibiting spontaneous or induced variation, or might be obtained by the introduction of specific genetic information through genetic recombination technology and subsequent plant regeneration.

In spite of the relative success of selection attempts with cultured higher plant cells, there is little information concerning the genetic basis for much of the observed variation. Although it was assumed earlier that variation among cultured plant cells was due largely to mutant genotypes, the apparent high frequency and quasi-stable nature of many variants has led to the postulation of numerous genetic mechanisms (3, 12). Before the potential use of cell culture techniques, *e.g.* selection and gene transfer, for crop improvement can be realized, the physiological and genetic basis for the characters of interest must be understood more.

We have reported earlier the selection of higher plant cells from suspension cultures which have increased tolerance to water stress imposed by PEG (4, 5, 8). We indicated that cells with increased tolerance to PEG-induced water stress obtained by *en masse* selection were the result of adaptation and not true selection of preexisting tolerant genotypes. We report here that clonal analyses of cell populations before and after selection indicate that spontaneous variation for water stress tolerance does exist in the cell population but that the adaptive ability of all the cells prevents the selection of preexisting tolerant cells by exposure to selection pressure (water stress). Nevertheless, stable tolerant and intolerant cells may be isolated by random selection, and the genetic basis for this tolerance difference might be distinguished from tolerance differences based on adaptive mechanisms.

MATERIALS AND METHODS

Cultured Cells. Cultured cells of tomato (*Lycopersicon esculentum* Mill cv VFNT-cherry) were obtained and maintained in culture as previously described (5).

Culture Medium. All culture maintainance and experiments were performed using tomato culture medium previously described (5). PEG 6,000 (mol wt 6,000–7,600) from J. T. Baker Chem. Co., Phillipsburg, NJ, was used in all experiments.

Isolation of Clones. Clones were obtained by methods similar to those described by Bergmann (2). Cell suspension cultures in stationary phase of growth were passed through sterile stainless steel screens of successively smaller mesh size with a final screening of 43 μm. Virtually all of the cells were contained in clumps of ten cells or less. Most of the clumps consisted of three or fewer cells (Fig. 1). Examination under the microscope revealed that the clumps appeared to consist of cells with common walls, although in the case of some clumps with higher cell numbers this could not be discerned.

Cells which passed through the 43-μm screen were concentrated by centrifugation at 200*g* for 8 min. Medium was preconditioned by incubation with stationary phase cells for 24 h at 40 g l^{-1}. The cell pellets were resuspended in preconditioned medium containing 0.8% agar at 38°C at a density of about 2 to 10 × 10^3 cell clumps ml^{-1}. Approximately 25-ml portions of the resulting suspension of cells were added to Petri plates and these plates were wrapped with Parafilm and kept at 26°C in a humidified chamber. Once the cell colonies reached about 1 mm in diameter, they were removed from the Petri plates and placed into culture tubes containing 10 ml of tomato agar medium.

Once the colonies had grown sufficiently, about 1 g mass, the tissue was transferred to liquid culture and incubated on a gyratory

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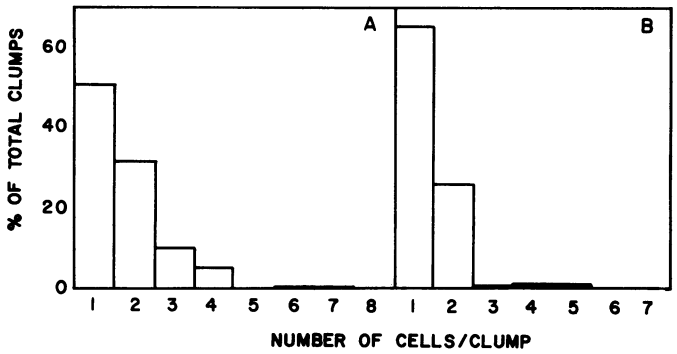

FIG. 1. Frequency distribution of cell clump size. Per cent of total clumps having various numbers of cells per clump in the cell suspension passing through the 43-μm screen is shown. A, Normal cell population growing in 0% PEG, $n = 216$; B, cell population growing in 25% PEG, $n = 233$.

shaker. Cultures were passed through two growth cycles on liquid medium before determination of water stress tolerance.

Determination of Tolerance of Clones. After isolation and establishment in liquid culture, clones were tested for tolerance to PEG-induced water stress. There were approximately 31 generations between isolation of clones and their tolerance measurements. Tolerance was tested by determining the growth after inoculation of stationary phase cells in medium with various concentrations of PEG. Growth was measured 17 d following inoculation using at least two replicate cultures in all cases. Tolerance was estimated as ID_{50}[2], which is defined as the per cent PEG required in the medium to inhibit weight gain of the cells by 50% of that gained by cells in medium without PEG, and as TI which is defined by the following formula: $TI = WG_0 + WG_5 \times 5 + WG_{10} \times 10 + WG_{15} \times 15 + WG_{20} \times 20$ where WG = weight gain of the cells in medium with per cent PEG denoted by the subscript. TI is a parameter which is much more sensitive to tolerance differences than ID_{50}. TI reflects also the ability to grow at higher PEG levels much more than ID_{50}.

Determination of Tolerance as a Function of Growth Cycle Stage. Tolerance of PEG-induced water stress was measured using cells of different ages (days following inoculation) as was described earlier (4). Cells were inoculated at a density of 8 g fresh weight l^{-1} into 2 L medium in 4-L flasks, and cells were collected by sterile filtration at various times following inoculation. These collected cells were then inoculated (8 g fresh weight l^{-1}) into 125-ml Erlenmeyer flasks containing 25 ml of medium with various concentrations of PEG. After 17 d, these cultures were harvested and fresh and dry weights of the cells were determined. All growth measurements were made using at least two replicate flasks.

