On the development of a mutant isolation scheme from cell and tissue cultures of Datura innoxia (Miller)

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On the development of a mutant isolation scheme from cell and tissue cultures of *Datura innoxia* (Miller)

by

Jerome Peter Ranch

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of

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For the Graduate College

Iowa State University
Ames, Iowa

1979
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<td>5-BrdUrd</td>
<td>5-bromodeoxyuridine</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>ethylene imine</td>
</tr>
<tr>
<td>EMS</td>
<td>ethyl methanesulfonic acid</td>
</tr>
<tr>
<td>5-FUra</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>5-FUrd</td>
<td>5-fluorouridine</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
</tr>
<tr>
<td>NSG</td>
<td>N-methyl-N-nitro-N-nitrosoguanidine</td>
</tr>
<tr>
<td>NAA</td>
<td>(\alpha)-napthaleneacetic acid</td>
</tr>
<tr>
<td>UV</td>
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ABSTRACT

A mutagenesis and selection scheme for the isolation of variants from plant cell and tissue cultures has been examined. Fresh petiole explants, and fragments of recently initiated callus and shoot cultures of monoploid and diploid *Datura innoxia* were treated with mutagenic agents. Subsequent selection of variants occurred at the cellular and organ level of organization with minimal exposure to the *in vitro* environment. Mutagenesis was directed at cells of known ploidy, and no apparent impairment of morphogenetic competency due to cultural conditions was observed. Variants resistant to a wide variety of anti-metabolites were sought. Five variants resistant to normally lethal concentrations of cycloheximide were selected at the shoot level of organization.

As an integral part of the selective scheme, the batch suspension culture of *D. innoxia* was further refined and characterized with respect to growth rate optimization and aggregate dissociation in medium lacking organic nitrogen. Cell separation was enhanced with superoptimal 2,4-D concentrations and was correlated with increased mitotic activity of smaller aggregates relative to large aggregates. Malic and succinic acids, although permitting rapid doubling times, caused a decrease in aggregate dissociation.

The use of turbidimetric methods in plant suspension cultures was investigated and appears to be an excellent non-destructive method of determining growth kinetics.
I. INTRODUCTION AND STATEMENT OF THE PROBLEM

The recovery of desirable phenotypes in higher plants (i.e. Angiosperms) through mutational breeding is a laborious process. Generally, mutagenized pollen is used for ovule fertilization, or seeds are treated with a suitable mutagen, and the \( M_2 \) is screened for the phenotype sought. The \( M_2 \) may consist of thousands of individuals, thus the selection procedure is lengthy and requires much space, particularly with important agronomic species such as corn, soybean, oats, and wheat. However, these procedures are efficient to the extent that desirable mutations can be obtained (Gaul, 1961; MacKey, 1961; Nybom, 1961; Quinby, 1961; Santos, 1969; Doll and Sandfaer, 1969; Konzak, 1972; Scarascia-Mugnozza, Bagnara, and Bozzini, 1972; International Atomic Energy Agency, 1976).

The large amount of labor necessary for the isolation of mutant phenotypes from higher plants is a consequence of an evolutionary trend in the plant kingdom toward reduction in the size, complexity, and duration of the gametophyte generation. Lower plants, such as mosses and fungi, possess a relatively long-lived, autonomous gametophyte, or haploid, generation (haplophase). Because of this, mutant selection schemes can be completed during haplophase, and since this generation is monoploid, all genetic lesions are immediately visible. In contrast, higher plants do not possess an autonomous gametophyte generation. Selection in higher plants is therefore usually confined to the sporophyte generation (diplophase), making it necessary to screen the progeny of sexual crosses. Diploid (and polyploid as well) material is wholly inadequate for mutational research with higher plants.
The mycelial fungus, *Neurospora crassa*, for example, is monoploid during much of its vegetative growth cycle (Figure 1.1). Because of this, it has become a favorite experimental organism for biochemists and geneticists (Beadle and Tatum, 1941; Beadle, 1945). Asexually produced conidiaspores are discharged from vegetative fruiting bodies, the conidia, and being monoploid, can be mutagenized and plated directly on selective medium. Only germinating spores are survivors of the selective conditions and hence are presumptive mutants (Srb, 1974). Fusion between sexually complementary conidiaspores and hyphae results in the development of a diploid zygote. Meiosis ensues, and the resulting monoploid ascospores can be used to determine linkage relationships through classical recombinational genetics.

Yeasts are unicellular diploid fungi that multiply by somatic budding (Figure 1.1). Environmental conditions unfavorable to growth can cause the formation of an ascus. The resulting ascospores are meiotic products and so are monoploid. Mutants can be isolated using techniques similar to those used with *Neurospora*.

In the Bryophyta, the mosses and liverworts, meiotically produced spores are released by the sporophyte (Figure 1.1). Germination of these spores results in a multicellular, haploid, gametophyte. Mutagenesis is directed at the spore, and selection is carried out in the gametophyte (Schieder, 1973). If a dioecious species is used, sperm from the male gametophyte fertilizes the egg of the female gametophyte, and a diploid sporophyte develops on the female. The spores can be used in recombinational studies. In dioecious species, male and female spore segregants can be cytologically recognized as sex determination is mediated by the
Figure 1.1 Life cycles of model plant species illustrating the phylogenetic trend toward modification of the gametophyte in alternation of generations.
LIFE CYCLE OF SACCHAROMYCES CEREVISIAE

LIFE CYCLE OF NEUROSPORA CRASSA
SPOROPHYTE GENERATION

SPORES (n)

MEIOSIS

XY (2N)

FERTILIZATION

Y(N) X(N)

GAMETOPHYTE GENERATION

LIFE CYCLE OF A DIOECIOUS MOSS

GAMETOPHYTE GENERATION

SPOROPHYTE GENERATION

MICROSPORE MOTHER CELL

2N

MEIOSIS

POLLEN (n)

2N

ZYGOTE (2N)

EMBRYO SAC

SEED

Figure 1.1 (continued)
presence of \( X \) (male), or \( Y \) (female) chromosomes.

Ferns, like the Bryophytes, are characterized by an autonomous gametophyte, the prothallus. The prothallus is derived from meiotically produced spores. Carlson (1969) has isolated several auxotrophic mutants of \textit{Osmunda cinnamomea} at the prothallus level using the BrdUrd enrichment technique (Puck and Kao, 1967; see also pages 17 to 21).

To shorten mutant isolation procedures in higher plants, it would be advantageous to use haploid (preferably monoploid) tissues, or to be able to screen the \( M_1 \) for mutational events. A most remarkable phenomenon makes this suggestion an alternative mutant isolation technique in higher plants. It has been observed that anthers, harvested at the proper stage of microsporogenesis, and cultured under the appropriate conditions, can undergo microspore embryogenesis resulting in the development of plants with the paternal gametophytic genome - haploid plants. This occurrence was first observed by Guha and Maheshwari (1964) with \textit{Datura innoxia}, and has been reported in an increasingly larger number of other species (Vasil and Nitsch, 1975; Reinert and Bajaj, 1977; Sink and Padmanabhan, 1977). Anther culture of \( M_1 \) plants, because of allelic segregation, allows immediate recovery of mutant phenotypes, and screening occurs in less time using less space (Melchers and Labib, 1970; Vyskot, Novak, and Havranek, 1974). A haploid plant with a desired phenotype can be somatically doubled (Jensen, 1974) and converted to a homozygous condition and normal sporophytic chromosome complement for sexual genetic studies and transfer of the desirable allele to other genetic stocks. Although this method is appealing, caution should be exercised, as haploid sporophytes of only judiciously chosen species (or cultivars) are truly mono-
ploid in chromosome complement, $2n=x$, thus containing genes in single dosages. Obviously, haploid plants from polyploid species are not monoploid, but it remains to be seen if such plants are functionally, or only structurally polyhaploid. In the former instance, only dominant mutations would be readily visible. If a recessive induced mutation would be present in perhaps the triplex condition, it would not, or rarely, visibly segregate in anther-derived plants.

The first report of sustained, serially subcultured growth of plant cells in suspension culture by Muir, Hildebrandt, and Riker (1954) provided another advance in the plant sciences which may enable more rapid plant breeding. Alone, the culture of plant cells is unimportant to the plant breeder, who, of course, wishes whole plants. Consequently, the reports by Steward, Mapes, and Mears (1958), and Stewart (1958) on embryogenesis in carrot suspension cultures, and the elegant demonstration by Skoog and Miller (1957) of adventitious shoot and root organogenesis from tobacco pith callus mediated by the relative and absolute concentrations of auxin and cytokinin, has enabled the tissue culturist to regenerate whole plants from cultured cells. Not only were these demonstrations of totipotency crucial to the development of mutant isolation technology from cell and tissue culture, but from a genetic and developmental viewpoint, indicated that somatic cells contain all the necessary genetic information required for a complete organism, and that development results from differential gene activity rather than by selective gene loss.

Researchers then attempted to show that individual plant cells in culture could be treated as microorganisms, and subsequently be regenerated to whole plants. Tissue from monoploid sporophytes (obtained through
anther culture) could be cultured, and screening for mutants of various types is simplified as the cells contain single gene copies. Mutations should thus be immediately observable (Binding, 1974).

Thus, plant cell and tissue culture was hoped to be of great value in the study of mutation in higher plants, and in the isolation of mutant phenotypes. At the cell culture level, millions of genotypes might be screened many times a year regardless of season. If the selective conditions allowed the proliferation of a desired cellular phenotype, and the cells were morphogenetically competent, whole plants with the desired phenotype might be obtained. Mutants may possess agronomically, pharmacologically, or ornamentally important phenotypes, and may be used for studies in biochemical genetics, developmental phenomena, and further our understanding of plant biology (Dulieiu, 1972; Street, 1973; Zenk, 1974; Carlson and Polacco, 1975; Green, 1977).

As will be described in the Integrative Perspective, the isolation of mutants from plant cell and tissue cultures was not, and still is not today a real alternative to classical methods. Many problems are found to be associated with the in vitro approach. Cultured plant cells do not respond as microorganisms, and there is no a priori reason to assume they should. Mutant isolation from plant cell and tissue cultures has generally followed techniques developed for microbial systems with the eventual outcome of poor efficiency of mutant isolation.

To be efficient, mutant isolation protocols in plant cell and tissue culture systems must include:

1. mutagenesis of cells with known ploidy - preferably monoploid,
2. high capacity for differentiation of the selected cells or
tissue,
(3) stability of the chromosome number,
(4) selection which allows proliferation only of the mutant phenotype,
(5) allowing an appropriate time for mutation fixation,
(6) expression of a phenotype in the whole plant in the desired fashion if selected at the cellular level, and
(7) use of an effective mutagen.

The use of a species which can easily be manipulated in vitro is, of course, particularly advantageous.

An efficient mutant isolation scheme from cultured plant cells, based on the criteria above, has yet to be described. The development of this mutant isolation scheme was initiated with the objective of satisfying the above demands of efficiency using *Datura innoxia* as a test species. Cells of known ploidy and high morphogenetic capacity should be mutagenized. Selection of mutant phenotypes should be made at a developmental level that insures the proper expression of the desired phenotype in the whole plant, with exposure to the in vitro environment being kept to a minimum.

At some juncture during a mutant isolation scheme from cultured plant cells, suspension cultures may be used either for rapid propagation of large numbers of cells, or for the isolation of single cells for cloning purposes. Aggregation, the failure of daughter cells to separate and become single cells, occurs to an extent in all plant cell suspension cultures. Cells comprising an aggregate are genetically the same since they are derived from the same parental cell. However, as de Jong, Jansen, and Olson (1967), and Verma and VanHuystee (1970) have demon-
strated, all cells in an aggregate are not the same metabolically. It is probable this heterogeneity of gene expression is due to differing cell environments within an aggregate. It is reasonable to postulate, then, that plant cells cultured as single, non-aggregated entities would not exhibit heterogeneity of gene expression. If a mutant isolation scheme involves the use of suspension cultures, it is desirable to use cells which are physiologically and metabolically homogeneous to reduce variability of response to the selective agent. It is necessary, therefore, to define cultural conditions favorable to aggregate dissociation while permitting rapid growth. Maximal aggregate dissociation, to the limit of single cells, would minimize cross-feeding, allow uniform exposure of exogenously supplied substances to all cells, and assist in the isolation of single cells for cloning purposes.

An additional goal would be the development of plant cell culture media lacking organic nitrogen which allows rapid growth and aggregate dissociation. Organic nitrogen, particularly in the form of amino acids, must be omitted from culture media if amino acid analog resistant mutants, or amino acid auxotrophs are sought.

*Atropa inoxia* was chosen for this study because of its well-characterized plasticity in cell and tissue culture. Suspension and callus cultures are easily initiated and maintained (Engvild, 1974), and adventitious shoots from stem pith explants can be initiated and propagated in large numbers (Engvild, 1973). Protoplasts from suspension cultures (Furner, King, and Gamborg, 1978), and leaf mesophyll (Schieder, 1975) can be isolated, will regenerate a cell wall, proliferate, and organize into whole plants. Haploid sporophytes can be obtained through anther
culture (Nitsch, 1972), and are, significantly, monoploid in chromosome complement. Because of these advantages, and after the initiation of this work, the use of *D. innoxia* as a model somatic cell genetic system was proposed (Maliga, 1976). *D. innoxia* being a member of the Solanaceae, is related to tomato and potato, and thus results from this work will be readily applicable to these important crop plants.
II. FACTORS AFFECTING MUTANT SELECTION FROM CULTURED PLANT CELLS - AN INTEGRATIVE PERSPECTIVE

A. Genetic and Epigenetic Variability

A mutant is characterized by a phenotype departing, in some respect, from a wild or standard type. Several alternative, but not mutually exclusive, criteria may be used to define such deviations:

1. gross phenotypic disturbances,
2. alteration in gene product, either in structure or function,
3. change in the DNA sequence,
4. stability of the phenotype in the absence of a selective agent,
5. appearance of the phenotype at low frequency, with increased frequency of occurrence after mutagenic treatment, and
6. heritability through mitosis and meiosis.

A mutation may be considered a point or deletion type lesion, but chromosome rearrangements and loss can be considered mutations providing a heritable change occurs. In the strictest sense, any deviant phenotype should be termed "variant" until a genetic lesion can unequivocally be demonstrated. From this viewpoint, many variants isolated from plant cell and tissue cultures have not yet been demonstrated to be true mutants. In some cases the phenotype is transient, or, as this phenomenon is termed, epigenetic. Carlson (1978) has estimated that 90% of the putative mutants isolated in his laboratory are epigenetic as they lose their variant characteristics. Indeed, many of the variants isolated from plant cell and tissue culture have been show to be epigenetic in nature. These phenotypes are lost upon differentiation to whole plants (Jacques and Sung, 1978), meiotically not transmitted (Dix, 1977), or are
unstable in the absence of the selective agent (Maliga, Lazar, Svab, and Nagy, 1976).

Epigenetic variability, in the sense used here, is that phenotypic variability arising from non-genetic (i.e. non-mutational) events. It is well known, for example, that plant cell suspension cultures exhibit marked metabolic differences from cell to cell (de Jong, Jansen, and Olson, 1967; Verma and VanHuystee, 1970). Some epigenetic variability may be derived from these differences in metabolic status, and if reflected in differential gene activity, might influence the recovery of variants if the differences are maintained through the selection process (Binns and Meins, 1973; Meins and Binns, 1977).

Certain epigenetic variants may be generated through gene amplification in the presence of a selective agent. Alt, Kellems, Bertino, and Schimke (1978) have isolated murine Sarcoma 180 cells resistant to the folic acid analog, methotrexate, which possess approximately 200-fold more copies of the dihydrofolate reductase gene. Some resistant lines stably retained this selective gene amplification in the absence of methotrexate, while others reverted to the normal gene dosage.

