QUANTITATIVE MEASUREMENT OF THE ABILITY OF DIFFERENT MUTAGENS TO INDUCE AN INHERITED CHANGE IN PHENOTYPE TO ALLOW MALTOSE UTILIZATION IN SUSPENSION CULTURES OF THE SOYBEAN, *GLYCINE MAX* (L.) MERR.

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ABSTRACT

Using a newly developed plating system, we have measured cell survival and the frequencies of variation in an inherited trait after treatment of soybean cell suspensions with different mutagens: ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG), hycanthone (1-{[2-(diethylamino) ethyl] amino}-4-(hydroxymethyl)-9H-thioxanthen-9-one and ultraviolet light (UV).——The heritable variation selected for displays a phenotype of rapid growth on maltose as carbon source. The marker is stable in the absence of maltose, and prolonged growth of variant cells on sucrose has not shown reversions to slow growth. Doubling time in suspension cultures is decreased from 100 hr to *ca.* 30 hr by the mutation. Both wild-type and variant cells grow on sucrose with a 24-hr doubling time. Thus, lethality after mutagen treatment can be estimated rapidly by growth on sucrose, whereas variants are scored on maltose medium. The spontaneous frequency of variants was 1.2×10^{-7}; induced frequencies ranged from a low of 3.6×10^{-5} for EMS to a high of 10^{-3} for hycanthone. The high frequency of variants induced by hycanthone, a frameshift mutagen, and the observation that UV induces variants in haploid cells with much higher frequency than in diploid cells suggests a recessive mutation.

CELLS from many plants can be grown in tissue culture as cell suspensions. In many cases, such suspensions can be used to produce a callus that, in turn, can regenerate a plant (KURZ and CONSTABEL 1979). Despite the fact that these techniques have been known for many years, only a few successful genetic experiments have been carried out with cell-suspension material. In general, the lack of mutants available in different plant systems has delayed progress in somatic-cell genetics using cell-suspension cultures. Soybeans have been grown in cell suspension culture for more than 10 years (GAMBORG 1966); however, almost no variants are known. Though protoplast-fusion techniques have been developed (KAO and MICHAYLUK 1974; COCKING 1978) whereby complementation studies could be carried out and, in many systems, it is possible to regenerate

plants from cell suspensions, few genetic experiments have taken advantage of these powerful techniques.

Two related obstacles have prevented a more intense attack on mutant production. The first has been the lack of rapid-plating techniques adequate to insure the survival of mutant cells despite severe selection pressures used to kill wild-type populations. The second has been an inadequate knowledge of the effect of different mutagens on plant suspension cultures. Studies from whole plants have not been useful in making predictions about mutagenesis in cell suspensions. In most of these studies, either seeds or pollen have been subjected to mutagens. These materials are difficult to treat and may also react in a rather specialized way since the cells are not dividing.

Several laboratories have isolated variants from suspension cultures or from protoplast suspensions obtained from plant tissues (MALIGA 1978). Two studies have dealt quantitatively with the effects of mutagens on suspended cells. In one study (SUNG 1976), cell suspensions of soybeans, *Glycine max* (L.) Merr., and carrots (*Daucus carota* L.) were treated with mutagens. A rough estimate of frequency of variants was obtained for carrots (10-fold increase over spontaneous). In the other, mesophyll protoplasts of haploid *Datura innoxia* Mill. were treated with mutagens and killing curves obtained. Variant frequency for one selected mutagen dose was reported, but no extensive study of the frequency of variants was carried out (SCHIEDER 1976).

Recently, we have developed a new plating system for selecting plant cells in suspension culture and growing them rapidly into clones on membrane filters (WEBER and LARK 1979). Using this system, we can select clones within one week under severe selection conditions. The system makes use of a feeder suspension layer separated from the cells under selection by a membrane filter. The cells on the membrane filter can be transferred from one selection medium to another and from one feeder population to another. These cells are protected from possible effects of toxins produced by wild-type cells dying on top of the membrane filter, since toxic products diffuse away from the population of cells. Similarly, by transferring the cells under selection frequently onto fresh feeder cells (just brought into contact with a selecting agent), it is possible to maintain a healthy feeder population during the period of selection.