RESULTS

Clonal Variation within the Cell Population for Tolerance to PEG-Induced Water Stress. Clones selected from the cell population at random were tested for tolerance to PEG-induced water stress by measuring their growth in media containing various amounts of PEG, and their ID_{50} values can be determined from the data in Figure 2. In addition, TI was calculated for each clone and these values are given in Table I. Six independent replicate measurements of the ID_{50} and TI of stationary phase cells of clone 27 and of clone 43 were made. The mean ID_{50} for clone 43 replicates was 5.4% PEG, while the mean ID_{50} for clone 27 replicates was 17.2% PEG. ID_{50} variation was not different between these clones by the F test (clone 43 $\sigma^2 = 1.3$ and clone 27 $\sigma^2 = 0.6$). Assuming the ID_{50} variance between replicates of these clones is typical of the variance for all the clones, an ID_{50} difference of 1.0% PEG between clones is significant at the 0.05 level by a t test (Fig. 2). The mean TI of replicates of clone 27 was 75, while the mean TI of clone 43 replicates was 41. The variations in TI between replicates of clone 43 ($\sigma^2 = 144$) and between replicates of clone 27 ($\sigma^2 = 119$) also were not different from each other by the F test. Again, if these variances are typical of all of the clones, a TI difference of 10.8 between clones is significant at the 0.05% level by the t test (Table I).

In addition, the variation in TI between all of the nonselected cell population clones tested ($\sigma^2 = 4{,}761$; see Table I) and the variation in TI between several replicates of dose response tests of the nonselected cell population before isolating clones ($\sigma^2 = 196$) were highly different. The variation between clones was much higher (Table I). Thus, there was considerable variation between the clones for tolerance of cell growth to the low water potential caused by the presence of PEG. It appears from these results that natural variation for water stress tolerance exists within the normal cell population.

Inasmuch as there was variation among the clones for growth rate in medium without PEG, their tolerance to PEG was compared to their growth on medium without PEG. Many clones which grew very rapidly without PEG showed little tolerance and there was a low correlation ($r = 0.2$) between tolerance to the PEG and rapid growth in the absence of PEG although the correlation was positive.

Tolerance to PEG-Induced Water Stress: Clonal Variation Which Is Stable in the Absence of Stress. Four clones taken from the original population of randomly selected clones were chosen for further analysis: two relatively tolerant clones, clone 27 and clone 41, and two relatively intolerant clones, clone 6 and clone 43. Inasmuch as we knew from previous work (4) that tolerance varied greatly depending on the growth cycle stage, the tolerance of these four clones was determined at various stages throughout their entire growth cycle. Tolerance of these clones is shown in Figure 3 as the ID_{50} and TI. It is clear from Figure 3, B, C, E, and F, that both clones 27 and 41, which originally appeared tolerant, required greater levels of PEG to inhibit their growth by 50% and had higher TI values than did the originally intolerant clones, 43 and 6, at all stages of the growth cycle. However, all four clones showed growth cycle-dependent tolerance to PEG. This is in contrast to selected or adapted cells (4) which show growth cycle-independent tolerance in medium which is not more stressful than that to which the cells have adapted. The enhanced tolerance shown by clones 27 and 41 compared to clones 6 and 43 (Fig. 3, B, C, E, and F) is quite stable in the absence of any selection pressure for many generations (>100).

If these clones are adapted to grow in 20% PEG for about 25 generations and their tolerance to stress is again tested at various growth cycle stages, it is found that they become indistinguishable in their tolerance to stress (Fig. 3, A and D) compared to the difference in tolerance that they show before being grown in 20% PEG (Fig. 3, B, C, E, and F). Furthermore, after adapting to 20% PEG, their tolerance is growth cycle independent (Fig. 3, A and D). Thus, all of the clones have adapted to the level of stress present during their growth in culture. Any selection procedure which attempts to discriminate against the originally intolerant clones by the application of stress will likely fail to do so because the intolerant clones can simply adapt and become indistinguishable (with respect to growth in the presence of stress) from the originally tolerant clones. This can be seen also from Figure 4 where the growth response of these clones is compared at various levels of PEG. Figure 4B shows that clones 6 and 43 are clearly less tolerant than clones 27 and 41. Once allowed to grow in 20% PEG medium, however, the intolerant clones 6 and 43 become as tolerant as the tolerant clone 27 (Fig. 4A). The only way such selection pressure could separate these clones would be on the

[2] Abbreviations: ID_{50}, 50% inhibiting dose; TI, Tolerance Index.