Gene expression is known to vary during a growth cycle (King and Street, 1977), as well as during the cell cycle (Mitchison, 1971). Under these conditions, the response of certain cells to a selective agent may be different. For example, the selective agent may be biocidal during specific physiological and biochemical states, but biostatic in others. Removal of the selective agent would allow proliferation of non-mutant cells maintained in a static condition by the selective agent (Dix and Street, 1976; Aviv and Galun, 1977b).
A second type of epigenetic variability, the superposition of developmental events on the expression of a true mutant phenotype possibly was observed by Jacques and Sung (1978). In this instance, carrot cell lines resistant to 5-FUra were selected in suspension culture and the trait remained stable in the absence of the selective agent. Plantlets organized from these resistant cell lines, however, were sensitive to 5-FUra, while suspension cultures initiated from these plants remained resistant to the analog. It is conceivable that the carrot plantlets were more sensitive to 5-FUra than suspension cultures, or a metabolic event not active *in vitro* was inhibited by 5-FUra *in vivo*. Alternatively, an isozyme may be expressed *in vitro* different from that active *in vivo*. Thus, an isozyme modified to confer resistance to 5-FUra may be expressed *in vitro* (which was the initial level of selection), while the 5-FUra sensitive isozyme was active in the whole plant. This concept has been given impetus by the observations of Widholm (1978b) with regard to certain 5-methyltryptophan resistant cell lines of *Nicotiana tabacum*. Anthranilate synthetase is inhibited by 5-methyltryptophan, and cultured cells resistant to this analog posses an altered enzyme. Plants regenerated from these resistant cultures, however, had an unaltered anthranilate synthetase. Cell cultures of these plants again expressed the altered enzyme. These results illustrate that expression of a phenotype is influenced by the ontogenetic state. If a certain phenotype is desirable in the whole plant, based on these observations, it might be expedient to select for that phenotype at the whole plant level rather than at some less differentiated organizational level. The fact that a number of variants have been isolated in cell and tissue culture (Table 2.1)
have maintained a stable phenotype through to the whole plant, and in some cases, have been demonstrated to be meiotically transmitted, could be merely fortuitous.

Hormone habituation (prototrophy) is an often reported and intensely investigated epigenetic variant. It is a condition in which cultured plant cells no longer require an exogenous supply of hormones for growth. This phenotype is lost when prototrophic cells are regenerated to plants (Sacristan and Wendt-Gallitelli, 1971; Meins, 1974), can be reversed to hormone dependence under specific conditions (Binns and Meins, 1973), and has been observed to occur with very high frequency - for example, $10^{-3}$/cell/generation for cytokinin habituation (Binns and Meins, 1973), and $10^{-4}$/cell/generation for auxin habituation (Syono and Furuya, 1974).

A review of the literature concerning epigenetic variability in plant and animal systems is beyond the scope of this review (see Siminovitch, 1976). It is sufficient to maintain that any variant isolated from cultured cells must be characterized fully before elevating the variant to mutant status. Perhaps the best criterion of a variant being a true mutant is meiotic transmission or the trait. Epigenetic variability is not transmitted meiotically (Maliga, 1976), but if the phenotype is due to a mutation, sexual hybridization would further allow localization of the lesion to nuclear or cytoplasmic linkage groups, determination of the relationship of the mutant allele to the wild type allele (i.e. dominant versus recessive), and allow the transfer of the mutant allele to other genetic stocks.
B. Classification of Possible Mutant Types and Methods of Selection

All classical types of mutants might be obtained from plant cell and tissue culture. Lines resistant to a chemical or physical factor could be selected. Chemical selective agents might be metabolic analogs or drugs, while physical factors include drought, disease (which may also be a chemical selection if a pathogen produces a toxin), or temperature.

Resistance variants may be selected recurrently - selecting, over time, cells resistant to increasingly higher, but sub-lethal concentrations of the selective agent. Selections of this type may be mediated by induced or spontaneous mutation prior to exposure to the selective agent. In plant cell and tissue cultures, resistance to 2,4-D (Zenk,1974; Oswald, Smith, and Phillips, 1977), 8-azaguanine ( Lescure, 1973; Bright and Northcote, 1975), and Helminthosporium maydis pathotoxin (Gegenbach and Green, 1975), have been obtained using a recurrent selection protocol. Recurrent, or incremental, selection, might be useful for isolation of phenotypes based on the modification of more than one gene (alteration of more than one biochemical or physiological process to confer resistance).

Resistance variants may also be selected stringently. After mutagenesis, cells are cultured in the presence of a normally lethal concentration of the selective agent. Only genotypes conferring resistance permit survival. One-step selections such as this might be reserved for mutant phenotypes based on a single gene. Examples of stringent selections in plant cell and tissue cultures include resistance to methionine sulfoximine (Carlson, 1973), streptomycin (Maliga, S.Breznovits, and Marton, 1973b), and Phytophthora infestans pathotoxin
In the broadest sense, one may classify variants capable of growth in the presence of a normally non-utilized (but not lethal) substance as a resistance variant. Thus, the *N. tabacum* cell lines able to utilize glycerol (Chaleff and Parsons, 1978), and the galactose utilizing lines of *Saccharum* (Maretzki and Thom, 1978), may be considered glycerol- and galactose-resistant phenotypes respectively.

At the gross phenotypic level variants may be selected visually, either at the cell or organ level of organization. Nishi, Yoshida, Mori, and Sugano (1974) have isolated several lines of cultured carrot cells with different pigmentation characteristics, and a number of pigment-deficient *D. innoxia* plants have been selected in shoot cultures (Schieder, 1976).

Auxotrophic plant cell lines have been sought, but generally have been difficult to obtain. Carlson (1970) used the 5-BrdUrd enrichment technique of Puck and Kao (1967) with suspension cultures of allodihaploid *N. tabacum* and reported the recovery of several leaky amino acid and vitamin auxotrophs. Essentially, suspension cultures were mutagenized, and any mutation fixed by subsequent incubation in fully supplemented medium for one to two generations. The suspensions were thoroughly washed, and incubated in minimal medium to allow the normal cells to proliferate while exhausting the intracellular pools of any auxotroph. Treatment with 5-BrdUrd, which was incorporated into the DNA on the growing wild type cells, but not into the DNA of the static auxotrophic cells, followed. Exposure of the cells to light caused photolysis of the DNA in the normal cells thereby killing them. Auxotrophic cells were not killed
by the light treatment because 5-BrdUrd was not incorporated into their DNA. Shifting the cells to fully supplemented medium allows the recovery of the auxotrophs.

Techniques used to isolate mutants in bacterial and mammalian cell culture systems should be carefully scrutinized before assuming they may be of value in plant cell culture. For example, although Carlson (1970) has apparently used the 5-BrdUrd enrichment technique to successfully recover auxotrophic plant cell lines, Zyrd (1976) reported that 5-BrdUrd was poorly incorporated into Acer pseudoplatanus suspension cultured cells. With the aim of permitting enhanced 5-BrdUrd incorporation into Acer DNA, 5-FUrd was used, with or without Urd, to specifically inhibit thymidylate synthetase. The results of these experiments indicated that after a 72 h incubation (about 1.5 cell generations), 5-(6-^3H)-BrdUrd alone replaced only 0.5% of the thymidine residues. Maximal 5-BrdUrd replacement occurred when 5-BrdUrd with 5-FUrd, or 5-BrdUrd with 5-FUrd plus Urd, were added fractionally (four times at 18 h intervals). In this case, respectively, 8.5% and 23% of the thymidine was replaced by 5-BrdUrd. In a test of the selective technique, an auxin-requiring cell line was washed free of auxin, and resuspended in auxin-free medium. The cells were incubated with fractional additions of 5-FUrd and Urd with 5-BrdUrd in concentrations of 0 to 5 X 10^{-5} M. Control cells were exposed to the same treatments, but 2,4-D was added to the medium. After treatment, the cells were washed, resuspended in 2,4-D-containing medium, and plated. Cells exposed to the treatment without 2,4-D, regardless of the 5-BrdUrd concentration, exhibited a plating efficiency of 50%. Cells exposed to 2,4-D during the treatment exhibited a linear decrease in
the plating efficiency from 50% with no 5-BrdUrd, to 0% with $5 \times 10^{-5}$ M 5-BrdUrd. This modification of the 5-BrdUrd enrichment technique might be useful for the isolation of auxotrophic mutants from cultured Acer cells and perhaps from cultures of other plant species.

Although the 5-BrdUrd enrichment technique has been used successfully in the isolation of auxotrophs from mammalian cell cultures, even here, Chu, Sun, and Chang (1972), using Chinese hamster line V79, have demonstrated that 5-BrdUrd functions as a mutagen rather than a selective agent. They attempted the rescue of auxotrophic mutants from mixtures of normal (ght+) and a mutant cell line (ght−; multiple growth requirement for glycine, hypoxanthine, and thymidine). As an initial control, ght+ cells were treated with $10^{-5}$ M or $10^{-4}$ M 5-BrdUrd for 24 h, with or without prior starvation for glycine, hypoxanthine and thymidine for 24 to 48 h. After incubation with 5-BrdUrd, the cells were irradiated with "black-light" for 1.5 h. This treatment should be lethal to these wild type cultures. However, approximately 100 of every $10^6$ cells plated survived. Prior starvation had no effect on this frequency. Thus, if this technique were used to isolate auxotrophs, and the frequency of mutation to auxotrophy was $10^{-6}$/cell/generation, about 100 colonies would need to be screened for the mutational event - not a very efficient recovery. Starvation of mixtures of ght+ and ght− cells prior to the selective treatments did not increase the frequency of colony formation; starvation of these mixtures should increase the frequency of colony formation as the ght− cells should not be killed by the selective treatment. This experiment was repeated with several other auxotrophic markers with similar results. They additionally were able to induce and isolate galac-
tose auxotrophs and 8-azaguanine resistant variants in high frequencies with 5-BrdUrd treatments with or without prior mutagenesis with EMS.

Another selective technique which might be useful in the recovery of auxotrophic mutants is the use of metabolic analogs, which when anabolized, are converted into a biocidal substance. Such an approach has been taken by Müller and Grafe (1978) in the isolation of reduced nitrogen-requiring *N. tabacum* cell lines. Mutagenized cells were cultured in a medium containing chlorate, a nitrate analog. Wild type cells convert chlorate to chlorite to cause death. Mutant cells possessed a modified nitrate reductase incapable of this conversion, and were unable to grow on medium containing nitrate, or nitrate and ammonium, as the sole nitrogen source. Wild type cells are capable of proliferation in both of these media. The chlorate-resistant cells were able to grow on medium with amino acids, even if a lethal concentration of chlorate was included. The mutants were shown to possess no nitrate reductase activity.

Unexpectedly, methods used in the isolation of auxotrophic mutants from mammalian cell cultures have not as yet been extrapolated to plant cell culture systems. Besides the use of 5-BrdUrd, which has already been described, other DNA "poisons", such as cytosine arabinonucleoside (Thompson, Manbrood, Baker, Till, Simitovitch, and Whitemore, 1970), 5-FUrd (Meiss and Basilico, 1972), and high specific activity 3H-thymidine (Thompson et al., 1970), have not been utilized. Aviv and Galun (1977a) used 5-FUrd in an attempt to recover auxotrophs from *N. tabacum* protoplasts, but were unsuccessful. Another technique useful for the selection of auxotrophic mutants is the "thymidine-less death" method. Mutagenized
cells are exposed to aminopterin, a folic acid antagonist which blocks the synthesis of thymidine from uridine. Thymidine is not included in selective minimal medium, but amino acid and protein synthesis proceeds normally in wild type cells until the lack of thymidine causes cell death. Mutant cells, usually amino acid auxotrophs, cannot synthesize proteins in the minimal medium, DNA synthesis is not initiated, and the mutant can be recovered by transfer to supplemented medium. Glutamine auxotrophs of Chinese hamster cells have been isolated using this enrichment technique (Chu et al., 1969), but it has not yet been used in plant cell culture. The general topic of mutant isolation from mammalian cell cultures has been capably reviewed by Thompson and Baker (1973).

Various auxotroph enrichment methods used in bacterial systems are based on the same basic principle as the 5-BrdUrd scheme - surviving cells in minimal medium utilize a biocidal substance and are killed, while mutants unable to proliferate in minimal medium are not killed by the poison. Penicillin has been used successfully in this way (Davis, 1948; Lederberg and Zinder, 1948), and, no doubt, other antibiotics may be equally effective. Bal, Balbin, and Pieniazek (1974) used the polyene antibiotic, N-glycosyl polifungin, to select auxotrophic Aspergillus nidulans. Surviving cells in minimal medium incorporate this analog into plasma membranes and are killed.

In bacterial systems the use of replica plating (Lederberg and Lederberg, 1952) has largely superseded other methods for the isolation of auxotrophic mutants. Briefly, a bacterial suspension is evenly plated on the surface of solid supplemented medium. After the formation of colonies, a circle of velveteen is pressed firmly in contact with the
surface of the plate (the master), and fragments of the bacterial colonies become attached to the pile of the velveteen. The surface of the velveteen with attached bacteria is then pressed upon the surface of replica plates containing minimal medium, thus transferring the bacteria. Careful attention must be paid to the orientation of the velveteen on the replica plate relative to the master, for colonies proliferating on the master plate, but not on the replica plates are presumptive auxotrophs for the nutrient lacking in the medium of the replica plate. Plated plant cell suspensions are not amenable to this method of replica plating because of their low friability and inability to adhere to velveteen or other material. Schulte and Zenk (1977) have developed a replica plating technique for use in plant cell culture. In their experiments, suspensions of *Morinda citrifolia* were sprayed onto agar plates as an aerosol. After 10 to 14 days growth, a small-pore mesh nylon cloth cut to fit into the petri dish was placed over the surface of the plated cells. During an additional 20 days growth, the colonies which developed were channeled through the pores of the cloth. The side of the nylon mesh directed toward the lid of the master plate was placed in contact with a replica plate. Approximately 80% of the colonies were transferred to the replica plate. This efficiency of transfer, unfortunately, is not good enough for expedient mutant isolation. If, for example, $10^5$ colonies developed on the master plate, 20,000 colonies would apparently exhibit no growth on the replica, minimal medium, plates. All these colonies would need to be screened, for it would be difficult to determine by this method if lack of growth on the replica plate was due to a mutational event to auxotrophy, or because of a failure of the colony to
be transferred.

The period subsequent to mutagenesis, but prior to exposure to selective conditions in which cells are allowed to proliferate freely in non-selective medium is termed mutation fixation. An analysis of the effect of the timing of the mutation fixation period has not, as yet, been accomplished with plant cell and tissue cultures, but the importance of the mutation fixation period has been demonstrated in mammalian cell culture. Hsie, Brimer, Mitchell, and Gosslee (1975) treated Chinese hamster line $K_1^1-BH_4$ with EMS and selected for variants resistant to 6-thioguanine. If selection occurred during the first 3 days post-mutagenesis no resistant variants were recovered. Selection after 4 to 7 days, however, produced an increase in the recovery of resistant variants, with a maximum occurring at 7 days post-mutagenesis. The doubling time of this cell line was approximately 12 h, so about 14 generations passed before a maximum in selection frequency could be attained.

There is no reason to assume that after a mutational event within a specific cell that the cell should immediately commence proliferation at the same rate as an un-mutagenized cell. Lag in growth after mutagenic treatment may be caused by recovery from the mutational event itself, or may be due to a poor growth rate of a fixed mutant. Sufficient time should be allowed for the variant to establish itself as a proliferating, unique genotype.