In this paper, we have carried out a quantitative study of the effects of different mutagens on soybean cell suspension cultures. Five mutagenic agents have been studied: ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG), ultraviolet light (UV), and the frame-shift mutagen, hycanthone (HARTMAN *et al.* 1971). All of these mutagens produced killing of cells as well as variants at high frequency. To do this, we have made use of a variant cell line that results in the ability of cells to grow rapidly using maltose as a carbon-energy source due to increased permeability to maltose (LIMBERG, CRESS and LARK 1979). This variant was selected in cell suspension culture by growing cells on maltose. Under these conditions, wild-type cells grew exponentially with an eight-day generation time, as opposed to the normal one-day generation time on sucrose. After a long

period of selection and continued growth at the low rate in batch culture, a new population of cells arose which grew with a four-day generation time. This population is stable and maintained its four-day doubling time on maltose despite hundreds of generations of growth in nonselective medium (LIMBERG, CRESS and LARK 1979). If growth on maltose was continued, other populations arose; these had growth rates of 38-hr or 24-hr doubling times. These were stable, as revertants were not detected after prolonged nonselective growth. The selection of these different cell types has been repeated several times which has led us to the conclusion that the growth rate on maltose is increased by sequential heritable changes (possibly mutations). This assumption was supported by experiments attempting to produce rapidly growing mutants by plating and selecting directly on maltose medium. Under these conditions, we were unable to select very rapidly growing cells directly from wild-type populations. However, when a population of cells able to grow on maltose with a generation time of four days was used as starting material, we could select spontaneous variants that grow on maltose even more rapidly. All of the rapidly growing clones that arose were stable and no phenocopies were encountered. Reconstruction experiments already published (WEBER and LARK 1979) demonstrated that the rapidly growing forms would plate with the same efficiency in the presence or absence of a 1000-fold larger population of wild-type cells. This system has been used to measure frequencies of variants after treatment with different mutagens.

Nothing is known about genetic characteristics of the maltose-utilizing variant except that in some aspects it resembles a recessive mutation. The stability of its inheritance and the spontaneous occurrence of variants have made it useful as a model system for measuring doses of mutagens required to obtain heritable changes in plant cell suspension cultures. Preliminary experiments have indicated that these doses of mutagens were useful in selecting other heritable traits, such as resistance to chlorate, 8 azaguanine or the ability to survive selection with 5-bromo-2′-deoxyuridine and light.

The data presented below should prove useful to others who wish to induce mutants in plant cell cultures.

MATERIALS AND METHODS

Cell cultures and media: All cell lines used are listed in Table 1. SB1 wild-type strain originally was obtained from O. L. GAMBORG, Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada. A haploid callus culture of soybean was provided by W. D. BEVERSDORF, University of Guelph, Guelph, Ontario, Canada, from which a suspension culture was initiated in this laboratory. All cultures were maintained on gyratory shakers (150 rpm) as described by CHU and LARK (1976).

Experiments involving plating used a system of feeder plates described in detail elsewhere (WEBER and LARK 1979). Briefly, cells were plated in petri dishes on membrane filters over exponentially growing feeder suspension cultures of SB1 or M24 cells. Colonies of 2 mm diameter could be scored after 1 week at 33° with a plating efficiency of 50%. To insure continuous growth under selective conditions, the membrane filters with the plated cells were transferred to fresh feeder cells every 2 days. Murashige and Skoog medium (MURASHIGE and SKOOG 1962) was used containing 1 μg/ml 2.4 dichlorophenoxyacetic acid and 0.2% caseinhydrolysate (caseinhydrolysate, acid, vitamin and salt free, ICN Pharmaceutical Inc., Cleve-

TABLE 1

Cell lines used in experiments

Cell line	Derived from	Characteristics	Reference
SB1	*Glycine max* (L.) Merr. cv. Mandarin	wild type soybean $2n-3=37$ (KAO *et al.* 1970) doubling times in sucrose: 24 hr; maltose: 200 hr.	GAMBORG (1966)
M100	SB1	$2n-3=37$ doubling times: sucrose 24 hr; maltose 100 hr.	LIMBERG, CRESS and LARK (1979)
M24	M100	$2n-3=37$ doubling times: sucrose 24 hr; maltose 24 hr.	LIMBERG, CRESS and LARK (1979)
S1n	*Glycine max* (L.) Merr.	Male sterile mutant (ms_1) of *Glycine max* (L.) Merr. $n=20$ haploid line derived from doubling times: sucrose 24 hr; maltose 200 hr.	W. D. BEVERSDORF

land, Ohio). Two percent sucrose (Sigma Chemical Co., St. Louis, Missouri) or 2% maltose (J. T. Baker Chemical Co., Phillipsburg, N.J.) was used as sole carbon sources (WEBER and LARK 1979).

Cell numbers were counted in a Neubauer hemacytometer after converting cell suspensions into protoplasts. Cell viability was determined by use of the vital stain, tryptophan blue (WEBER and LARK 1979).