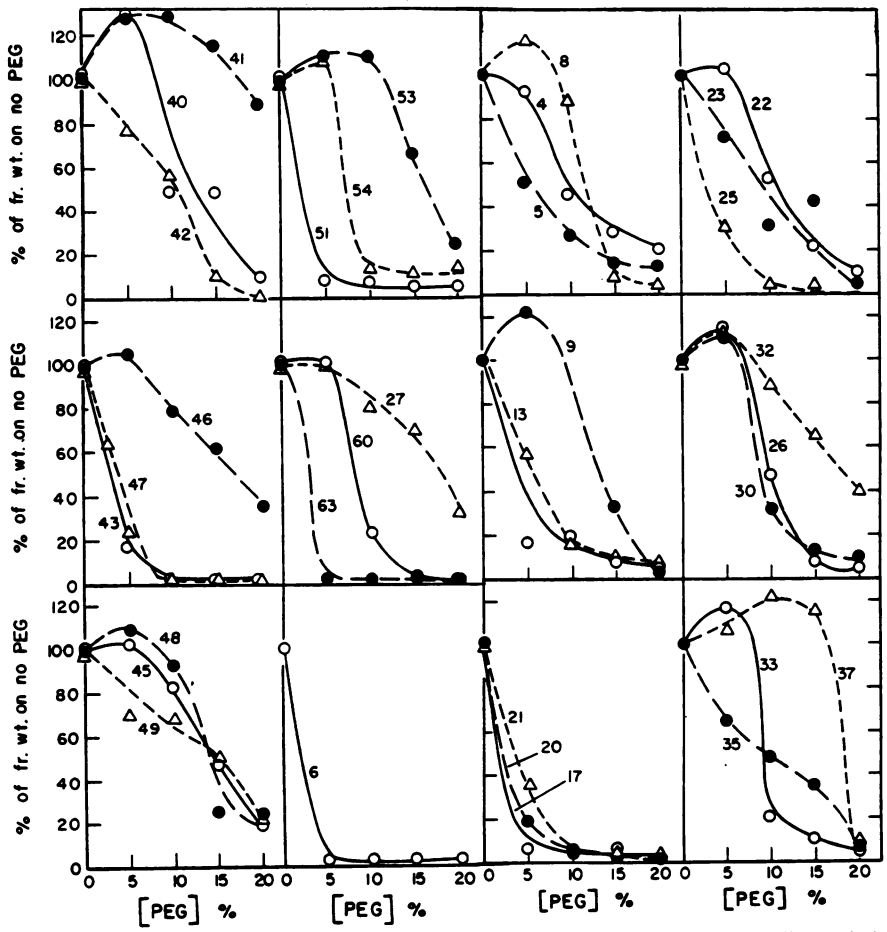

FIG. 2. Tolerance to PEG-induced water stress of clones isolated at random from the nonselected tomato cell population. Growth as per cent of growth on 0% PEG is shown as function of PEG concentration. Cells from stationary growth phase were used as inoculum. Cultures were harvested when cells on 0% PEG reached stationary phase about 17 d after inoculation. Clone designation numbers are indicated.

basis of differences in rate of adaptation between clones. If differences between rates of adaptation are not large, enrichment of the culture with the more tolerant clones during exposure to selection pressure could be negligible, however.

Population Analysis of Tolerant and Intolerant Clones. From Figures 3 and 4 it can be seen that clones 27 and 41 have significantly greater tolerance to water stress than do clones 43 and 6 even after examining tolerance at all stages of the growth cycle. Clones 43 and 6 have a mean ID_{50} of about 8% PEG, while the mean ID_{50} of clones 41 and 27 is about 17% PEG. The mean TI values of clone 43 and 6 are 98 and 83, respectively, whereas those of clones 27 and 41 are 167 and 192, respectively (Fig. 3). These clones retain their relative tolerances after several (at least 100) generations of growth in the absence of any selection pressure. These clones might exhibit such tolerance differences even if each clone itself is composed of a mixture of cell types with different tolerances. Therefore, several subclones were isolated from cell populations of both clone 27 and clone 43 and their frequency distribution for TI was examined. In addition, clones were isolated from populations of cells which had been grown for over 100 generations in the presence or absence of 25% PEG (Table I). The frequency distributions for TI of individual clones taken from all of these populations are shown in Figure 5. The mean growth response at various PEG concentrations of all the clones isolated from these populations can be seen in Figure 6. The mean growth on 15% PEG of the clones of the unadapted cell population was 22% of the growth on 0% PEG and the ID_{50} was 9% PEG (Fig. 6B), while the mean growth on 15% PEG of the clones from the cell population after adaptation on 25% PEG was 29% of that on 0% PEG and the mean ID_{50} was also 9% PEG (Fig. 6B). The mean TI of the clones of the original cell population was 87 ± 16 (95% confidence interval) while the mean TI of the clones from the cell population adjusted to growth on 25% PEG was 71 ± 15 (95% confidence interval). These means are not different at the 0.10 level by a t test before or after transformation of the values by $\log_{10}$ (since the apparent distributions of these populations were not normal; Fig. 5). Selection by exposure of the original cell population to 25% PEG did not seem to alter significantly the mean tolerance of the population or, apparently, the proportions of cells of various tolerances as seen from the frequency distributions in Figure 5, C and D.

The mean growth of the subclones of clone 43 on 15% PEG was 19% and their mean ID_{50} was 5% PEG, while the mean growth on 15% PEG of the subclones of clone 27 was 51% of their growth on 0% PEG and their mean ID_{50} was 15% PEG (Fig. 6A). The frequency distributions for TI of those subclones (Fig. 5, A and B) show populations of individuals with both tolerant and intolerant phenotypes compared to each parent clone population phenotype. These populations appear to have overlapping and bimodal distributions suggesting that two distinct phenotypes may exist and that the relative frequency of these phenotypes in the population could yield various population phenotypes as seen in Figure 2 and Table I. If one assumes that each clone originated from a single cell, then it appears that the phenotypes of clones can spontaneously change during culture at a very high rate. This phenotypic change would have to have occurred within the 31 generations between first isolating the clones and the testing of the subclones. Alternatively, the clones first isolated may have originated from

Table I. *Tolerance Index Values for Replicate Samples*

Samples are from the normal (nonselected) cell populations (controls), normal cell population clones, clones from cells adjusted to growth on 25% PEG medium, subclones of the relatively intolerant clone 43, and subclones of the relatively tolerant clone 27.