C. Choice of Cells or Tissues for Use in Mutant Isolation

1. Suspension and callus culture

The source of cultured cells used for mutant isolation should be carefully chosen. Suspensions are useful because large numbers of cells
can be propagated in a small area. In general, cultured plant cells maintained as suspensions or callus characteristically exhibit changes in ploidy and chromosome structure (d'Amato, 1977; Sunderland, 1977).

Sacristan (1971), for example, examined the chromosome number of serially propagated callus cultures of monoploid and diploid Crepis capillaris for approximately 1 year. *C. capillaris* is an ideal species for this type of investigation because of its low chromosome number, 2n=6, and easily differentiated chromosomes. After the third passage, 40% of the cells derived from monoploid tissue were diploid, and upon further subcultures, the frequency of monoploid metaphases continued downward to about 20%. In contrast, diploid-derived tissues appeared more stable since at the fifteenth passage, 70% of the cells screened remained diploid. The degree of chromosome structural changes in these *Crepis* cultures was found to vary with the ploidy level. While 9% of the diploid cells from a monoploid-derived culture showed chromosomal aberrations, 16% of the tetraploid cells in the same culture displayed similar structural alterations.

The factors which influence the stability of chromosome number in cultured plant cells remain unknown. For suspension and callus cultures to be of use in mutant isolation in higher plants, it does appear that subculturing regime, culture media, and phytohormone concentrations must be empirically determined for each species to effect a stable chromosome number, particularly if the cultures are derived from monoploid tissues.

Frequent subculture of suspension cultures, every 3 to 4 days, thus maintaining the cells in continuous exponential growth, has been
observed to dramatically stabilize the chromosome number at the initial ploidy level. Singh and Harvey (1975), for example, initiated suspension cultures from 122-day old callus cultures of *Haplopappus gracilis* (2n=4), and while after 7 days in culture approximately 53% of the cells contained 4 chromosome, the frequency of 4-chromosome cells increased to 93% after 258 days of frequent subculturing.

More recent attempts to experimentally maintain a stable chromosome number in plant suspension cultures has been reported by Bayliss (1977a; 1977b). In this instance, diploid carrot cells were found to increase in ploidy under a 14-day passage subculturing regime. When the cells were subjected to 7-day passages, the frequency of tetraploid cells decreased from 3% to 0.2% in 2 passages. The apparent cause for this difference in ploidy between subculturing regimes was a selective advantage of polyploid cells in the 14-day passages. Phosphate was found to be the growth limiting nutrient in the medium used (Murashige and Skoog), and because of the long passage period, phosphate was utilized to near exhaustion from the medium. When phosphate was the growth limiting nutrient, polyploid cells were able to compete more effectively than diploid cells thus increasing the frequency of polyploid cells.

Furner, King, and Gamborg (1978) employed a frequent subculturing regime with monoploid *D. innoxia* suspension cultures. After several months, 57% of the cells remained monoploid.

Singh (1975) examined stability of chromosome number and structure of *H. gracilis* suspension cultures during a frequent subculturing regime in B5 (Gamborg et al., 1968) or Murashige and Skoog (1962) medium with \(2.25 \times 10^{-6} \text{ M } 2,4\text{-D}\). After 286 days in culture, cells in Murashige
and Skoog medium were observed to exhibit a higher frequency of polyplloid cells (6.2%) than cells grown in B5 medium (1.9%). The frequency of chromosomal bridges, fragments, and laggards during anaphase was also greater in cells from Murashige and Skoog medium (16.6%) than those from B5 medium (11.7%).

Relative and absolute hormone concentrations, particularly of auxin and cytokinin, have been shown to exert an influence on the stability of the chromosome number. Bennici, Buiatti, d'Amato, and Pagliai (1971) examined the chromosome complement of *H. gracilis* callus cultures grown on a series of Murashige and Skoog media with different concentrations of NAA and kinetin. After 30 days, cells on a medium with 0.02 ppm kinetin + 1 ppm NAA exhibited only polyploid mitoses, while callus proliferating on a medium with 0.02 ppm kinetin + 4 ppm NAA displayed only 20% polyploid mitoses.

It might be reasoned that cultured cells maintained as suspensions or callus for only a short period from the time of initiation from an explant would be less subject to undergo aberrations in chromosome number or structure. Mastrangelo, Epp, and Smith (1974) have studied this possibility with cultures from monoploid *D. innoxia*. Fresh explants were callused, and the chromosome number of suspensions derived from this callus was examined in the initiation passage. Even though the cells were exposed to the *in vitro* environment for a short time, only 50% of the cells screened were monoploid.

Based on the observations of Day and Jones (1971) on the p-fluorophenylalanine-mediated haploidization of the fungus *Ustilago violacea*, Gupta and Carlson (1972) used this analog in an attempt to stabilize the
chromosome number of allodiploid *N. tabacum* callus cultures. Their data indicated that exposure to 9 \( \mu g/ml \) killed 2n callus while n callus proliferated freely. Cytologically, they reported maintenance of the n chromosome number in callus grown in the presence of p-fluorophenylalanine. Dix and Street (1974) repeated this experiment using callus and suspension cultures derived from monoploid and diploid *N. sylvestris* (2n=2X=24). Diploid callus died on exposure to 37.5 \( \mu g/ml \) p-fluorophenylalanine, but monoploid callus grew vigorously. Callus cultures initiated from different monoploid plant isolates displayed differences in sensitivity to p-fluorophenylalanine - in one case, monoploid callus died only on exposure to 75 \( \mu g/ml \). These differences in sensitivity were maintained in suspension cultures. Suspensions were initiated from monoploid-derived callus cultures and were Feulgen stained after 3 passages in a medium containing 37.5 or 50 \( \mu g/ml \) p-fluorophenylalanine. The cells were analyzed by microdensitometry, and the data indicated that although p-fluorophenylalanine was present in these suspension cultures, a large proportion of the cells were polyploid. For example, in the presence of 37.5 \( \mu g/ml \) p-fluorophenylalanine, cultured cells of the monoploid plant H9 exhibited 48% of the cells 2c or greater. In the absence of p-fluorophenylalanine, cultures of the same derivation showed 36% of the cells 2c or higher. It was concluded that p-fluorophenylalanine did not permit preferential growth, or segregation of cells with a haploid chromosome complement, but because the tissues used in these experiments were initially obtained from different anther-derived plants, segregation of genotypes more resistant to p-fluorophenylalanine occurred.

If regeneration of plants from callus or suspension cultures is
desired, does the instability of the ploidy level in any way affect the efficiency of plant regeneration or the chromosome number of the regenerated plants? Torrey (1967) has observed a progressive loss in morphogenetic competency of tobacco callus with increased polyploidy and aneuploidy. The loss of organ forming potential was therefore correlated with increasing variation in chromosome number. Sacristan and Melchers (1969), however, have reported no impairment in morphogenetic competency of polyploid or aneuploid tobacco callus cultures, and were able to regenerate a large number of polyploid and aneuploid plants. Mastrangelo et al. (1974) using freshly initiated suspensions from monoploid D. innoxia have observed the chromosome number of plants regenerated from these cultures reflected the chromosome number distribution in the progenitor suspensions. In this case, there was no apparent loss in morphogenetic capability with increased chromosome number, and morphogenetic competition between cells of different ploidy did not occur. It still remains to be seen, however, if the chromosome number of cultured plant cells is the sole determinant of morphogenetic potential, or if "epigenetic" changes in response to the in vitro environment, over time, modify morphogenetic competency. Differences in morphogenetic potential with genotype have been observed with Zea mays (Green and Phillips, 1975), but this does not infer that a specific locus controls the expression of totipotency. It is probable that a combination of certain alleles or blocks of genes, either structural or regulatory, can be more conducive to growth in vitro and expression of totipotency.

2. Protoplasts

At this time, the best source of cells for use in mutant isolation
in higher plants are mesophyll protoplasts. Although protoplasts can also be isolated from suspension cultures, these protoplasts must also suffer from the same problems of chromosomal instability as cultured cells. Mesophyll protoplasts may be isolated from plants with a known chromosome complement, and, as in vivo stabilization factors preclude any drastic change in the chromosome complement, a ready source of cells with known ploidy is always available. Mutagenesis is generally directed at protoplasts immediately after isolation and the protoplasts are cultured in a medium conducive to cell wall formation and cell division. Although protoplasts usually exhibit low plating efficiencies (proportion of those protoplasts which eventually form callus colonies), Potrykus, Harms, and Lörz (1976) have demonstrated that this is only a technological problem. They reported that under concentration gradients of 7 auxins and 4 cytokinins, conditions were found that resulted in a plating efficiency of 90% for mesophyll protoplasts of haploid N. tabacum; a published standard medium permitted only a 10% plating efficiency. It is advantageous to use sterile shoot cultures as a source of leaf tissue for mesophyll protoplasts (Binding, 1975). In this way, variables associated with isolation of mesophyll protoplasts from greenhouse grown plants are eliminated.

Unfortunately, the use of protoplasts in mutant isolation is restricted to a few species. Compared to the number of species that can be cultured in vitro, cell proliferation from protoplasts has been reported much less frequently, and regeneration of the protoplasts to whole plants even less frequently (Potrykus et al., 1976; Bajaj, 1977).

The most visible obstacle to the use of protoplasts for mutant
isolation is the increase in ploidy associated with the manipulation. One might expect spontaneous fusions during protoplast isolation or culture to increase the chromosome number. In much of the literature, the chromosome number of plants regenerated from mesophyll protoplasts have not been reported - phenotypic normality has been used instead as a criterion of "true breeding" from protoplasts. Takebe, Labib, and Melchers (1971) have reported that of 195 plants organized from mesophyll protoplasts of N. tabacum, only 7 of 36 were found to possess the normal chromosome number, and the overwhelming majority of the plants obtained were morphologically abnormal.

Similarly, Schieder (1976) isolated 11 pigment-deficient plants from X-irradiated mesophyll protoplasts of monoploid D. innoxia (2n=x=12). Of these 11 variants, 10 plants had 24 chromosomes, and 1 possessed 48 chromosomes. Of 183 plants regenerated from non-irradiated protoplasts, only 7.7% were monoploid, while 78.1% were diploid, 5.5% triploid, and 8.7% tetraploid. Earlier work by Schieder (1975) in the regeneration of plants from mesophyll protoplasts of monoploid D. innoxia, 50% of the calli were monoploid, and all plants originating from monoploid calli were monoploid. If the pigment-deficient variants of D. innoxia (Schieder, 1976) prove to be inherited as simple nuclear recessive lesions, endomitosis after mutation fixation probably occurred to increase the chromosome number, thus indicating these plants are homozygous. The spontaneous fusion frequency of D. innoxia mesophyll protoplasts was found to be approximately 8%, thus fusion was probably not the major cause of ploidy increases in this system. Additionally, recessive lesions should not be expressed if fusion occurred, as the mutation frequency is
low, and fusion of any mutant protoplast would most likely be with a normal protoplast, thus resulting in a heterozygous, normal phenotype. Indeed, Furner et al. (1978) detected diplochromosomes in many protoplasts from monoploid D. innoxia suspension cultures, and suspensions derived from these protoplasts contained only 9.5% monoploid cells (the parental suspension cultures contained 57% monoploid cells, see page 25). The frequency of spontaneous fusion in this system was estimated to be 9%, thus agreeing quite well with Schieder's observation of 8%.

Although ploidy increases seem to be an unavoidable consequence of protoplast isolation and culture, this occurrence does not rule out their use in mutant isolation. As previously discussed, endoreduplication is probably the most common means to increased ploidy during protoplast isolation and culture. If mutagenesis is directed at protoplasts prior to endoreduplication, then, theoretically, the allelic status of the cells will be homozygous. If haploid tissue is used, endoreduplication may lead to the attainment of the normal 2n chromosome complement, and chemically-mediated somatic chromosome doubling of the regenerated plants, necessary for sexual hybridization, is not obligatory. Because protoplasts can be cultured as single cells, each proliferating colony of cells obtained from protoplast culture is a clone. Therefore, the growth of unique, individual genotypes can be followed, and each mutant isolated using protoplasts can be considered as arising from a unique mutational event.

3. Adventitious shoots, meristem culture, and somatic embryogenesis

In general, adventitious shoots initiated directly from fresh tissue explants exhibit minimal phenotypic variability and high chromosomal
stability (Narayanaswamy, 1977). There are few reports of variant selection at the level of adventitious shoots. Only gross morphological (Malepszy, Grunewaldt, and Maluszynski, 1977), and albino (Malepszy and Maluszynski, 1978; Schieder, 1976) variants have been selected at this level. Variants of these types must be selected visually at the plant level (or plantlet level in shoot cultures) as they are not expressed at the cell level.

The culture of shoot apices (meristem culture) is currently the most efficient means of plant propagation through tissue culture. Since the first report of shoot tip culture with orchids (Morel, 1960), meristem culture has been successfully applied to a wide variety of horticulturally and agronomically important plant species (Murashige, 1974; Quak, 1977). Because of the genetic stability of meristematic regions, shoot tip cultures provide a means of rapid plant propagation while maintaining phenotypic and chromosomal constancy (d'Amato, 1977; Reinert, Bajaj, and Zbell, 1977). Meristem culture has also been used to develop virus-free plants (Quak, 1977). There are no reports of induced mutant isolation from cultured meristems, with perhaps the only notable exception being the H. maydis pathotoxin-resistant maize lines isolated from cultures of immature embryos by Gegenbach and Green (1975).

Under certain circumstances, plant suspension cultures have been observed to undergo somatic embryogenesis, and generally the ontogenetic sequence in vitro mirrors that found in vivo (Reinert, Bajaj, and Zbell, 1977; Narayanaswamy, 1977). Although somatic embryogenesis is a widely studied phenomenon, the selection of variants at the level of the somatic embryo have not been reported.
4. Androgenetic haploid plants

Another in vitro manipulation not widely used for the isolation of mutants in plants is the selection of variants at the level of haploid sporophytes by inducing androgenesis in anthers cultured directly in selective medium. Two, perhaps unique, processes are evident during microspore androgenesis. First is the initial response of microspores in the induction of embryoid formation, with the growth of these embryoids to whole plants following. The selective conditions used in variant selection from anther cultures may have different effects in the two circumstances. It is conceivable that the selective conditions may inhibit the initial induction of embryoid formation even though the capacity for growth as an embryo under the selective conditions is present. It seems best to allow embryoid formation to occur in non-selective medium, and then transfer the developing embryoids to selective medium.

Although isolating variants in the whole plant using anther culture is seen as an ideal method, many problems are currently associated with this technique. The method is not generally applicable as proper conditions eliciting the androgenetic response have been defined for only a few species (Vasil and Nitsch, 1975; Clapham, 1977; Reinert and Bajaj, 1977; Sink and Padmanabhan, 1977; Sunderland and Dunwell; 1977). In species which can be induced to undergo microspore androgenesis, the response has been found to be highly variable. Generally, anthers are excised from floral buds and cultured at a point when the microspores are about to undergo the mitotic division to generative and vegetative cells (Sunderland, 1977) - late uni-nucleate to pollen mitosis. Stages of
pollen development can be correlated to anther, bud, or corolla length (Sunderland and Dunwell, 1977). However, due to variables, such as age and growth state of the donor plant, these relationships are inconstant (Sunderland, 1977), and it is difficult to harvest large numbers of anthers at the same developmental stage in order to conduct quantitative experiments. The importance of anther excision and culture at the proper developmental stage was indicated by Engvild, Linde-Laursen, and Lundqvist (1972) using *D. innoxia*. Anthers cultured at the uninucleate stage only produced haploid plants, while culture of anthers at later stages resulted in the production of diploid, triploid, and tetraploid plants.