The following mutagens were used to treat cell suspensions: EMS (Eastman-Kodak, Rochester, N.Y.) and MMS (Aldrich Chemical Co., Milwaukee, Wisconsin) were dissolved in 10-fold excess of dimethylsulfoxide. MNNG (Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in distilled water and filter sterilized. Aliquots were frozen at —20°. After thawing they were used immediately. Hycanthone (Sterling-Winthrop Research Institute, Rensselaer, N.Y.) was dissolved in 1N HCl; the pH was then adjusted to 4.3. The filter-sterilized solution was stored at —20°. For chemical mutagenesis, cultures were treated 1 day after subculture with various doses of mutagens for 17 hr. Normal growth conditions were maintained (33°; shaking). After washing the cells 3 times in appropriate culture medium, they were plated.

When using UV as the mutagen, cells were plated on membrane filters without feeder cells. A 15W General Electric germicidal lamp was used as a UV source. The UV flux at the surface of the cells was 30 erg/mm^2/sec. After treatment, the feeder cells were added and the plates grown as described above.

Cells were treated with mutagens under nonselective conditions. The survival after mutagenesis was determined by plating dilutions of cells on feeder plates containing sucrose. All experiments were repeated several times. The killing by different doses of mutagen was measured as a decrease of the number of colonies scored 1 week after plating dilutions of the cells on sucrose medium with SB1 feeder cells. The dose response was expressed as the ratio of surviving cells to nontreated controls. Variant frequencies were determined by plating at least 10^6

cells on plates with maltose as the sole carbon source. Every 2 days, the membrane filters were transferred to new plates with fresh feeder cultures. After 6 to 7 days, colonies were scored.

In order to determine whether photoreactivation could occur after UV irradiation, cells were treated with UV and incubated in the dark or under a fluorescent light source known to kill soybean cells after pretreatment with 5-bromo-2'-deoxyuridine (CHU and LARK 1976) or to photoreactivate *E. coli*.

RESULTS

Spontaneous mutation frequencies and the effects of different mutagens: Cell suspension cultures from a single clone of M100 cells were grown in sucrose and then plated at different times on maltose medium. Variants were selected and counted. A spontaneous frequency of around 10^{-7} was observed in cultures growing in nonselective sucrose medium (see Table 2). During growth in selective

TABLE 2

Survival of M100 cells and frequency of fast-growing variant cells after treatment with different mutagens*

Mutagen	Dose	N/N_0† (%)	Frequency of variants	Average variant frequency
No treatment	—	100	6.8×10^{-8}	
			9.2×10^{-8}	1.2×10^{-7}
			1.0×10^{-7}	
			2.0×10^{-7}	
MMS (μl/100 ml)	7	62	24.0×10^{-6}	24.0×10^{-6}
	10	1.0	140.0×10^{-6}	
		1.5	200.0×10^{-6}	150.0×10^{-6}
		0.7	100.0×10^{-6}	
EMS (μl/100 ml)	100	6.5	2.6×10^{-6}	2.6×10^{-6}
	200	0.5	36.0×10^{-6}	36.0×10^{-6}
		0.8	35.0×10^{-6}	
MNNG (μg/ml)	10	39	7.7×10^{-6}	6.4×10^{-6}
		58	5.1×10^{-6}	
	30	5	100.0×10^{-6}	105.0×10^{-6}
		6	110.0×10^{-6}	
Hycanthone (μg/ml)	2	49	11.0×10^{-6}	8.7×10^{-6}
		51	6.4×10^{-6}	
	5	1	990.0×10^{-6}	1000.0×10^{-6}
		0.5	1000.0×10^{-6}	
UV (erg/mm²)	900	14	21.0×10^{-6}	42.0×10^{-6}
		11	62.0×10^{-6}	
	2250	0.2	380.0×10^{-6}	390.0×10^{-6}
		0.8	390.0×10^{-6}	

* Variant frequencies were determined by plating 10^8 or more cells on plates with maltose as sole carbon source. The variant frequencies were obtained in independent experiments. Twenty to 25 plates were scored for rapidly growing clones in each experiment for individual mutagen doses.

† Relative survival of M100 cells subjected to given mutagen dose. N_0 = number of colonies per plate arising from nontreated cells.

medium, variants accumulated exponentially in a manner predicted from their more rapid growth rate and their subsequent selective advantage in this medium.