Control 1 Normal Cell Population Replicates	Control 2 Normal Cell Population Replicates	Normal Cell Population Clones	Clones of 25% PEG-Adjusted Cells	Subclones of Clone 43	Subclones of Clone 27
54	57	27	80	147	197
46	80	21	105	64	90
37	54	99	16	179	134
42	66	9	42	79	129
47	58	5	128	60	75
29	57	15	191	166	139
51	34	54	138	165	79
44	48	74	225	181	116
51	49	229	66	13	179
43	41	222	196	182	158
44	53	152	184	53	174
45	50	136	192	9	125
52	47	228	86	11	139
45	25	173	8	28	175
42	58	109	47	133	178
50	63	186	15	12	94
48	77	45	28	22	201
55	71	184	28	33	95
41	67	124	41	10	39
40	57	125	51	15	139
50	46	23	41	41	138
43	66	114	35	85	207
34	68	112	104	12	188
46	100	152	81	29	36
50	39	35	78	81	177
47	71	218	51	41	108
50	50	80	132	22	119
42	68	69	30	11	129
48	59	9	54	7	79
44	60	67	15	51	128
44	81	38	42	45	109
54	51	246	126	14	157
60	53	42	90	43	79
46	65	35	17	76	157
48	55	91	10	18	78
34	55	49	20	49	108
31	49	91	10	62	38
53	53	14	20	177	39
29	54	54	41	142	39
55	55	20	41	103	106
		44	29	94	79
$n = 40$	$n = 40$	8	40	25	117
mean = 45	mean = 58	13	51		198
SD = 7	SD = 14	70	148	$n = 42$	147
SE = 2.7	SE = 3.7	72		mean = 62	138
$\sigma^2 = 49$	$\sigma^2 = 196$	9	$n = 44$	SD = 57	148
		115	mean = 71	SE = 7.6	88
			SD = 60	$\sigma^2 = 3249$	46
		$n = 47$	SE = 7.7		118
		mean = 87	$\sigma^2 = 3600$		158
		SD = 69			38
		SE = 8.3			39
		$\sigma^2 = 4761$			19
					166
					117
					188
					107
					$n = 57$
					mean = 119
					SD = 51
					SE = 7.1
					$\sigma^2 = 2601$

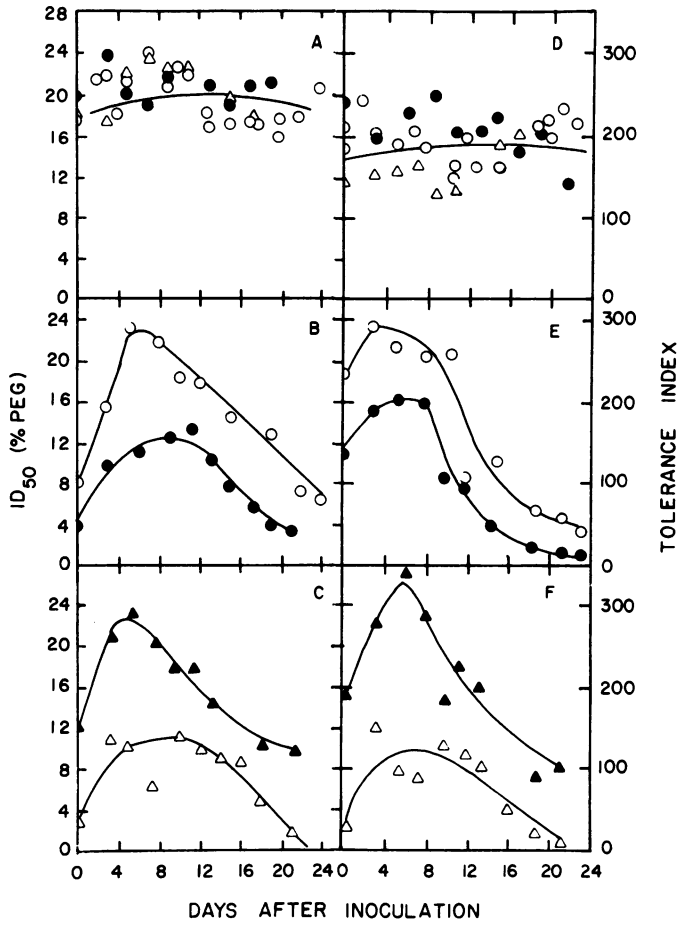

FIG. 3. Tolerance of clones 43, 41, 27, and 6 shown as ID_{50} (B, C) or as tolerance index (TI: E, F) as a function of growth cycle stage (days after inoculation) when the clones are maintained on 0% PEG medium. Clone 43 (●): mean ID_{50} = 8.5, mean TI = 98; clone 27 (○): mean ID_{50} = 14.5, mean TI = 167; clone 41 (▲): mean ID_{50} = 16.1, mean TI = 192; clone 6 (△): mean ID_{50} = 7.9, mean TI = 83. Tolerance after growth in 20% PEG medium for approximately 25 generations is shown in A and D. Clone 27 (○): mean ID_{50} = 18.3, mean TI = 208; clone 43 (●): mean ID_{50} = 20.4, mean TI = 182; clone 6 (△): mean ID_{50} = 23.1, mean TI = 203.

two or more genotypically distinct cells, thus yielding a subclone population not normally distributed around the tolerance level of the parent clone population but having a mean tolerance level similar to that of the parent clone population.