Whole anthers are usually cultured on a solid medium, but recently, Nitsch (1974) cultured isolated microspores of *D. innoxia* and *N. tabacum* in a newly developed liquid medium. Under these conditions, for example, 6,984 haploid plants per floral bud (5 anthers/bud) of *N. tabacum* could be obtained. Considering each plant furnishes 50 to 100 floral buds, 3.5 X 10^5 to 7 X 10^5 haploid plants can be obtained from each donor plant. Nitsch (1974) also reported that exposing the floral buds of *D. innoxia* or *N. tabacum* to a 4°C cold treatment for 48 to 72 h prior to anther culture greatly enhanced the embryogenic response. Cold pre-treatment of floral buds prior to culture is now an almost universally used technique to increase the androgenetic response.

Culture of isolated microspores is a tedious process, particularly in the disruption of anthers and the harvesting of the microspores. Another technique developed by Sunderland and Roberts (1977) and Wernicke and Kohlenbach (1976) involves the floating of properly staged anthers
on a liquid medium. In comparison to culture on solid medium upon which 27% of *N. tabacum* anthers produced plantlets, 80% of the anthers cultured on liquid medium produced plantlets (Wernicke and Kohlenbach, 1976).

After the initial dehiscence of the anthers when cultured on liquid medium, many plantlets and embryoids in various stages of development are released. The anthers are then removed to fresh liquid culture medium, reserving the plantlets already released from the anthers. This serial subculture of anthers to fresh medium enhances the androgenetic response, and after many such subcultures, large numbers of haploid plants can be recovered (Sunderland and Roberts, 1977; Wernicke and Kohlenbach, 1976).

The direct use of anther-derived plants in mutant isolation has not been fully exploited. Early attempts by Nitsch (1969), Nitsch, Nitsch, and Peraeus-Leroy (1969), and Devreux and Saccardo (1971) to isolate mutants in anther culture by irradiation of *Nicotiana* anthers resulted in large numbers of plants with abnormal morphological features and various flower colors. EMS was also used by Nitsch (1969). Fertility of these haploid plants after colchicine doubling, and heritability of the apparent mutants have not been reported.

Vyskot et al. (1974) treated seeds of *N. tabacum* with N-methyl-N-nitrosourea, and cultured anthers from the plants derived from these seeds. The anther-derived plants were screened for chlorophyll-deficient phenotypes. With mutagen concentrations of 0.1 mM, no chlorophyll variants were found, but at concentrations of 0.2 mM to 0.5 mM, a high frequency of the plants obtained were pigment-deficient. With a 0.5 mM treatment for 24 h, for example, 40% of the plants from cultured anthers were chlorophyll-deficient. Some dwarf plants which flowered when 20 to
30 cm in height (plants usually flower when 70 cm in height) were also isolated.

In certain species, the use of anther culture in mutant isolation appears to be an excellent alternative to classical methods. Use of anther culture, even in easily manipulated species, is predicated by the occurrence of an androgenetic response, reproducible staging, and cultural conditions allowing proliferation of large numbers of individuals. Generally, these conditions have been met only in a few species, mostly within the Solanaceae. Improvements should be particularly directed toward minimizing the variability in anther staging. Perhaps a technique will be developed in which floral organ formation can be effected in vitro. Hopefully this would reduce variability due to the constancy of cultural conditions. Toward this end, Hillson and LaMotte (1977) have shown that an apparent floral gradient exists in mature N. tabacum plants. Culture of apically located stem explants on a medium with the proper hormonal constitution resulted in the formation of many floral organs.

D. Choice of Mutagen

The propensity for particular mutagens to cause certain types of mutations (mutational spectrum) has been observed in mammalian cell cultures. Friedrich and Coffino (1977) reported on the efficiency of different mutagens to generate S49 mouse lymphoma cell lines resistant to a variety of cell poisons. Mutagen specific responses were observed. Ouabain-resistance was more effectively induced by NSG than ICR 191, while ICR 191 was more effective than NSG in the recovery of lines resistant to 6-thioguanine.
Very few different mutagens have been used in the isolation of mutants from plant cell and tissue cultures (Table 2.1). Dose-response relationships, particularly with respect to the efficiency of different mutagens, have not been widely investigated in vitro either. A comparative study of different mutagens has been recently reported by Nijkamp, Colijn, and Kool (1978) in plant suspension cultures. A number of different mutagens were used to treat Petunia hybrid suspension cultures. Mutagenized cells were washed and plated on solid fresh medium containing lethal concentrations of various anti-metabolites or poisons. Although NSG supplied at 50 μg/ml for 4 h resulted in near 100% survival, the frequency of variants resistant to HgCl₂, for example, was 1.4 X 10⁻⁵/cell. Higher concentrations of NSG to 40 μg/ml, although increasing mortality, were not more effective at inducing HgCl₂-resistant phenotypes. Resistance to a number of other anti-metabolites and poisons followed a similar pattern. None of the other mutagens tested were as effective as NSG in inducing variant phenotypes with high survival frequencies.

Sung (1976a) has also reported that with soybean and carrot suspension cultures, NSG was a more efficient mutagen than EMS for the production of 5-FUrđ and cycloheximide-resistant phenotypes.

In whole plants, the mutation spectrum caused by certain mutagens, mutagen specificity, and efficiency of mutagens have been studied extensively (Brock, 1969; Doll and Sandfaer, 1969; Gustafsson, 1969; Kawai, 1969; Mikaelson, 1969; Narayanan and Konzak, 1969; Wickham, Narayanan, and Konzak, 1969; Ehrenberg, 1971; Gaul, Frimmel, Gichner, and Ulonska, 1972; Nilan, 1973; Auerbach, 1976).

Kawai (1969), for example, reviewed the efficiency of chemical and
physical mutagen treatment of seeds on the production of chlorophyll mutations in barley in relation to seed survival, and the sterility and frequency of chlorophyll mutations in the $M_1$ and $M_2$ generations. X- and $\gamma$-irradiation, thermal neutrons, 22 alkylating agents, 9 nitroso compounds, and 2 base analogs were examined. Chlorophyll mutations are easy to screen, and it is reasoned that the frequency of their occurrence should be similar to the frequency of occurrence of other mutations. Based on the above criteria, EMS proved to be the best mutagen by far for seed treatments.

The efficiency of alkylating agents is dictated by a number of factors (Narayanan and Konzak, 1969; Milan, 1973): properties of the biological system, physical and chemical properties of the mutagen, concentration of the mutagen, duration of treatment, temperature, pH, catalytic agents, pre-treatments, and post-treatments. Similarly, factors affecting the efficiency of ionizing radiation include properties of the biological system, $O_2$ concentration, temperature, pH, and water content of the tissues (Milan, 1973; Auerbach, 1976).

Mechanisms of mutagen action, factors affecting mutagen effectiveness and specificity, other aspects of mutagens and methods of mutagen treatment can not be discussed here as the literature on these subjects is extensive. The reader is advised to consult the previously cited references, as well as reviews and monographs on these subjects (Drake, 1970; Chu, 1971; Ehrenberg, 1971; Freese, 1971; Roth, 1974; Auerbach, 1976).
E. Overview

The rationale for the use of plant cell and tissue culture technology in the isolation of mutant phenotypes arises from the proposition that individual plant cells can be treated as microorganisms. A desired phenotype at the cellular level can then, under the proper conditions, be differentiated to complete plants with the same phenotype (Carlson et al. 1973; Maliga, 1976; Nabors, 1976; Smith, 1974, Widholm, 1978a). These assertions are unreasonable, for it is presumptuous to infer that a phenotype at the cellular level can be expressed identically in the whole plant (Table 2.1).

Aside from the described instability of chromosome number and morphogenetic competency of cultured plant cells, the lack of basic knowledge concerning metabolic pathways and their regulation in plants has severely hampered the effort to devise suitable selection techniques. Because of this, nutritional mutants have not unequivocally been isolated from higher plant systems. Notable exceptions are the thiamine auxotrophs of Arabidopsis (Redei and Acedo, 1976), and the proline-requiring lines of maize (Gavazzi, Nava-Racchi, and Tonelli, 1975). Both of these, however, were selected in the whole plant. The arginine-requiring lines of Ginkgo biloba (Tulecke, 1959) were isolated from pollen, but formed callus only, and biochemical studies were not reported. Without nutritional mutants, biochemical pathways are difficult to elucidate, and without sufficient knowledge of biochemical pathways, proper selection schemes can not be devised. Li, Redei, and Gowens (1967) reviewed the phylogenetic spectrum of nutritional mutants from bacteria to higher plants. Many auxotrophs exist in bacteria, but with increasing
organismal complexity, the frequency of auxotrophic mutants decreases rapidly.

There is rapidly accumulating evidence that all amino acid biosynthesis, as well as ammonium assimilation and nitrate reduction occurs in the chloroplasts (Miflin and Lea, 1977). It can not be stated with certainty that the chloroplast genome is not complex or large enough to code for all or some enzymatic steps in amino acid biosynthesis. If indeed amino acid biosynthetic enzymes are coded in the chloroplasts, nutritional mutants would be quite difficult to obtain because of the sheer number of chloroplast genomes per cell.

Because so little is truly known about the effects of in vitro culture on the behavior of plant cells, this review is a critical examination of variables which are influential in the maintenance of stability in the cultured state. Essentially, the quest for mutants in plant cell and tissue culture has been reductionist in viewpoint. However, as this review indicates, there is no need to concentrate solely on the cellular level as several levels of organization are available for mutant selection in vitro. The various shortcomings and advantages in using specific ontogenetic states in mutant isolation have have been discussed, with efficiency of mutant isolation the prime motive.
Table 2.1 Variants isolated from cell and tissue cultures of higher plants

<table>
<thead>
<tr>
<th>Variant phenotype</th>
<th>Species; ploidy</th>
<th>Mutagenized cell type; mutagen; selection level; selection type; frequency of occurrence</th>
<th>Plant; stability; inheritance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>arginine-requiring</td>
<td>Ginkgo biloba; 2x</td>
<td>pollen; spontaneous; callus; stringent; nd</td>
<td>no; nd; nd</td>
<td>Tulecke, 1959</td>
</tr>
<tr>
<td>various morphological variants</td>
<td>Nicotiana tabacum; 2x</td>
<td>anthers; EMS, γ-ray, spontaneous; whole plant; visual;</td>
<td>yes; nd in plant; nd</td>
<td>Mitsch, 1969; Mitsch et al., 1969</td>
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<tr>
<td>streptomycin-resistance</td>
<td>Petunia hybrida; x</td>
<td>callus; spontaneous; callus; stringent; nd</td>
<td>no; nd; nd</td>
<td>Binding et al., 1970; Binding, 1972</td>
</tr>
<tr>
<td>hypoxanthine-, biotin-, p-aminobenzoic acid-, arginine-, lysine-, proline-requiring</td>
<td>Nicotiana tabacum; 2x</td>
<td>suspensions; EMS; suspension plating; BrdU enrichment; nd</td>
<td>hypoxanthine-, and lysine-requiring, yes, others, no; all stable; hypoxanthine-, and lysine- requiring as single Mendelian factors</td>
<td>Carlson, 1970; Carlson et al. 1973; Chaleff and Carlson, 1974</td>
</tr>
<tr>
<td>Symbol</td>
<td>Term</td>
<td>Definition</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>a</td>
<td>mutagenized cell type</td>
<td>source of cell, tissue, or organ used in variant isolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>selection level</td>
<td>level of organization at which selection was performed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>plant</td>
<td>differentiation of variant into whole plant</td>
<td></td>
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</tr>
<tr>
<td>d</td>
<td>stability</td>
<td>stability of the variant phenotype in vitro and in vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>ploidy</td>
<td>initial chromosome complement of the mutagenized cell type used in variant isolation, where x is the basic chromosome number of the species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>f</td>
<td>selection type</td>
<td>method of exposure to selective agent, either stringent or recurrent, or by BrdU enrichment; visually if no selective agent is present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g</td>
<td>frequency of occurrence</td>
<td>frequency of occurrence of the variant phenotype relative to the total number of individuals screened</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>inheritance</td>
<td>if differentiated into whole plant, mode of inheritance, either Mendelian or cytoplasmic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i</td>
<td>nd</td>
<td>not determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>j</td>
<td>na</td>
<td>data not available</td>
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Table 2.1 (continued)