When cells were treated with the five different mutagens (EMS, MMS, MNNG, UV or hycanthone), killing of cells was observed, as well as a higher frequency of variants able to grow rapidly on maltose. The results of these experiments are summarized in Table 2. Figures 1a, b, c and d present detailed results for the killing of cells and the production of variants by four of the different mutagens. In all experiments, 10 to 25 fast-growing variant colonies were isolated. They were grown under nonselective conditions for approximately six to eight generations. Upon testing on maltose as the sole carbon source, all of them had retained their ability to grow fast. In every case, the rise in frequency was offset by increased killing at high doses. Only one mutagen, EMS, appeared to be inefficient. The frequencies of variants induced by the different mutagens ranged from a low for EMS of about 3.6 per 10^5 survivors to a high of one per 10^3 survivors for hycanthone, the frame-shift mutagen. With the exception of EMS, the four other mutagens appear to be quite adequate for producing variants at doses in which the killing is not extravagant. Because hycanthone is a frame-

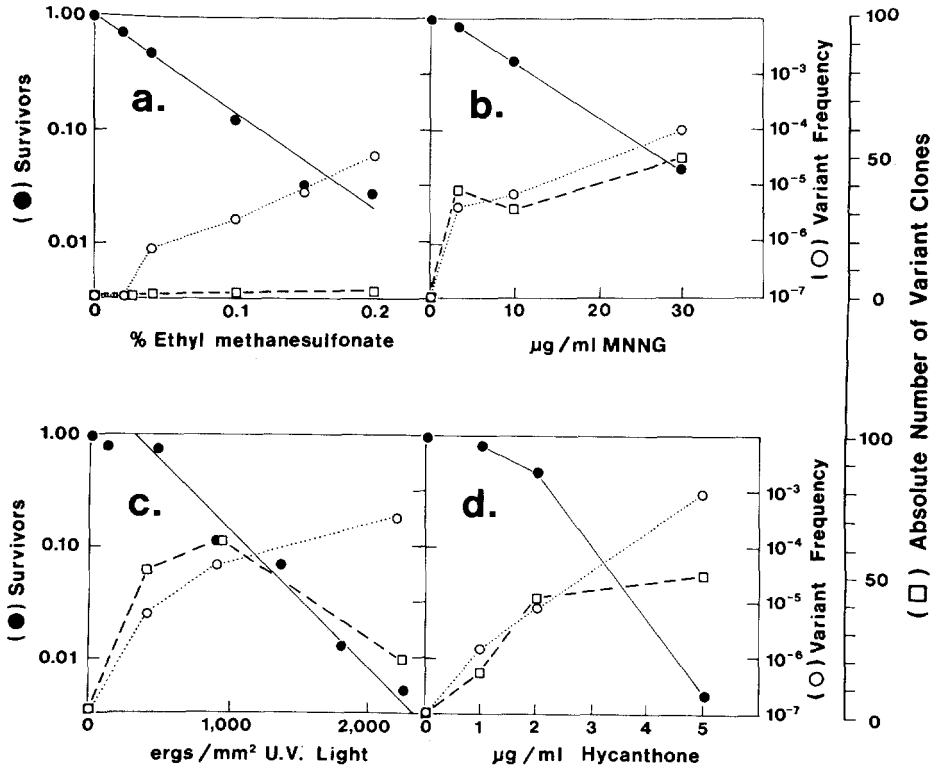

FIGURE 1.—Comparison of killing and frequencies of variants at different doses of EMS (a), MNNG (b), UV (c), hycanthone (d). Abscissa: Dose of mutagen. Ordinate: fraction of surviving clones; absolute frequency of variant clones; ratio of variant to total clones.

TABLE 3

Frequencies of fast-growing variant clones growing on maltose after treating haploid and diploid cells with UV*

	No treatment	UV 1000 erg/mm²
S1n	3.5×10^{-7}	1.3×10^{-5}
(haploid)	2.0×10^{-7}	7.4×10^{-5}
SB1	$< 5 \times 10^{-8}$	1.3×10^{-7}
(diploid)		8.0×10^{-8}

* Variant frequencies reported are the results of two independent experiments. The data shown represent the average of 20 plates being sampled in each experiment.

shift mutagen, it was thought that rapid growth on maltose might result from a recessive mutation. This was somewhat surprising to us since the phenotype of this variant results in permeability to maltose and its subsequent utilization for growth (LIMBERG, CRESS and LARK 1979). Therefore, we decided to investigate the effect of mutagenesis on maltose utilization in haploid and diploid populations of soybean cell suspensions. We found that the frequency of variation in the wild-type, diploid SB-1 strain was far lower than in the haploid strain (Table 3). This result is in agreement with the finding that hycanthone produces a high frequency of variation. Together they suggest that the variation could be due to a mutation.