Relationship between Inoculum Density and Tolerance to PEG-Induced Water Stress: Clonal or Spontaneous *versus* Adaptive Tolerance. It was obvious from our earliest experiments that inoculum density could effect greatly the ability of the cells to grow in the presence of PEG. This was not altogether surprising inasmuch as raising inoculum density was known to affect cell growth patterns (17). Of particular interest to us was whether increasing inoculum density could specifically alter the tolerance of the cells to water stress. This we determined by inoculating PEG-free medium and medium containing 15% PEG with increasing densities of cells. The amount of growth of the cells after 17 d on the stress medium divided by their growth over the same period on non-stress medium provides a measure of their tolerance. The tolerance of nonselected tomato cells increases dramatically with increasing inoculum density (Fig. 7). However, cells selected and maintained in 15% PEG (100 generations) did not decline dramatically in tolerance at low inoculum densities. These cells grew nearly equally well in stress (15% PEG) and non-stress (0% PEG) medium at all inoculum densities tested. Nonselected cells clearly

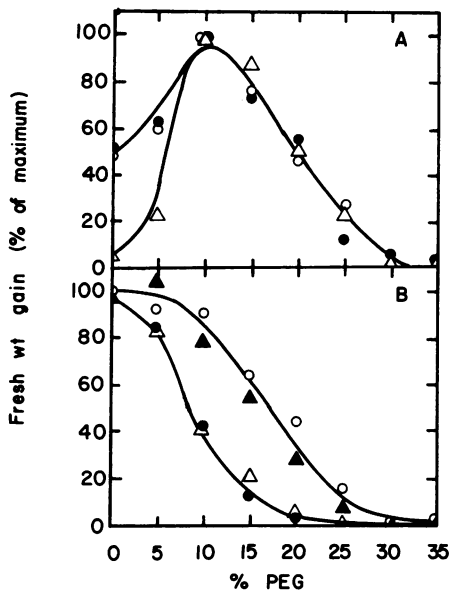

FIG. 4. Shown are the tolerant clones 27 and 41 compared to the intolerant clones 43 and 6 for their ability to gain fresh weight in the presence of different levels of PEG. Values are the average of fresh weight gains of cells of different ages (all the times after inoculation given in Fig. 3) for each PEG concentration shown. A, The clones after growing in 20% PEG for 25 generations. B, The clones maintained in medium without PEG. (○), clone 27; (▲), clone 41; (●), clone 43; (△), clone 6.

are able to grow better in stress medium at higher inoculum densities (Fig. 7) indicating that increased cell density helps the cells to adjust rapidly to the stress environment. The selected cells are clearly preadjusted to the 15% PEG medium and increasing the inoculum density has little effect on their ability to adjust and grow in the stress medium (Fig. 7).

Clones which differ in their tolerance to water stress behave altogether differently. Two clones which differed in tolerance to the stress (Fig. 2), clone 27 and clone 43, were compared at different inoculum densities. The more tolerant clone did show greater growth on 15% PEG relative to growth on 0% PEG when compared to the less tolerant clone. However, this greater tolerance which is exhibited by clone 27 was evident only over a narrow range of inoculum densities (Fig. 7, C and D). At low densities, both clones were unable to adjust rapidly to the stress and grow, whereas at high densities both clones adjusted rapidly and showed fresh weight growth under stress of 70 to 80% of the fresh weight gain on 0% PEG medium. Dry weight gain responses were similar except that at high inoculum densities both clones gained more dry weight in stress medium than in medium without stress. It appears that neither clone is more preadapted to a higher stress environment than is the other clone. Rather, clone 27 appears to be able to adjust more quickly than clone 43 and thus requires less inoculum density to begin gaining both fresh and dry weight under stress at rates comparable to growth in the absence of stress. Yet it appears from our population analyses that exposure of a cell population containing both of these types of cell clones to PEG-induced water stress does not enrich substantially the population for clones which adjust more quickly (Figs. 5, C and D, and 6).

The results indicate that the clones differ in tolerance from each other because of some stable mechanism which affects the ability of the cell to adapt to PEG-induced water stress. Because we cannot regenerate plants from these cells it is not possible to test the genetic basis of these tolerance differences using regenerated plants and their progeny. In such instances, the Luria-Delbruck (13) fluctuation test has proved useful (16). With this test, spon-