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<tr>
<th>Variant phenotype</th>
<th>Species; ploidy</th>
<th>Mutagenized cell type; mutagen; selection level; selection type; frequency of occurrence</th>
<th>Plant; stability; inheritance</th>
<th>Reference</th>
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<tr>
<td>L-threonine-</td>
<td>\textit{Nicotiana tabacum}; 4x</td>
<td>suspensions; NSG; suspensions; recurrent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Heimer and Filner, 1970</td>
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<td>resistance (with</td>
<td>various</td>
<td>anthers; X-ray; whole plant; visual; nd</td>
<td>yes; nd; nd</td>
<td>Devreux and Saccardo, 1971</td>
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<td>nitrate as the sole</td>
<td>morphological</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>nitrogen source)</td>
<td>variants</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>DL-5-methyl-</td>
<td>\textit{Daucus carota}; 2x</td>
<td>suspensions; spontaneous; suspensions; stringent; $2 \times 10^{-7}$</td>
<td>yes; stable in vitro</td>
<td>Widholm, 1972a; 1974</td>
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<td>tryptophan resistance</td>
<td></td>
<td></td>
<td>plants are not resistant, but cultures initiated from these plants are resistant; nd</td>
<td></td>
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<tr>
<td>DL-5-methyl-</td>
<td>\textit{Nicotiana tabacum}; 4x</td>
<td>suspensions; spontaneous; suspensions; stringent; $1.6 \times 10^{-6}$</td>
<td>yes; stable in vitro</td>
<td>Widholm, 1972b; 1978</td>
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<tr>
<td>tryptophan resistance</td>
<td></td>
<td></td>
<td>plants are not resistant, but cultures initiated from these plants are resistant; nd</td>
<td></td>
</tr>
<tr>
<td>Mutant Type</td>
<td>Species</td>
<td>Somatic Target</td>
<td>Resistance Type</td>
<td>Source</td>
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</tr>
<tr>
<td>Methionine/sulfoximine</td>
<td>Nicotiana tabacum; 2x</td>
<td>mesophyll protoplasts</td>
<td>yes; stable in vitro and as plant; Mendelian</td>
<td>Carlson, 1973</td>
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<tr>
<td></td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; NSG; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Lescure, 1973</td>
</tr>
<tr>
<td>5-bromodeoxyuridine</td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; NSG; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Lescure, 1973</td>
</tr>
<tr>
<td>8-azaguanine</td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; NSG; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Lescure, 1973</td>
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<td>Streptomycin</td>
<td>Nicotiana tabacum, Nicotiana sylvestris; 2x and x</td>
<td>callus; spontaneous; shoot cultures; stringent; 10^-6</td>
<td>yes; stable in vitro and as plant; cytoplasmic</td>
<td>Maliga et al., 1973b; Maliga et al., 1975; Yurina et al., 1978</td>
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<tr>
<td>5-bromodeoxyuridine</td>
<td>Nicotiana tabacum; 2x</td>
<td>callus; spontaneous; callus; stringent; nd</td>
<td>yes; stable in vitro and as plant; Mendelian</td>
<td>Maliga et al., 1973a; Marton and Maliga, 1975; Kandra and Maliga, 1977</td>
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<tr>
<td>5-bromodeoxyuridine</td>
<td>Acer pseudoplatanus; 8x</td>
<td>suspensions; spontaneous; suspensions; recurrent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Bright and Northcote, 1974</td>
</tr>
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<td>Altered callus pigmentation</td>
<td>Daucus carota; 2x</td>
<td>suspensions; NSG; suspension plating; visual; nd</td>
<td>no; stable in vitro; nd</td>
<td>Nishi et al., 1974</td>
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<tr>
<td>Variant phenotype</td>
<td>Species; ploidy</td>
<td>Mutagenized cell type; mutagen; selection level; selection type; frequency of occurrence</td>
<td>Plant; stability; inheritance</td>
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<td>5-bromodeoxyuridine resistance</td>
<td><em>Glycine max</em>; 4x</td>
<td>suspension protoplasts; NSG; callus; stringent; $4 \times 10^{-5}$</td>
<td>no; stable in vitro; nd</td>
<td>Ohyama, 1974; 1976</td>
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<td>2,4-dichlorophenoxyacetic acid resistance</td>
<td><em>Nicotiana sylvestris</em>; x</td>
<td>suspensions; spontaneous; suspensions; recurrent; nd</td>
<td>no; nd; nd</td>
<td>Zenk, 1974</td>
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<tr>
<td>sodium chloride resistance</td>
<td><em>Nicotiana sylvestris</em>; x</td>
<td>suspensions; spontaneous; suspensions; recurrent; nd</td>
<td>no; nd; nd</td>
<td>Zenk, 1974</td>
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<tr>
<td>ethionine resistance</td>
<td><em>Nicotiana sylvestris</em>; x</td>
<td>suspensions; spontaneous; suspensions; recurrent; nd</td>
<td>no; nd; nd</td>
<td>Zenk, 1974</td>
</tr>
<tr>
<td>sodium chloride resistance</td>
<td><em>Nicotiana tabacum</em>; 4x</td>
<td>suspensions; EMS; suspensions; recurrent; nd</td>
<td>no; nd; nd</td>
<td>Nabors et al., 1975</td>
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<tr>
<td>sodium chloride resistance</td>
<td><em>Nicotiana sylvestris</em>; x and 2x</td>
<td>suspensions; spontaneous; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Dix and Street, 1975</td>
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<td>Sodium chloride resistance</td>
<td>Capsicum annum; 2x</td>
<td>suspensions; spontaneous; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Dix and Street, 1975</td>
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<tr>
<td>p-fluorophenylalanine resistance</td>
<td>Daucus carota; 2x</td>
<td>suspensions; spontaneous; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Palmer and Widholm, 1975</td>
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<tr>
<td>p-fluorophenylalanine resistance</td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; spontaneous; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Palmer and Widholm, 1975</td>
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<td>Helminthosporium maydis Race T pathotoxin-resistance</td>
<td>Zea mays; 2x</td>
<td>callus; spontaneous; callus; recurrent; nd</td>
<td>yes; stable in vitro and as plant</td>
<td>Gegenbach and Green, 1975; Gegenbach et al., 1977</td>
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<tr>
<td>8-azaguanine</td>
<td>Acer pseudo- platanus; 8x</td>
<td>suspensions; NSG; suspension plating; recurrent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Bright and Northcote, 1975</td>
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<td>Cycloheximide resistance</td>
<td>Nicotiana tabacum; 2x</td>
<td>mesophyll protoplasts; EMS; callus; stringent; $4.3 \times 10^{-6}$</td>
<td>yes; unstable in vitro and as plant; nd</td>
<td>Maliga et al., 1976</td>
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<td>Chilling resistance</td>
<td>Nicotiana sylvestris; 2x</td>
<td>suspensions; EMS; suspension plating; stringent; nd</td>
<td>yes; stable in vitro; not sexually transmitted</td>
<td>Dix and Street, 1976; Dix, 1977</td>
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<tr>
<td>Chilling resistance</td>
<td>Capsicum annum; 2x</td>
<td>suspensions; EMS; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Dix and Street, 1976</td>
</tr>
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<td>Variant phenotype</td>
<td>Species; ploidy</td>
<td>Mutagenized cell type; mutagen; selection level; selection type; frequency of occurrence</td>
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<tr>
<td>p-fluoro-phenylalanine-resistance</td>
<td>Acer pseudoplatanus; 8x</td>
<td>suspensions; spontaneous; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Gathercole and Street, 1976</td>
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<td>altered pigment patterns</td>
<td>Datura innoxia; x</td>
<td>mesophyll protoplasts; X-ray; shoots; visual; 10^-4</td>
<td>yes; stable as plant; nd</td>
<td>Schieder, 1976</td>
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<td>cycloheximide-resistance</td>
<td>Daucus carota; 2x</td>
<td>suspensions; spontaneous, NSG, EMS; suspension plating; 4.4 X 10^-6 to 10^-8</td>
<td>no; variable; nd</td>
<td>Sung, 1976a</td>
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<td>5-fluorouracil-resistance</td>
<td>Daucus carota; 2x</td>
<td>suspensions; spontaneous, NSG, EMS; suspension plating; 1.2 X 10^-7 to 4.5 X 10^-9</td>
<td>no; variable; nd</td>
<td>Sung, 1976a</td>
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<td>aminoethyl-L-cysteine-resistance</td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; EMS; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Widholm, 1976</td>
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<td>δ-hydroxylysine-resistance</td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; EMS; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Widholm, 1976</td>
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<tr>
<td>Characteristic</td>
<td>Species/Genus</td>
<td>Phenotype</td>
<td>Description</td>
<td>Cited Source</td>
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<tr>
<td>ethionine-resistance</td>
<td>Daucus carota; 2x</td>
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<td>hydroxy-L-proline-resistance</td>
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<td>suspensions; EMS; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Widholm, 1976</td>
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<td>isopropyl-N-phenylcarbamate-resistance</td>
<td>Nicotiana tabacum; 4x</td>
<td>mesophyll protoplasts; EMS; callus; stringent; nd</td>
<td>yes; stable in vitro and protoplasts from plants remain resistant; nd</td>
<td>Aviv and Galun, 1977b</td>
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<td>Phytophthora infestans pathotoxin-resistance</td>
<td>Solanum tuberosum; 2x</td>
<td>callus; spontaneous; callus; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Behnke, 1977</td>
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<td>chilling-resistance</td>
<td>Picea excelsia; 2x</td>
<td>callus; spontaneous; callus; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Tumanov et al., 1977</td>
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<td>various morphological variants</td>
<td>Nicotiana sylvestris; x</td>
<td>callus; EMS, EI; whole plant; visual; nd</td>
<td>yes; stable as plant; sexually heritable</td>
<td>Malepszy et al., 1977</td>
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<td>herbicide-resistance; 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, 4-(2,4-dichlorophenoxy)butyric acid</td>
<td>Trifolium repens; 2x</td>
<td>suspensions; spontaneous; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Oswald et al., 1977</td>
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Table 2.1 (continued)

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<th>Variant phenotype</th>
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<th>Plant; stability; inheritance</th>
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<td>amitrole-resistance</td>
<td>Nicotiana tabacum; 4x</td>
<td>callus; spontaneous; callus and shoots; stringent; nd</td>
<td>yes; stable in vitro</td>
<td>Barg and Umiel, 1977</td>
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<td>Helminthosporium sacchari pathotoxin</td>
<td>Saccharum officinarum; na</td>
<td>suspensions; MMS, X-ray; suspension plating; stringent; nd</td>
<td>yes; nd; nd</td>
<td>Heinz et al., 1977</td>
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<td>Fiji disease (virus)</td>
<td>Saccharum officinarum; na</td>
<td>na; na; na; na</td>
<td>yes as stable plant; nd</td>
<td>Heinz et al., 1977</td>
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<td>aminopterin-resistance</td>
<td>Datura innoxia; x</td>
<td>suspensions; spontaneous; suspension plating; stringent; $5.4 \times 10^{-6}$ to $10^{-7}$</td>
<td>yes; stable in vitro and cultures from regenerated plants remain resistant; nd</td>
<td>Mastrangelo and Smith, 1978</td>
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<td>L-valine-resistance</td>
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<td>mesophyll protoplasts; UV; callus; stringent; nd</td>
<td>yes; nd in vitro, cultures from regenerated plants remain resistant; Mendelian</td>
<td>Bourgin, 1978; Thom, 1978</td>
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<td>galactose-resistance</td>
<td>Saccharum sp.; nd</td>
<td>suspensions; spontaneous; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Maretzki and Thom, 1978</td>
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<td>Trait</td>
<td>Organisms</td>
<td>Methods</td>
<td>Results</td>
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<td>Glycerol-resistance</td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; spontaneous; suspensions; stringent; nd</td>
<td>yes; nd in vitro, cultures from regenerated plants remain resistant; Mendelian</td>
<td>Chaleff and Parsons, 1978</td>
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<td>Reduced nitrogen-requiring</td>
<td>Nicotiana tabacum; 2x</td>
<td>suspensions; NSG; suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Muller and Grafe, 1978; Mendel and Muller, 1978</td>
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<td>&quot;Albina&quot; (chlorophyll-deficient)-</td>
<td>Nicotiana sylvestris; x</td>
<td>callus; EMS, EI, BrdU; shoot; visual; nd</td>
<td>yes; stable as plant; nd</td>
<td>Malepszy and Maluszynski, 1978</td>
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<td>Variants</td>
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<td>callus; EMS, EI, BrdU; whole plant; visual (biochemical analysis); nd</td>
<td>yes; nd; nd</td>
<td>Malepszy et al., 1978</td>
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<td>Nicotiana tabacum; 2x</td>
<td>suspensions; UV; suspension plating; stringent; nd</td>
<td>yes; stable in vitro and cultures from regenerated plants remain resistant; nd</td>
<td>Berlyn and Zelitch, 1978</td>
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<td>Iso-nicotinic acid hydrazide-resis-</td>
<td>Glycine max; 4x</td>
<td>suspensions; spontaneous; suspensions; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Limberg et al., 1978</td>
</tr>
<tr>
<td>tance</td>
<td>Tobacco; na</td>
<td>suspensions; spontaneous; suspension plating; BrdU enrichment; nd</td>
<td>no; stable in vitro; nd</td>
<td>Malmberg, 1978</td>
</tr>
<tr>
<td>Variant phenotype</td>
<td>species; ploidy</td>
<td>Mutagenized cell type; mutagen; selection level; selection type; frequency of occurrence</td>
<td>Plant; stability; inheritance</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>--------------------------------------------------------------------------------------</td>
<td>---------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>aminoethyl-L-cysteine-resistance</td>
<td>Daucus carota; 2x</td>
<td>na; na; na; na; na</td>
<td>yes; stable in vitro; nd</td>
<td>Bright and Miflin, 1978</td>
</tr>
<tr>
<td>aminoethyl-L-cysteine-resistance</td>
<td>Hordeum sp.; na</td>
<td>embryo; na; na; na; na</td>
<td>yes; stable in vitro; nd</td>
<td>Bright and Miflin, 1978</td>
</tr>
<tr>
<td>L-lysine + L-threonine-resistance</td>
<td>Zea mays; 2x</td>
<td>callus; na; callus; stringent; nd</td>
<td>yes; stable in vitro; nd</td>
<td>Hibberd et al., 1978</td>
</tr>
<tr>
<td>selenocystine, and selenomethionine-resistance</td>
<td>Nicotiana tabacum; 4x</td>
<td>suspensions; na; suspension plating; stringent; nd</td>
<td>no; unstable in vitro; nd</td>
<td>Flashman, 1978</td>
</tr>
<tr>
<td>aluminum-resistance</td>
<td>Lycopersicon sp; na</td>
<td>callus, suspensions; spontaneous; callus, suspension plating; stringent; nd</td>
<td>no; stable in vitro; nd</td>
<td>Meredith, 1978</td>
</tr>
<tr>
<td>kanamycin-resistance</td>
<td>5-fluoro-uracil-resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana sylvestris; X Nicotiana tabacum; 4X</td>
<td>Daucus carota; 2X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>suspensions; spontaneous; suspensions; stringent; nd</td>
<td>callus; NSG; callus; stringent; nd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no; stable in vitro; nd</td>
<td>yes; stable in vitro, but plants are sensitive, while cultures initiated from these plants are resistant; nd</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dix et al., 1977
Umiel and Goldner, 1976; Umiel et al., 1978; Zamski and Umiel, 1978
Jacques and Sung, 1978
III. MATERIALS AND METHODS

A. Cell and Tissue Culture

1. Isolation and chromosome analysis of haploid sporophytes

Eleven haploid plants of *D. innoxia* were isolated in 1973 by the anther culture method of Nitsch (1972), and have since been propagated vegetatively. Chromosome verification of haploidy, 2n=12, was determined by chromosome counts of preparations from corollas using a method modified after Burns (1964). Floral buds, when 13 mm to 35 mm long, were removed from vigorously growing presumptive haploid *D. innoxia* plants and the corolla dissected out. The corollas were treated with 0.003 M 8-hydroxyquinoline at 16°C for 4 h. After rinsing the treated corollas with tap water several times, the tissue was simultaneously fixed and hydrolyzed with 5 N HCl at 25°C for 10 min. The corollas were rinsed with tap water to neutral pH. Two mm$^2$ sections of the corollas were macerated with a dissecting needle in a drop of 10% modified carbol fuchsin (Kao, 1975) on a microscope slide. After lowering a cover slip, the corolla tissue was further disintegrated by gently tapping over tissue masses with a glass rod. The preparations were observed, after squashing with heavy thumb pressure, under bright-field, and the chromosomes in a visibly intact cell counted and photographed (Figure 3.1). The description of this simplified method is currently being prepared for publication.

2. Callus culture initiation and maintenance

Young petioles of greenhouse-grown monoploid and diploid *D. innoxia* were thoroughly washed with detergent to remove a naturally occurring oily secretion. The petioles were sterilized for 15 min in 10% Clorox (commercial bleach containing 5.25% sodium hypochlorite) containing
Figure 3.1 Photomicrograph of metaphase chromosomes from corolla tissue of monoploid *D. innoxia* plant X(5). X 6552
0.1% Tween 80. The petioles were rinsed with four changes of sterile distilled water and cut transversely into 2 mm segments.

Cultures were grown on a series of Murashige and Skoog (1962) media, adjusted, as listed in Table 3.1, to contain different concentrations of ammonium and nitrate. Medium DM-1 contains the same mM equivalents of ammonium and nitrate as does standard Murashige and Skoog medium. Potassium chloride was added to DM-2 to replace potassium nitrate as a source of potassium. The pH was adjusted with NaOH. During certain seasons, particularly the autumn, because of excessive bacterial contamination of initial explants, Gentamicin (Schering Corp.) was added at 50 μg/ml to culture media prior to autoclaving. No undesirable effects of this antibiotic on callus initiation or proliferation was observed at this concentration. Only the initiation passage was conducted in the presence of Gentamicin. Media were steam sterilized for 20 min at 16 lb/in^2.

Callus cultures were initiated and maintained under cool white fluorescent lamps at 500 lux with a 16 h light and 8 h dark cycle for two weeks. The cultures were then transferred to darkness for an additional two weeks. All subsequent passages were subcultured at two week intervals by transferring approximately 20 mg of callus to fresh solid medium and maintained in the dark. Callus cultures were grown at 27°C, under ambient humidity, in 100 X 15 mm disposable bacteriological petri dishes sealed with 'Parafilm M' tape.