Our cell population might contain a subpopulation of cells resistant to mutagenic treatments that might also have the capability of growing much more rapidly on maltose. To test this, we carried out an experiment in which rapidly growing clones were selected on maltose after treatment with MMS or MNNG. Suspensions of cells from these clones were then tested for their sensitivity to killing by different doses of the same or another mutagen. In every case, the rate of killing was comparable to that of M100 parent cells. Similarly, cells were mutagenized for different lentghs of time to select mutagen-resistant populations that might be capable of growing more rapidly on maltose. Again no resistant population was obtained.

Upon UV treatment, no photoreactivation could be observed in our experiments. When cells were irradiated with UV to a survival of either 30% or 5%, no increase in colonies occurred despite 24-hr exposure to visible light (see MATERIALS AND METHODS). This result was surprising, but may be explained by the fact that our cells are continually grown in the dark and may have lost an adaptive response to make photoreactivating enzymes.

DISCUSSION

Our results indicate that all of the tested mutagens were capable of increasing the frequency of heritable change. Since it was not possible to regenerate variant cells into whole plants, it was not possible to characterize the inheritance of the

new phenotype. However, rapidly growing cells have been grown for hundreds of generations under nonselective conditions on sucrose (our own data, not presented; also see LIMBERG, CRESS and LARK 1979). No lag in growth was observed upon return to selective medium (maltose) unlike other variants that have been reported (MALIGA *et al.* 1976). This observation suggests a permanent change in the metabolism of the cells. Furthermore, our results demonstrated that the frequency of variants increased with increasing mutagen dose. Both findings are compatible with the assumption that changes in the genetic material (mutations) have taken place.

Although all of the mutagens tried were capable of inducing variants in soybean cells in suspension culture, a surprising result was the low frequency of variants induced by EMS and the high frequency induced by the frame-shift mutagen, hycanthone. The low frequency of variants obtained with EMS may be due to the fact that these cells are rapidly dividing. Thus, EMS may kill cells before mutations can be induced. If cells were arrested and not allowed to divide, it is possible that the killing rate would drop, allowing longer exposure to the mutagen and possible higher frequencies of variants.

As indicated above, the heritable change to maltose utilization was originally observed as a stepwise process. The fact that maltose utilization could be induced by treating M100 cells with the frame-shift mutagen, hycanthone, indicates that, if this variation is due to mutation, it could be a recessive mutation suggesting that the M100 cell line may have only one functional locus or be heteroploid for this marker.

Cytogenetic studies of the soybean and related subgenera have suggested that *Glycine max* (L.) Merr. may be tetraploid (HADLEY and HYMOWITZ 1973). As yet, it has been impossible to obtain chromosome-deficient plants ($2n - 1 = 39$, etc.), but trisomics $2n + 1$, 2 or 3 have been obtained, and the high frequency of trisomic transmission is compatible with tetraploidy (SORRELLS and BINGHAM 1979). However, a recent comparative survey of the relative frequency of dominant and recessive mutations isolated from the soybean and from other crops indicates that more recessive alleles have been found in the soybean than in rice, alfalfa, cotton or wheat. Corn had a higher proportion of recessives (RECK SOBEL, personal communication). This suggests that, if the soybean is a tetraploid, it is an allotetraploid (BINGHAM, BEVERSDORF and CUTTER 1976). Our finding that UV induced a much higher frequency of variants in gametic haploid cultures ($n = 20$) than in the diploid line, SB1 ($2n - 3 = 37$), supports the concept of allotetraploidy. Moreover, recent attempts to use our mutagenesis techniques to detect other recessive phenotypes has yielded an apparent high frequency of such variants (see below). Thus, a large proportion of the haploid genome ($n = 20$) may be unique.

The concentration of mutagens needed to produce variants in plant cells is high. With the exception of hycanthone, all of the agents are required in doses approximately 10 times the level required to produce variants in animal cells. However, the relative frequency of variants for the number of viable cells re-

maining after treatment with mutagen must be viewed with some latitude. In all probability, the frequency of variants per surviving cells was higher than measured because the soybean cells grow as clumps in cell suspension, and it is necessary to destroy most of the cells in a clump before the clump ceases to produce a colony. Thus, the frequency of survivors decreases as a multihit curve.

Our results have encouraged us to search for variants with recessive phenotypes, using a sibling selection technique. Using UV, we have recently obtained variants from our S1n line, which require casamino acids for growth. These have been recovered with a frequency of 1%.

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