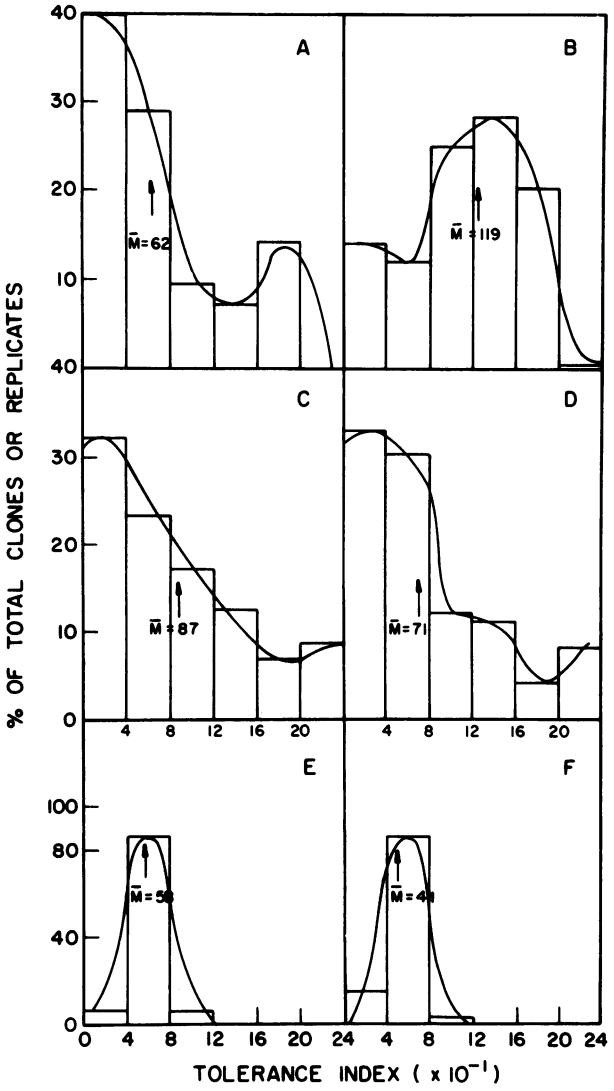

FIG. 5. Frequency distribution of clones for tolerance to PEG-induced water stress. Tolerance is shown as TI. Tolerance categories are in intervals of 40 TI units. The percent of total clones tested falling into each category is shown. A, Subclones of clone 43 (n = 42). B, Subclones of clone 27 (n = 57). C, Clones of nonselected tomato cell population (n = 47). D, Clones of cell population which has grown on 25% PEG for about 80 generations (n = 44). E, Replicate control 2 in which replicate inocula were taken from 40 separate flasks each containing stationary nonselected cells approximately two generations after their inoculation (n = 40). F, Replicate control 1 in which replicate inocula were taken from a single flask containing stationary phase nonselected cells (n = 40).

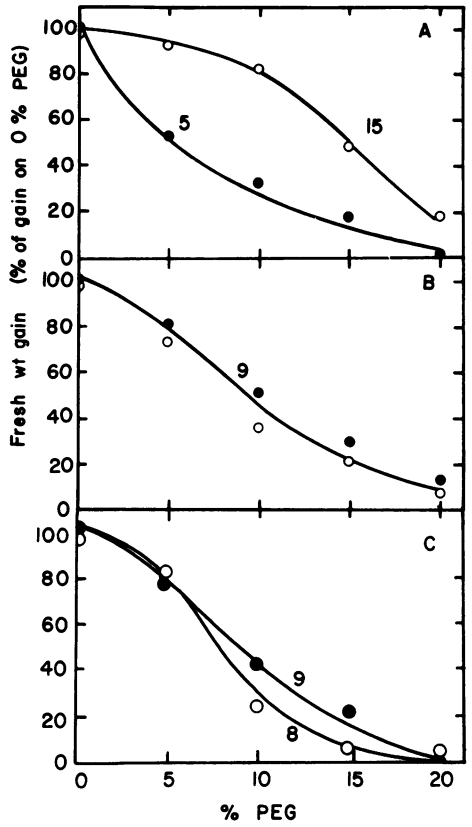

FIG. 6. Average growth response of all the clones and replicate inocula of the normal nonselected cell population tested at various levels of PEG-induced water stress. Their growth response is shown as the fresh weight gain as per cent of fresh weight gain on 0% PEG medium. The variation in TI which exists within these populations can be seen in Figure 5 or Table I. A, (○), Average of the subclones of clone 27 (n = 57); (●), average of the subclones of clone 43 (n = 42). B, (●), Average of the clones of the nonselected cell population (n = 47); (○), average of the clones of cells grown on 25% PEG (n = 44). C, (○), Average of replicate control 1; (●), average of replicate control 2. Indicated next to each curve is the ID_{50}.

taneous variants can be distinguished from those due to acquired traits by comparing the distribution of phenotypic variation exhibited by isolated clones to the variation found between repeated measurements of the character in the population as a whole. An experiment was conducted to determine the distribution of TI values obtained from several replicate samples taken from a nonselected cell population. The cumulative frequency distribution of these values was then compared to the cumulative distribution of TI values of clones isolated from the normal nonselected cell population. All TI values were obtained using stationary phase cells as inoculum. The values for the replicate population samples (control 2 in Table I) fit a Poisson distribution around the mean ($P > 0.10$) relatively well, while the values from clones (normal cell population clones in Table I) appeared to deviate substantially from the Poisson distribution around the mean ($P < 0.01$) (Fig. 8). According to the original Luria-Delbruck (13) test, this suggests that the differences between the clones result from spontaneous phenotypic changes (mutations) and not from induced or acquired phenotypic alterations due to the environment. Two separate control analyses of replicate samples of the nonselected cell population were performed (control 1 and 2 in Table I; Fig. 5, E and F). In one control, the inocula were taken from a single flask and placed into media containing various PEG levels to determine TI (control 1). In the second control, 40 separate flasks of cells were grown from an inoculum density of 1 g to around 4.5 g (representing approximately two generations until stationary phase), and then inoculum was taken from each of the 40 flasks and used to determine 40 separate TI values. The first control which is less analogous to the methods used to measure clone TI values fit the Poisson distribution around the mean to a lesser extent ($P > 0.04$) than this second control.

DISCUSSION

The genetic basis for the phenotypic variability observed among cells of cultured plant tissues has been the subject of considerable interest and speculation (12) although there is relatively little experimental evidence which bears directly on this subject. Aside from the numerous observations and reports concerning the general subject of phenotypic variability associated with 'tissue cul-