3. **Suspension cultures**

   a. **Initiation, maintenance, and determination of growth rates**

   Suspension cultures of *P. innoxia* were initiated by placing approxi-
Table 3.1 Media used for the culture of *D. innoxia* callus and suspension cultures

<table>
<thead>
<tr>
<th>Medium components</th>
<th>DM-1</th>
<th>DM-2</th>
<th>DM-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal salts:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(NH_4)_2SO_4$ (mg/liter)</td>
<td>1360</td>
<td>1360</td>
<td>0</td>
</tr>
<tr>
<td>$KNO_3$ (mg/liter)</td>
<td>3980</td>
<td>0</td>
<td>3980</td>
</tr>
<tr>
<td>$KCl$ (mg/liter)</td>
<td>0</td>
<td>1500</td>
<td>0</td>
</tr>
<tr>
<td>other macronutrients</td>
<td>as in standard Murashige and Skoog medium (see Appendix A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>micronutrients</td>
<td>as in standard Murashige and Skoog medium (see Appendix A) except where otherwise noted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic constituents:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sucrose</td>
<td>30 g/liter for all media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>succinic or L-malic acids</td>
<td>20-40 mM for all media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vitamins</td>
<td>after Engvild for all media (see Appendix A)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>casein hydrolyzate (Sheffield 'N-Z-Amine', enzymatic digest)</td>
<td>1 g/liter</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2,4-D</td>
<td>$7.5 \times 10^{-7}$ M to $2.5 \times 10^{-6}$ M; $2.5 \times 10^{-6}$ M: $10^{-5}$ M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>agar (Difco Noble or Gibco Phytagar I)</td>
<td>0.7% for all media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>5.6 for all media</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
mately 100 mg of callus into 30 ml liquid medium. The composition of media used are listed in Table 3.1. Suspensions were initiated from callus growing on medium of the same composition except suspensions in DM-2 and DM-3, which, because of the poor growth of callus on these media, were subcultured from suspensions growing in DM-1. Cell suspensions were grown in 125 ml DeLong culture flasks, with or without turbidimetric (nephelometric) side-arms, at 135 rpm on a gyratory shaker (New Brunswick), in the dark at 27°C. The flasks were plugged with polyurethane foam plugs and capped with Morton stainless-steel closures. Suspensions were subcultured at 15 to 45 day intervals, depending on the growth rate, by transferring 2 ml of a late log culture into 30 ml of fresh medium with a wide-tip pipette. Growth kinetics were monitored turbidimetrically at 420 nm with a Klett-Summerson colorimeter (Sung, 1976b). Growth rates of suspension cultures were determined after five passages in any specific medium. Doubling times are means of 3 to 5 replicates, and are based on the regression line fitted to the log-linear phase of growth (see Appendix B). Packed cell volume was determined by collecting cultures at various Klett values in graduated conical centrifuge tubes at 2000 x g for 5 min, and was expressed as cellular volume/ml of culture volume. Dry weight/ml, and fresh weight/ml were related to Klett units by harvesting the total volume of a flask at various Klett values on a pre-weighed (wet and dry) circle of Miracloth held in a Millipore filter apparatus. Fresh weight was determined from freshly harvested cells, and the filters were dried under vacuum at 60°C for 24 h to determine dry weight.

As a control, the growth rate of *D. innoxia* was also monitored by
dry weight accumulation. Suspensions were initiated by transferring 2 ml of a Klett 500 suspension grown in DM-1 with \(2.5 \times 10^{-6} \text{M} \) 2,4-D into 30 ml medium of the same composition in 125 ml DeLong culture flasks. At 3 to 5 day intervals, the entire volume of 3 flasks were individually harvested, and the dry weight/ml of each culture was determined as described. Each data point represents the mean dry weight/ml of 3 cultures.

b. Aggregation assay  Separation of aggregates was accomplished by the sequential filtering of an entire suspension culture through 30 mesh (0.57 mm square pores), and 100 mesh (0.14 mm square pores) stainless steel cloth filters (Small Parts, Inc., Miami). The fractions obtained were termed 30 mesh residue, 100 mesh residue, and 100 mesh filtrate respectively. At each filtration step, the filter containing the cellular residue was gently rinsed with 3-150 ml washes of distilled water, the filtrates and washes being combined. Cell residues and filtrates were collected and pre-weighed Miracloth filters held in a Millipore filter apparatus. The Miracloth filter with the retained cells was then dried, under vacuum, at 60°C for 24 h.

c. Mitotic index determinations  Suspension fractions were collected on Miracloth filters, and resuspended in 5 volumes of 5 N HCl at 25°C in a test tube, and incubated for 10 min. The cells were collected by centrifugation at 200 x g for 5 min, the supernatant removed by aspiration, and the pellet washed 3 times with 70% ethanol. The final pellet was resuspended in a small volume of 70% ethanol. A portion of the cells was placed on a clean microscope slide, the alcohol allowed to evaporate partly and a drop of 10% modified carbol fuchsin added. A cover slip was lowered, the cells squashed, and the mitotic index deter-
mined by counting the cells in any recognizable stage of mitosis (prophase to telophase), and the total number of cells screened. Mitotic index was expressed as number of cells in mitosis/number of total cells screened. Two counts of 2000 cells each was performed upon each fraction from 2 independent experiments.

d. Chromosome analysis Solid colchicine was added to a final concentration of 0.1% to a Klett 200 to 300 suspension culture. After a 2 h incubation with no shaking at 25°C, the suspension was collected at 200 x g for 5 min. The pellet was washed 3 times with distilled water, and the final pellet treated with 5 N HCl at 25°C for 10 min. The cells were washed with distilled water to neutral pH. For chromosome staining, the washed pellet was resuspended in 2 volumes of Feulgen reagent (Darlington and LaCour, 1976), and allowed to stain for 6 h in the dark at 25°C. The cells were squashed in a drop of aceto-carmine or propiocarmine under a cover slip. Alternatively, the cells were squashed in a drop of 10% modified carbol fuchsin without prior Feulgen staining. The preparations were observed under bright-field, and chromosomes of visibly intact cells were counted and photographed. Additional details are included in Figure 4.7.

e. Suspension plating Two ml of a Klett 500 to 600 suspension culture were spread evenly over the surface of solid DM-1 medium in plastic petri dishes. Addenda to DM-1 medium used for suspension platings and subsequent cultural conditions for the plates are described in Section III. B. 3.
4. **Shoot culture initiation, maintenance, and whole plant propagation**

Petiole segments obtained as described (Section III. A. 2.) were cultured on the medium specified in Table 3.2. The cultures were maintained in plastic petri dishes under a 16 h light and 8 h dark photoperiod at 5000 lux, 27°C, and ambient humidity. After a 5 to 6 week initiation period, the resulting tissues were subdivided and subcultured to fresh solid medium of the same composition. Shoot cultures were routinely subcultured at 3 week intervals to the same medium and physical environment. Gentamicin at 50 µg/ml was added to media only during the initiation passage for control of bacterial contamination.

To obtain rooted plants, proliferating shoots were transferred to shoot culture medium lacking hormones. The shoots were cultured in plastic petri dishes, and maintained under subdued light (approximately 1000 lux). In approximately 3 weeks, the shoots formed roots, were transplanted to a soil mix, maintained under intermittent high humidity for 2 weeks to permit hardening-off, and then transferred to greenhouse conditions.

Table 3.2 Culture medium used for the initiation, maintenance, and rooting of adventitious shoot cultures of *D. innoxia*.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal salts:</td>
<td>after Murashige and Skoog (1962)</td>
</tr>
<tr>
<td></td>
<td>(Appendix A)</td>
</tr>
<tr>
<td>Vitamins:</td>
<td>after Engvild (1973)</td>
</tr>
<tr>
<td></td>
<td>(Appendix A)</td>
</tr>
<tr>
<td>Sucrose:</td>
<td>30 g/liter</td>
</tr>
<tr>
<td>Casein hydrolyzate:</td>
<td>1 g/liter</td>
</tr>
<tr>
<td>6-BAP:</td>
<td>10^-6 M</td>
</tr>
<tr>
<td>Agar:</td>
<td>0.7%</td>
</tr>
</tbody>
</table>
B. Variant Isolation

1. **Determination of selective conditions**

   Before one can attempt the selection of anti-metabolite resistant phenotypes, the minimum concentration of an anti-metabolite lethal under the selective conditions used must be determined. As an initial approximation for *D. innoxia*, these determinations were made under acute exposure in suspension cultures. Replicate 125 ml DeLong turbidimetric flasks containing 30 ml of DM-1 with $2.5 \times 10^{-6}$ M 2,4-D were inoculated with 2 ml of cells from a late log (Klett 500-600; DM-1; $2.5 \times 10^{-6}$ M 2,4-D) stock culture. Media may contain casein hydrolyzate depending on the anti-metabolite tested (Table 3.3). During early to mid log, 2 ml of different concentrations of an anti-metabolite, filter-sterilized in fresh culture medium, were added. Sterile culture medium was added to control flasks. The kinetics of growth were monitored for all flasks. Ten days after the addition of the anti-metabolite, cultures exhibiting a modified growth pattern were harvested by centrifugation at 200 x g for 5 min and washed 3 times with fresh culture medium lacking anti-metabolites. The cells were diluted to Klett 500 with anti-metabolite-free culture medium, and plated on DM-1 with $10^{-6}$ M 2,4-D and no anti-metabolites, and cultured in darkness. Growth of any colonies was monitored for 2 months. The minimum concentration of any anti-metabolite causing no growth of the plated suspensions was chosen as the selective concentration.

   To determine the lethal concentrations of anti-metabolites under chronic exposure at the callus and shoot level of organization, suspension cultures at Klett 500 to 600 were plated on, or shoot cultures were divided and subcultured to, solid DM-1 medium containing different concentra-
Table 3.3 Previous genetic and metabolic uses of the anti-metabolites used to isolate resistance variants from *D. innoxia* cell and tissue cultures.

<table>
<thead>
<tr>
<th>Anti-metabolite</th>
<th>Species</th>
<th>Use as a metabolic or genetic probe</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cycloheximide</td>
<td><em>Neurospora crassa</em> cultured Chinese hamster cells</td>
<td>inhibits protein synthesis at the ribosome; variants have altered ribosomes; variants selected with 10 ( \mu g/ml )</td>
<td>Neuhauser et al., (1970); Pongratz and Klingmüller, (1973)</td>
</tr>
<tr>
<td>cytosine arabinoside</td>
<td>cultured leukemia cells</td>
<td>as above; resistant variants selected with 0.14 ( \mu g/ml )</td>
<td>Poche et al., (1975a; 1975b)</td>
</tr>
<tr>
<td>8-azaguanine</td>
<td>cultured Chinese hamster cells, cultured human cells</td>
<td>inhibits 2'‑deoxycytidine kinase; resistant variants lack the enzyme; resistant variants selected with 0.28 ( \mu g/ml )</td>
<td>Chu and Fischer, (1965)</td>
</tr>
<tr>
<td></td>
<td>cultured <em>A. pseudoplatanus</em></td>
<td>inhibits HPRT; resistant variants lack the enzyme; variants selected with 0.1 ( \mu g/ml )</td>
<td>Chu et al., (1969)</td>
</tr>
<tr>
<td>2,6-diaminopurine</td>
<td>yeast</td>
<td>inhibits HPRT; resistant variants have diminished HPRT activity; variants selected at 0.1 to 10 ( \mu g/ml )</td>
<td>Bright and Northcote, (1975)</td>
</tr>
<tr>
<td></td>
<td><em>Salmonella typhimurium</em> cultured murine cells</td>
<td>variants selected for enhanced purine phosphoribosyl transferase activity; resistant variants lack adenine phosphoribosyl transferase as above; resistant variants selected with 100 ( \mu g/ml )</td>
<td>Lomax and Woods, (1969) Kalle and Gots, (1961) Atkins and Gartler, (1968)</td>
</tr>
<tr>
<td>Compound</td>
<td>Organism</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------</td>
<td>-----------------------------------------------------------------------------------------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>2-thienylalanine</td>
<td>E. coli</td>
<td>false feedback inhibition of DAHP synthetase (phenylalanine sensitive isozyme); resistant variants had an altered DAHP synthetase, no longer sensitive to phenylalanine; variants selected with 0.1 µg/ml</td>
<td>Ezekiel, (1965)</td>
</tr>
<tr>
<td></td>
<td>B. subtilis</td>
<td>false feedback inhibition of prephenate dehydrogenase; resistant variants possess prephenate dehydrogenase not inhibited by phenylalanine; variants selected with 100 µg/ml</td>
<td>Coats and Nester (1967)</td>
</tr>
<tr>
<td></td>
<td>S. typhimurium</td>
<td>inhibits IGP dehydratase; growth stops at 20 mM as above; resistant variants lack IGP dehydratase; variants selected with 20, 50, and 100 mM</td>
<td>Hilton et al., (1965)</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>cells accumulate imidazole glycerol with amitrole, reversed by histidine, but not with adenine</td>
<td>Burke and Pattee (1972)</td>
</tr>
<tr>
<td></td>
<td>P. zopfii,</td>
<td></td>
<td>Casselton, (1966)</td>
</tr>
<tr>
<td></td>
<td>C. vulgaris</td>
<td>cultured Paul's Scarlet rose cells accumulates IG and IGP with amitrole</td>
<td>Siegel and Gentile, (1966)</td>
</tr>
<tr>
<td></td>
<td>mammal</td>
<td>inhibits DOPA decarboxylase in brain tissue</td>
<td>Davies, (1971)</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>inhibits DAHP synthetase (tyrosine sensitive isozyme)</td>
<td>Tellman, (1956)</td>
</tr>
<tr>
<td></td>
<td>S. typhimurium</td>
<td>inhibits IGP dehydratase; growth stops at 20 mM as above; resistant variants lack IGP dehydratase; variants selected with 20, 50, and 100 mM</td>
<td>Hilton et al., (1965)</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
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</tr>
<tr>
<td></td>
<td>P. zopfii,</td>
<td>cultured Paul's Scarlet rose cells accumulates IG and IGP with amitrole</td>
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</tr>
<tr>
<td></td>
<td>C. vulgaris</td>
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</tr>
<tr>
<td></td>
<td>mammal</td>
<td>inhibits DOPA decarboxylase in brain tissue</td>
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</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>inhibits DAHP synthetase (tyrosine sensitive isozyme)</td>
<td>Tellman, (1956)</td>
</tr>
<tr>
<td></td>
<td>S. typhimurium</td>
<td>inhibits IGP dehydratase; growth stops at 20 mM as above; resistant variants lack IGP dehydratase; variants selected with 20, 50, and 100 mM</td>
<td>Hilton et al., (1965)</td>
</tr>
<tr>
<td></td>
<td>S. aureus</td>
<td>cells accumulate imidazole glycerol with amitrole, reversed by histidine, but not with adenine</td>
<td>Burke and Pattee (1972)</td>
</tr>
<tr>
<td></td>
<td>P. zopfii,</td>
<td>cultured Paul's Scarlet rose cells accumulates IG and IGP with amitrole</td>
<td>Casselton, (1966)</td>
</tr>
<tr>
<td></td>
<td>C. vulgaris</td>
<td>cultured Paul's Scarlet rose cells accumulates IG and IGP with amitrole</td>
<td>Siegel and Gentile, (1966)</td>
</tr>
<tr>
<td></td>
<td>mammal</td>
<td>inhibits DOPA decarboxylase in brain tissue</td>
<td>Davies, (1971)</td>
</tr>
<tr>
<td></td>
<td>E. coli</td>
<td>inhibits DAHP synthetase (tyrosine sensitive isozyme)</td>
<td>Tellman, (1956)</td>
</tr>
</tbody>
</table>
Table 3.3 (continued)

<table>
<thead>
<tr>
<th>Anti-metabolite</th>
<th>Species</th>
<th>Use as a metabolic or genetic probe</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>allyl alcohol</td>
<td>Schizosaccharomyces pombe</td>
<td>ADH converts allyl alcohol to acryl aldehyde and kills cells; cells lacking ADH survive; variants selected with 1 mM as above; treat pollen with vapor</td>
<td>Megnet, (1967)</td>
</tr>
<tr>
<td></td>
<td>Zea mays</td>
<td></td>
<td>Schwartz and Osterman, (1976)</td>
</tr>
</tbody>
</table>
tions of an anti-metabolite. Callus medium contained $10^{-6}$ M 2,4-D, while the test medium for shoot cultures contained $10^{-6}$ M 6-BAP. Callus and shoot cultures were maintained as described (Section III. A. 2.; III. A. 3. a.). Growth was monitored for 2 months, and the minimum concentration of an anti-metabolite causing a lack of growth was chosen as the selective concentration.

Because of the possibility of spontaneous mutation to anti-metabolite resistance in monoploid tissues, or segregation of a genotype in any one monoploid plant isolate conferring resistance to an anti-metabolite, only diploid-derived tissues were used for toxicity determinations. The anti-metabolites used are listed in Table 3.3.

2. Mutagenic treatment

Petiole segments were treated with 0.2% or 1% EMS (v/v) for various times in a liquid support medium consisting of 0.5 X Murashige and Skoog medium (Appendix A) without hormones at pH 6.0. The incubations were carried out aseptically in 9 cm diameter petri dishes with 25 rpm gyratory shaking (Junior Orbit Shaker, Labline), in darkness. After EMS treatment, the segments were removed from the treatment medium and washed with fresh support medium for 0.5 h. The segments were cultured on callus or shoot medium. Petiole segments were also treated with EMS after a variable pre-culture period on callus or shoot medium. Pre-cultured segments, after mutagenesis, were returned to medium of the same composition upon which they were initiated.

Petiole segments were also treated with $10^{-5}$ M KCN for varying times. Potassium cyanide was chosen as a control treatment to test for cross-feeding effects, and at the concentration used, causes cell death while proba-
ly not being mutagenic (Lilly, 1958; Freese, 1971). Potassium cyanide treated segments were treated exactly as EMS treated segments, but were cultured only on shoot medium.

Whole petioles were treated for varying times with gamma irradiation from a $^{60}$Co source using a flux of 100 R/min. No filters were used. After treatment, the petioles were surface sterilized, cut into segments, and cultured on callus or shoot medium.

3. Selection procedures

Cell suspensions derived from mutagenized petiole explants were initiated by placing the entire callus originating from a single petiole segment into 30 ml of DM-1 with $2.5 \times 10^{-6}$ M 2,4-D in 125 ml DeLong flasks. The callus pieces were approximately 1 cm in diameter when used as inoculum for suspensions - about 3 to 8 weeks post-mutagenesis. The propagation of cells in liquid non-selective medium allows the growth of variant and non-variant phenotypes. At late log, about 15 days after the initiation of the suspension, 2 ml of the suspension were plated on DM-1 medium containing the determined selective concentration of an anti-metabolite. Each suspension was used to inoculate several plates with selective concentrations of different anti-metabolites. The solid selective medium contained $10^{-6}$ M 2,4-D or $10^{-6}$ M 6-BAP. Growth of colonies, which would be presumptive resistant variants, was monitored for 2 months. Suspensions plated on 2,4-D containing medium are tan when grown in the dark, but become green when in the light. Thus, as an additional aid in the localization of a possible variant capable of growth under selective conditions, two platings were prepared for each anti-metabolite tested. One plate was cultured in darkness, and the other cultured under a 16 h
light and 8 h dark photoperiod at 5000 lux. In the latter case, proliferating cells should become green, while the non-proliferating (dead) cells remain tan or become black.

Suspensions plated on medium with 6-BAP, like those plated on 2,4-D containing medium, become green in the light while remaining tan in the dark. However, in contrast to 2,4-D, 6-BAP promotes the organogenesis of shoots either in the dark or light. Because organogenesis is a more rigorous response than callus formation, it was reasoned that a genotype resistant to the selective agent may be able to initiate differentiation in darkness, but not in the light. Suspensions plated on selective medium containing 6-BAP were thus cultured in the dark, or under a 16 h light and 8 h dark photoperiod. This procedure should allow the recovery of variant phenotypes not viable when exposed to organogenesis-inducing conditions in the light under selective conditions.

Mutagenized petiole segments were also cultured directly on non-selective shoot medium. Shoot formation occurred 4 to 8 weeks post-mutation. At this point, each responding explant was divided into smaller pieces and subcultured to shoot medium containing the selective concentration of an anti-metabolite, and cultured in the dark, or under a 16 h light and 8 h dark photoperiod under the rationale described above.

Occasionally, non-mutagenized petiole segments were carried through the entire selection scheme to provide controls and further characterization. A schematic of the selection scheme is shown in Figure 3.2.
Figure 3.2 Schematic of selection scheme used to isolate variants from cell and tissue cultures of *D. innoxia*.
Petiole explants of monoploid or diploid *Datura innoxia*

Mutagenic treatment

**Callus formation on non-selective medium**
- Initiate suspension cultures in non-selective medium
- Plate suspensions on selective medium
- Variant proliferation

**Adventitious shoot formation on non-selective medium**
- Subculture pieces to selective medium
- Variant proliferation
VI. RESULTS

A. Growth and Aggregation in Batch Suspension Culture

1. Effect of micronutrient and 2,4-D concentration on growth rate

Suspension cultures of D. innoxia were initiated in 30 ml of DM-1 with 1 g/liter casein hydrolyzate and full- or 0.1-strength micronutrient concentration in 125 ml DeLong culture flasks. The doubling time of suspensions varied linearly over the 2,4-D concentration range, $10^{-6}$ M to $10^{-5}$ M, with no significant effect of micronutrient concentration ($t=0.08$ ns; 31 df) (Figure 4.1). The effect of micronutrient concentration on lag time after subculture was also negligible (Figure 4.2). The effect of micronutrient concentration on growth rate and lag time was not examined in medium lacking casein hydrolyzate. If the 2,4-D concentration was less than $10^{-6}$ M, growth was not observed upon extended subculture. Dry weight/ml, fresh weight/ml, and packed cell volume/ml increased linearly with respect to Klett units in the range 100 to 800 (Figure 4.3). At Klett values less than approximately 100, these three monitoring functions were observed to diverge from the linear relationship at Klett 100 to 800. Log phase, as determined turbidimetrically, begins at approximately Klett 100 which corresponds to about 0.5 mg dry weight/ml. A control experiment using dry weight/ml as a growth monitoring function showed a doubling time of $2.86\pm0.20$ days for cells growing in DM-1 with $2.5 \times 10^{-6}$ M 2,4-D (Figure 4.4). In this case, log phase was observed to begin at about 0.6 mg dry weight/ml, thus corresponding well to the initiation of log phase as determined turbidimetrically.

Because growth rates of monoploid and diploid derived suspensions were not significantly different in initial experiments, subsequent growth
Figure 4.1 Turbidimetrically determined doubling times of *D. innoxia* suspension in DM-1 with 1 g/liter casein hydrolyzate and full strength micronutrients (---), or one tenth strength micronutrients (–––), at different 2,4-D concentrations.
Figure 4.2  Effect of full strength micronutrients (—●—), or one tenth micronutrients (—○—) on lag phase subsequent to subculture of *D. innoxia* suspensions. Suspensions grown in DM-1 with 1 g/liter casein hydrolyzate and 2.5 $\times$ $10^{-6}$ M 2,4-D.
Figure 4.3 Calibration of Klett units to (a) mg dry weight/ml, (b) mg fresh weight/ml, and (c) cm$^3$ packed cells/ml for diploid D. innoxia suspensions growing in DM-1 with 2.5 x 10$^{-6}$ M 2,4-D. Two ml of a Klett 500 suspension culture were subcultured into replicate 125 ml DeLong turbidimetric flasks containing 30 ml medium. Initial Klett was 30. At intervals, a single flask was harvested, and the packed cell volume, fresh weight, and dry weight determined and expressed per ml of culture volume.
Figure 4.4  Growth of diploid D. innoxia suspensions in DM-1 with $2.5 \times 10^{-6}$ M 2,4-D as monitored by dry weight. Two ml of a Klett 500 suspension culture were subcultured into replicate 125 ml DeLong flasks containing 30 ml medium. At intervals, 3 flasks were individually harvested and the mg dry weight/ml for each flask was determined. Each point is the mean of 3 replicates.
experiments employed only diploid derived suspensions.

2. **Effect of inorganic nitrogen content and Krebs organic acids on growth rate and aggregation**

A series of experiments using L-malic or succinic acids as an organic supplement to DM-1, DM-2, and DM-3, in the absence of casein hydrolyzate, indicated that these organic acids exerted a great influence on doubling times (Table 4.1). However, the addition of Krebs organic acids caused an increase in aggregation (Plate 4.1). Equivalent concentrations of malic and succinic acids had similar qualitative effects on aggregation in DM-1, DM-2, and DM-3. There was no growth in DM-2 and DM-3 without the organic acids. Succinic acid apparently provided a better substrate for rapid growth than malic acid in DM-1 (Table 4.1). Generally, the absolute pH change of cultures at Klett 500 relative to the initial pH, was inversely proportional to the concentration of Krebs organic acids (Table 4.1). Media containing ammonium only, or ammonium and nitrate, became more acidic, while media containing nitrate only became more alkaline. Qualitatively, aggregation in DM-1 with or without casein hydrolyzate remained the same (Plate 4.1, A and E).

3. **Effect of 2,4-D concentration on aggregation and mitosis**

Aggregation was quantitatively examined using the aggregation assay between early and late log phase in cultures grown in DM-1 with 1 g/liter casein hydrolyzate and 2,4-D concentrations of $10^{-6}$ M to $10^{-5}$ M (Figure 4.5). At 2,4-D concentrations of $2.5 \times 10^{-6}$ M and higher, a dramatic decrease in the dry weight of the 30 mesh residue (largest aggregates), and an increase in the 100 mesh residue dry weight were observed. The pattern of 100 mesh filtrate dry weight accumulation remained essentially
Table 4.1 Growth rates of diploid *D. innoxia* suspension cultures with organic nitrogen lacking. All media contained $2.5 \times 10^{-6}$ M 2,4-D, with initial pH of 5.6. Doubling times are means of three to five replicates, and are based on the slope of the regression line fitted to the log-linear phase of growth (Appendix B).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Organic addenda</th>
<th>Mean doubling time (days) (± std. dev.)</th>
<th>Final pH Klett 500</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>succinic acid (mM)</td>
<td>malic acid (mM)</td>
<td>casein hydrolyzate (g/liter)</td>
</tr>
<tr>
<td>DM-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DM-1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DM-1</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DM-1</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DM-1</td>
<td>40</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DM-1</td>
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<td>DM-1</td>
<td>0</td>
<td>30</td>
<td>0</td>
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<tr>
<td>DM-1</td>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>DM-2</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>DM-2</td>
<td>20</td>
<td>0</td>
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</tr>
<tr>
<td>DM-2</td>
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<td>0</td>
</tr>
<tr>
<td>DM-2</td>
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<tr>
<td>DM-3</td>
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<tr>
<td>DM-3</td>
<td>20</td>
<td>0</td>
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<tr>
<td>DM-3</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DM-3</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*a* no proliferation after 2 months, but cultures remained a light yellow color

*b* cultures became brown-black and never proliferated
Plate 4.1 Macroscopic estimates of aggregate size and number from D. innoxia suspension cultures at Klett 500. Medium was DM-1 with 1 g/liter casein hydrolyzate at

(A) $7.5 \times 10^{-7}$ M 2,4-D,
(B) $10^{-6}$ M 2,4-D,
(C) $5 \times 10^{-6}$ M 2,4-D,
(D) $10^{-5}$ M 2,4-D, and in medium DM-1 with $2.5 \times 10^{-6}$ M 2,4-D with

(E) no casein hydrolyzate or organic acid supplement,
(F) 20 mM L-malic acid,
(G) 30 mM L-malic acid, and
(H) 40 mM L-malic acid
Figure 4.5 Quantitative estimation of aggregate size in suspension cultures of *D. innoxia*. Suspensions were grown in DM-1 with 1 g/liter casein hydrolyzate in turbidimetric flasks. At various Klett values, the suspensions were harvested, filtered, and the dry weight of aggregate fractions determined. Total dry weight (●), 30 mesh residue dry weight (○), 100 mesh residue dry weight (■), 100 mesh filtrate dry weight (□), at (a) $10^{-6}$ M 2,4-D, and (b) $10^{-5}$ M 2,4-D
Plate 4.2 Aggregates from *D. innoxia* suspensions grown in DM-1 with 1 g/liter casein hydrolyzate with $10^{-6}$ M 2,4-D (A, C, E), or $10^{-5}$ M 2,4-D (B, D, F). A and B, 30 mesh residue, C and D, 100 mesh residue, E and F, 100 mesh filtrate. Photographs do not represent absolute cell concentrations. Relative cell size, aggregate size, and cells/aggregate can be compared. Suspensions filtered at Klett 500.
the same over this 2,4-D concentration range. Plate 4.2 shows representative samples of cultures grown with $10^{-6}$ M 2,4-D or $10^{-5}$ M 2,4-D. At the higher hormone concentration, the cells were considerably larger, and aggregates consisted of fewer cells.

The mitotic index of the three aggregate fractions was determined at $10^{-6}$ M and $10^{-5}$ M 2,4-D between Klett 100 and 700 (Figure 4.6). At $10^{-5}$ M 2,4-D, the 100 mesh residue exhibited an absolute increase in the mitotic index over a similar fraction at $10^{-6}$ M 2,4-D; the 30 mesh residue mitotic index decreased under the same treatment. In $10^{-5}$ M 2,4-D, a relative increase in the mitotic index of the 100 mesh residue was observed with respect to the 30 mesh residue mitotic index. The mitotic index of the 100 mesh filtrate generally increased at $10^{-5}$ M 2,4-D relative to $10^{-6}$ M 2,4-D. Turbidimetrically determined early log phase was characterized by an increase in cell division, but the transient increase of the 100 mesh filtrate mitotic index in $10^{-5}$ M 2,4-D was not reflected by increased dry weight accumulation in this fraction.

Cells conditioned to $10^{-5}$ M 2,4-D were subcultured (2 ml into 30 ml of medium) into medium containing $10^{-6}$ M 2,4-D, and aggregation was assayed between Klett 100 and 800 (Figure 4.7). The kinetics of 30 mesh residue dry weight accumulation paralleled that of total dry weight accumulation, and evidently was not linear with respect to Klett units. More total, and specifically, 30 mesh residue dry weight amassed in this subculturing regime than when subculturing cells into medium of the same hormonal composition.
Figure 4.6 Mitotic index of aggregate fractions at various Klett values during the batch growth cycle. Suspensions were harvested, filtered, and the mitotic index of each fraction determined. Mitotic index of 30 mesh residue (---), 100 mesh residue (---), and 100 mesh filtrate (---), at (a) 10^{-6}M 2,4-D, and (b) 10^{-5}M 2,4-D. Pooled standard deviation of all mitotic index determinations was 0.3.
Figure 4.7  Quantitative estimation of aggregate size in suspension cultures of *D. innoxia* after subculture of $10^{-5}$M 2,4-D conditioned cells into $10^{-6}$M 2,4-D. At various Klett values, the suspensions were harvested, filtered, and the dry weight of each aggregate fraction determined. Total culture dry weight (●), 30 mesh residue dry weight (○), 100 mesh residue dry weight (■), and 100 mesh filtrate dry weight (□). Medium was DM-1 with 1 g/liter casein hydrolyzate.
B. Variant Isolation

1. Stability of chromosome number, and maintenance of morphogenetic competency in cultured cells

*D. innoxia* suspensions were initiated in DM-1 with 1 g/liter casein hydrolyzate and $10^{-6}$ M 2,4-D from monoploid plant isolate X(8). After 3 years of serial subculture, the chromosome number of this highly aggregated suspension culture was determined. Figure 4.8 shows the distribution of chromosome numbers in this suspension culture. No monoploid cells were observed in 500 cells screened, so the frequency of monoploid cells in this culture was less than 0.2%. This suspension was also plated on shoot medium, and although the cells proliferated and became green, the organization of the resulting callus into plants was minimal compared to control platings with freshly initiated suspensions. The shoots eventually derived from the plating of 3 year old suspensions were morphologically abnormal, and never formed roots in 3 months on rooting medium (Figure 4.9).