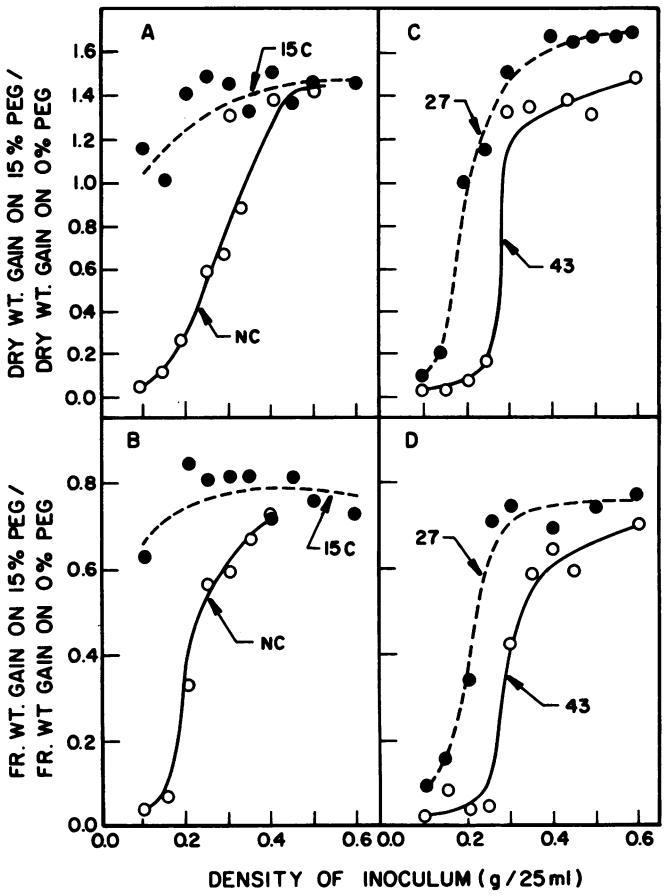

FIG. 7. Effect of inoculum cell density on tolerance to PEG-induced water stress. Tolerance is shown as either fresh or dry weight gain on 15% PEG as per cent of the gain on 0% PEG medium. Cells used for inoculum were taken from stationary phase cultures. (NC), Nonselected cell population; (15C), cell population adjusted to medium containing 15% PEG; (27), clone 27; (43), clone 43.

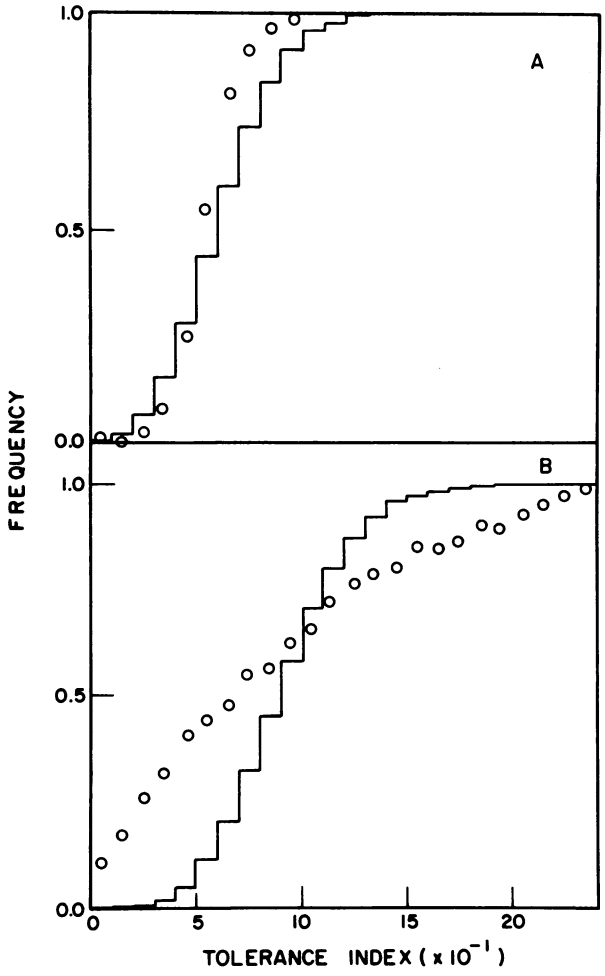

FIG. 8. Frequency distribution (as cumulative frequency ≦ no. shown) of TI values for (A) replicate samples (control 2) of inocula taken from 40 individual flasks at stationary growth phase. The 40 replicate inocula were separated from each other for approximately two generations before measurement of TI. B, Clones taken from the same population as in (A). Individual clones were separated from each other for approximately 31 generations before measurement of TI. The Poisson distributions shown are for the mean's of the respective sample populations. A, mean = 58; (B), mean = 87.

ture,' a number of investigators have demonstrated that clonal cell lines isolated from cultured plant (1, 7, 11, 14, 15, 18, 21–23) and animal (19) cell populations exhibit considerable phenotypic variability. As early as 1958, Hildebrandt observed differences among cells for color, growth rate, texture, morphology, nutritional requirements, and susceptibility to virus (10). Later, Sievert and Hildebrandt (20) noted clonal variation for the ability to grow on various carbon sources with tobacco cultures. Others have isolated clones which vary in their ability to produce anthocyanin (7), grow in the presence of ABA (22, 23), utilize urea as the only source of nitrogen (21), produce nicotine (18) and grow in the absence of growth regulators (14). Collectively, these studies essentially have shown that within a population of cultured cells there exist individuals with variant forms of a particular characteristic and are each able to pass on their own forms of the characteristic to their descendents. The different forms of these characters may be present either in the original population of cells placed into culture or may arise in culture. In the sense that these characters have certain properties, *e.g.* that they may be induced by the cells' external environment, they are often referred to as epigenetic (3). Epigenetic clonal variation in cell populations has been carefully examined by Meins and Binns for the cytokinin habituation character in tobacco cultures (14). They concluded that cytokinin habituation is passed through cell generations in culture, but the phenotype of any given cell can change quantitatively through a gradual process which can be influenced by the cells' environment. These quantitative changes in the degree of habituation can be in either direction, *i.e.* greater or lesser habituation, but over long-term culture (years) there is a gradual shift of the cell population distribution toward greater habituation. A very important observation was made concerning this slow transition. Increased habituation of the population occurred by a gradual shift of the entire population and not by an increasing proportion of a subpopulation as would be expected if the population changes occurred by simple selection of more highly habituated phenotypes. Similar conclusions were drawn by Skokut and Filner (21) and Yamaya and Filner (24) concerning changes in urease levels in cultured tobacco cells when grown with urea or NO_3 as the N source.

We know that if the original tomato cell population is placed under water stress by adding PEG to the medium the population of cells will increase its tolerance (ID_{50} of original population is ~8% PEG and that of cells growing in 5% PEG is ~26% PEG; see Ref. 4). However, if clones are isolated from the population of cells growing on 25% PEG and grown in the absence of PEG, there is no enrichment of the population for tolerant clones (Fig. 5D; Table I). There are a number of possible explanations for this. Inasmuch as we are not able to plate the clones on PEG medium

(agar medium containing PEG will not solidify), clones from the population growing on 25% PEG may undergo some back selection as they grow in the plates without PEG in the medium. However, because the growth of colonies from the 25% PEG population appeared to occur normally (plating efficiency was unaffected), any selection would have to have occurred on a substantial proportion of the population. The cell population growing on 25% PEG thus would have to be composed of a substantial number of cells with low tolerance. The more likely explanation is that individual cells are induced to change their level of tolerance by the stress level of the medium. During the plating and subsequent establishment in liquid culture, cell clones which had been tolerant when growing on 25% PEG must revert to a less tolerant phenotype. Thus, the population distribution or the mean for TI values of clones from the cell population growing on 25% PEG did not differ significantly from those of cells growing on 0% PEG as can be seen in Figure 5 and Table I. Since individual clones can change their tolerance after exposure to stress (Figs. 3 and 4) and the tolerance change induced by stress is distinct from tolerance differences between clones with respect to growth cycle dependence (Fig. 3) and density effects (Fig. 7), this seems the likely explanation for the observed population dynamics. It appears that applying selection pressure in this manner will not distinguish between tolerance which is independent of the presence of stress and tolerance which is gained by adaptation, because tolerance differences between clones (independent of the presence of stress) are not large enough to prevent the less-tolerant cell types from adapting and remaining a significant proportion of a 'selected' population. Reconstitution experiments using tolerant and intolerant clones with easily distinguishable markers should allow accurate measurement of the ability of selection pressure to enrich for the more stable tolerant clones.

When the mean TI values of the replicate samples of the stationary phase nonselected cell population are compared to the mean TI values of clones taken at random from this population, it appears that the clones have a higher mean TI (Fig. 5; Table I). This difference is significant at the 0.05 level by a t test but not significant by the same test when the TI values are transformed to $\log_{10}$ values. All of the clone analyses indicate that there is higher variability for tolerance between clones than between replicates of populations. This tendency suggests that the cells tend to behave (with respect to TI) less tolerant when they are together compared to when they are separated into individual clones. An interaction between clones affecting tolerance is suggested. Perhaps the clones with the higher TI values exhibit less tolerance when mixed with less-tolerant clones because of some cell-cell interaction.

It should be pointed out that, in the fluctuation analysis, the use of TI instead of number of variants for the construction of the frequency distributions places some constraints on the interpretation of the results. Very low frequencies of more tolerant genotypes should not influence the TI measurement since the less tolerant types also can adjust and grow in the stress medium in less time than would be required for an infrequent more-tolerant genotype to grow to a measureable mass. This means that the differences between clones for TI (if the interpretation of the fluctuation test results is that differences are due to spontaneous genotype changes) either result from high frequency spontanous genotypic changes or that the population from which the clones were taken contained a high frequency of both variants and nonvariants. In the latter situation, multi-cell clones would likely contain both variant and nonvariant cells in high frequencies relative to each other since clonal cell clumps contained few cells (*i.e.* ten or less). It is the ratio of variant to nonvariant cells within a clump which is selected as a clone which determines its relative tolerance in our tolerance measurements. Furthermore, the ratios of variant to nonvariant cells in the cell clumps would not likely be randomly distributed within a sample population of clones because of the ways in which plant cells divide and form clumps in culture (16). That is they tend to produce clumps consisting of mainly variant or nonvariant cells which would make the frequency distribution of the ratios of variant to nonvariant cells within such multi-cell clones tend to fit the distribution predictions of the fluctuation test for spontaneously occurring variants. The bimodal appearance of the subclone populations could be explained also by either of these possibilities, *i.e.* high frequency genotypic changes or clones containing a mixture of genotypes. Fluctuation tests of the subclones of clone 43 and clone 27 indicated deviation from the Poisson distribution for TI ($P < 0.01$). It may be difficult to use fluctuation analysis alone to analyze traits which are induced or acquired but also may undergo spontaneous changes in their ability to be induced or acquired.

Inasmuch as it appears now that the tolerance exhibited by the cell populations exposed to PEG-induced water stress results from an adaptation response, it might be questioned whether plants with enhanced tolerance to water stress ever could be recovered by cell cultures techniques. Clearly, it is not possible to draw such conclusions at this time since there seem to be more stable tolerance differences between cell clones in the populations, and it is not known exactly how the tolerance differences between clones is related to differences in tolerance due to adaptation. Although it appears that osmotic adjustment through the accumulation of several types of solutes is at least a part of the adaptive tolerance mechanism (9), we know very little of the tolerance mechanism responsible for the more stable clonal variation. If this mechanism is, as discussed earlier, based on differential adaptability through osmotic adjustment, then it might very well be expressed in whole plants as greater or lesser adaptability.

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