The use of long-term suspension cultures of *D. innoxia*, under the described cultural conditions, because of the observed ploidy instability and poor morphogenetic competency, precludes their use as a source of cells for variant isolation because of the required efficiency. Chromosome stability and morphogenetic capacity under short-term cultural conditions was not examined, but as Mastrangelo et al., (1974) have indicated, even in short-term suspension cultures of monoploid *D. innoxia*, only 58% of the cells screened were monoploid.

Chromosome counts of corolla preparations from 6 plants obtained from the initiation passage of shoot cultures of monoploid *D. innoxia* has shown
Figure 4.8 Distribution of chromosome numbers from suspension cultured cells of *D. innoxia* haploid isolate X(8) after 3 years in culture. Five hundred cells were screened.
them to be monoploid. Similarly, 6 plants regenerated from the initiation passage of diploid shoot cultures were diploid and fertile. Thus, rather than using suspension cultures as a source of cells in a variant isolation scheme, the scheme described in Materials and Methods was instituted.

Figure 4.9 Morphology of shoot cultures derived from 3 year old suspension cultures of monoploid suspension cultures of *D. innoxia*.

2. **Determination of selective conditions**

As an approximation to the minimum lethal concentrations of anti-metabolites at the callus and shoot level of organization, these determinations of toxicity were made using turbidimetric methods in suspension culture. Figure 4.10 provides an example of the type of data obtained using allyl alcohol as an example; cultures containing 6 μg/ml, 30 μg/ml, and 60 μg/ml allyl alcohol were harvested 10 days after the addition of the anti-metabolite, washed with fresh medium, and plated on medium without allyl alcohol. No growth of colonies from the 30 μg/ml or 60 μg/ml treated
Figure 4.10 Effect of allyl alcohol on the growth of D. innoxia batch suspension cultures as determined turbidimetrically. Medium was DM-1 with 1 g/liter casein hydrolyzate and 2.5 X 10^{-6}M 2,4-D. Control (●), 0.6 μg/ml (○), 3 μg/ml (■), 6 μg/ml (□), 30 μg/ml (▲), and 60 μg/ml (△) allyl alcohol. Arrow indicates point of allyl alcohol addition.
suspensions was observed after 2 months, but 6 μg/ml treated suspensions yielded many colonies after 1 week. Thus, 30 μg/ml was chosen as the selective concentration of allyl alcohol under acute conditions. All other anti-metabolites, except where noted, exhibited similar results - suspensions exposed to concentrations of an anti-metabolite causing a lack of growth, as determined turbidimetrically, did not give rise to colonies when plated on non-selective medium. Thus, under the exposure conditions in these experiments, the anti-metabolites were biocidal rather than biostatic. The minimum exposure time to anti-metabolites necessary to cause death in suspension cultures was not examined.

In certain cases, the color of the suspension culture changed upon addition of an anti-metabolite in such a way as to produce turbidimetric artifacts. Of the anti-metabolites tested, only 3-amino-L-tyrosine evoked such a response. Suspensions with 3-amino-L-tyrosine became progressively yellow, the intensity of which was apparently proportional to the concentration of the anti-metabolite. The combination of yellow color with the blue filter used in the colorimeter caused increased Klett values while visual observations indicated no increase in cell number had occurred. In this case, when control flasks reached Klett 500, all cultures were individually harvested on Miracloth filters and the dry weight/ml culture volume determined. Although Klett values were increased at high 3-amino-L-tyrosine concentrations, little or no dry weight accumulated. Suspension platings on media with 3-amino-L-tyrosine also became yellow, while shoot cultures subcultured to medium with this anti-metabolite became brown-black in the dark or light.

During initial selection experiments, suspensions plated on, and
shoot cultures subcultured to medium containing acutely-determined lethal concentrations of certain anti-metabolites grew with a frequency and vigor similar to control cultures on non-selective medium (Table 4.2). Suspensions and shoots derived from non-mutagenized petiole explants also grew on acutely-determined selective concentrations of these anti-metabolites. It was reasoned that neither mutation, nor an artifact in the turbidimetric determination of lethality was responsible for this effect. Rather, it was an expression of differences in the sensitivity of suspensions, callus, and shoot cultures to the anti-metabolites. Thus, it was necessary to determine the lethal concentration of these anti-metabolites at the callus and shoot level of organization (Table 4.2). Because of the close proximity of cells in callus and shoot cultures, determination of lethal concentrations of these anti-metabolites using acute exposure was technologically troublesome and lengthy. Lethal concentrations in callus and shoot cultures were determined chronically as described in Materials and Methods. The results of these determinations are listed in Table 4.2.

As indicated in Table 4.2, shoot cultures subcultured to medium containing 2,6-diaminopurine or 3-amino-L-tyrosine resulted in the medium becoming a darkened color when cultured in the light or dark. The color change originated around the tissues, and eventually the entire plate turned black. Because the reaction occurring was unknown, variants resistant to these anti-metabolites were not selected at the shoot level. Shoot cultures subcultured to medium containing 8-azaguanine survived on concentrations to 50 μg/ml upon repeated subculture. Concentrations of 8-azaguanine greater than 50 μg/ml resulted in a precipitate, probably
Table 4.2 Toxicity of anti-metabolites in cell and tissue cultures of diploid *D. innoxia*. Minimum lethal concentrations were determined acutely in suspension cultures using turbidimetry followed by suspension plating, and chronically in callus and shoot cultures. Differences in sensitivity between monoploid and diploid cells and tissues, and between different haploid isolates were not determined.

<table>
<thead>
<tr>
<th>Anti-metabolite</th>
<th>Lethal concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Suspensions</td>
</tr>
<tr>
<td>allyl alcohol(^a)</td>
<td>30</td>
</tr>
<tr>
<td>(\beta)-2-thienylalanine</td>
<td>100</td>
</tr>
<tr>
<td>DL-phenyllactic acid</td>
<td>500</td>
</tr>
<tr>
<td>8-azaguanine(^a,c)</td>
<td>5</td>
</tr>
<tr>
<td>L-methionine-DL-sulfoximine</td>
<td>100</td>
</tr>
<tr>
<td>L-ethionine</td>
<td>100</td>
</tr>
<tr>
<td>3-amino-1H-1,2,4-triazole (amitrole)(^c)</td>
<td>80</td>
</tr>
<tr>
<td>2,6-diaminopurine(^a)</td>
<td>115</td>
</tr>
<tr>
<td>cycloheximide(^a)</td>
<td>10</td>
</tr>
<tr>
<td>cytosine arabinonucleoside(^a)</td>
<td><em>(^f</em>)</td>
</tr>
<tr>
<td>3-amino-L-tyrosine(^c)</td>
<td>100</td>
</tr>
</tbody>
</table>
casein hydrolyzate added at 1 g/liter to medium containing this anti-metabolite

nd = not determined

indicates the minimum lethal concentration, acutely-determined in suspension culture, was not lethal to callus and/or shoot cultures; the lethal concentration of this anti-metabolite was thus determined chronically over a concentration range of the anti-metabolite in callus and/or shoot cultures

cultured shoots proliferated, apparently normally, for extended subcultures on concentrations to 50 μg/ml

nd = not determined; when shoot cultures were transferred to medium with this anti-metabolite, the medium became brown-black; variants resistant to this anti-metabolite were not selected at the shoot level of organization

see Table 4.3

plated suspensions proliferated, apparently normally, for extended subcultures on concentrations to 1000 μg/ml
of the 8-azaguanine due to its low solubility at the pH of the culture medium.

Suspension cultures challenged with cytosine arabinonucleoside proliferated at a faster rate than controls, thus precluding its use as a resistance-variant selective agent, or use in variant enrichment (Table 4.3).

Table 4.3 Turbidimetrically determined doubling times of diploid *D. innoxia* suspension cultures containing cytosine arabinonucleoside. Test medium was DM-1 with 1 g/liter casein hydrolyzate and $2.5 \times 10^{-6}$ M 2,4-D.

<table>
<thead>
<tr>
<th>cytosine arabinonucleoside (µg/ml)</th>
<th>doubling time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.48</td>
</tr>
<tr>
<td>30</td>
<td>1.41</td>
</tr>
<tr>
<td>150</td>
<td>1.41</td>
</tr>
<tr>
<td>300</td>
<td>1.31</td>
</tr>
<tr>
<td>3000</td>
<td>1.45</td>
</tr>
</tbody>
</table>

If the concentration of an anti-metabolite observed to be toxic in suspension cultures was also toxic to callus or shoot cultures, toxicity of the anti-metabolites in lower concentrations at the latter levels of organization were not tested.

Although diploid derived tissues were used for determinations of anti-metabolite toxicity, control experiments with monoploid callus and shoot cultures exhibited no growth when subcultured to medium containing lethal concentrations of the anti-metabolites. Toxicity to monoploid tissues at lower anti-metabolite concentrations was not tested.
3. **Tissue response to mutagenic agents**

During mutagenesis and selection experiments, some explants were reserved to characterize the effect of EMS and γ-irradiation treatments on the subsequent growth response of petiole explants. The effect of EMS on callus growth from monoploid and diploid explants was investigated by treating the explants with 0.2% EMS for 2 to 15 h, and inoculating 6 replica plates per treatment time with 5 explants per plate. The explants were cultured as described (Section III. A. 2.). Untreated explants served as controls. At 1 week intervals, for 6 weeks, the explants on a single plate per treatment time were harvested and the mean fresh weight/explant determined. Figure 4.11 illustrates the results from these quantitative growth studies. Monoploid tissue was more sensitive to EMS than diploid tissue as evidenced by the longer lag times exhibited by monoploid tissue relative to diploid tissues at the same EMS treatment times. The data from this experiment was manipulated using a back-extrapolation procedure (Appendix C) to yield the plot shown in Figure 4.12. Since the survival of single cells was not measured, but rather the response of a population of cells, the ordinate of Figure 4.12 was termed response frequency rather than mortality frequency. The back-extrapolation allows a better visualization of the response of monoploid and diploid tissues to EMS, and, as Figure 4.12 indicates, monoploid tissue, relative to diploid tissue, was more sensitive to EMS.

The callus growth kinetics of Figure 4.11 also indicated no difference in the doubling time between monoploid and diploid tissue during the initiation passage. Analysis of growth rates was not determined for succeeding passages. The slope of the regression lines fitted to the
Figure 4.11 Growth of callus from EMS treated petiole explants of (a) monoploid, and (b) diploid D. innoxia. Explants were treated with 0.2% EMS for 0 h (–●–), 2 h (–○–), 4 h (–■–), 8 h (–□–), and 15 h (–▲–) with gyratory shaking at 30rpm in the dark at 27°C. Explants were washed, and cultured on callus initiation medium.
Figure 4.12 Growth response of monoploid (—O—), and diploid (—•—) callus initiated from EMS treated petiole explants of D. innoxia. Data were computed by back-extrapolation of the log-linear growth phases shown in Figure 4.9.
logarithmic phase of growth of monoploid and diploid tissues were not significantly different ($t=0.0024$ ns; 10 df). Doubling times of monoploid and diploid callus were 6.23 and 6.16 days respectively. The slope of the regression lines fitted to the logarithmic growth phase of EMS treated explants were not significantly different than that of the controls. Differences in callus growth rate during the initiation passage by explants from different monoploid plant isolates were not examined.

In a second series of experiments, EMS treated monoploid and diploid explants were cultured on shoot medium. After 4 weeks culture, the frequency of surviving explants (those explants which grew), and the mean number of shoots/surviving explant were determined for EMS treatment times of 2, 4, and 6 h. It was reasoned that these data could be used as another measure of mortality due to mutation. As Figure 4.13 indicates, the survival of explants, and the number of shoots/surviving explant generally increased with EMS treatment. A 2 h EMS treatment increased the survival frequency of monoploid explants, while EMS treatments longer than 2 h resulted in a gradual decrease in the survival frequency to a value of 0% survival with an 8 h treatment. Diploid explants also displayed greater survival with EMS treatment. In contrast, diploid tissue did not exhibit a gradual decrease in survival with EMS treatments longer than 2 h, but rather, the survival frequency steadily increased to a precipitous drop between 6 h and 8 h EMS.

The number of shoots produced per monoploid or diploid explant also increased with EMS treatments (Figure 4.13). Approximately 80% more shoots /surviving monoploid explant were observed with a 4 h EMS treatment compared to untreated monoploid explants. Similarly, 160% more
Figure 4.13 Effect of EMS treatment on % explants forming shoots (---O---), and mean number of shoots/surviving explant (---●---), using petiole explants of (a) monoploid, and (b) diploid D. innoxia. Measurements were made 4 weeks post-treatment. Explants were cultured on shoot initiation medium in the light.
shoots/surviving diploid explant were initiated with a 6 h EMS treatment compared to untreated diploid explants. To determine the association of EMS treatment time with the number of shoots/surviving explant, a one-way classification analysis of variance was performed on the data. Statistically, $F=6.17^{**}$, for monoploid explants, and $F=12.67^{**}$, for diploid explants. Thus, the variation in shoots/surviving explant between treatments was statistically different than the variation within a treatment. No difference was observed in the growth response of monoploid explants from different monoploid plant isolates, so monoploid data represents information pooled from experiments using tissues from different monoploid plant isolates.

Shoots from EMS treated monoploid explants were rooted, and whole plants established under greenhouse conditions. To determine if the increased morphogenetic capacity of petiole explants after EMS treatment was somatically heritable, explants from four of these regenerated plants were cultured on shoot medium. Corolla chromosome counts indicated these plants were monoploid. The four plants originated from different mutagenized petiole explants. Explants from plants regenerated from non-mutagenized monoploid tissue served as the control. After 4 weeks culture, the number of surviving explants and shoots/surviving explant were determined (Table 4.4). Although there was considerable variability in the % explants surviving, the number of shoots/explant indicated no increase in the morphogenetic capacity of explants from plants originating from mutagenized tissue.

The increased shoot morphogenesis and survival of explants after EMS treatment may be due to cross-feeding of substances from dead cells.
Table 4.4  Morphogenetic capability of petiole explants of plants regenerated from mutagenized tissues of *D. innoxia*.

<table>
<thead>
<tr>
<th>Source of petiole tissue</th>
<th>% explants surviving</th>
<th>shoots/surviving explant</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>X(4)a</td>
<td>14.3 (35)b</td>
<td>21.7 (5)c</td>
<td>-</td>
</tr>
<tr>
<td>X(4)-1</td>
<td>42.9 (21)</td>
<td>10.2 (9)</td>
<td>2.13 ns</td>
</tr>
<tr>
<td>(0.2% EMS, 2 h)d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X(4)-2</td>
<td>14.8 (27)</td>
<td>9.3 (4)</td>
<td>1.71 ns</td>
</tr>
<tr>
<td>(0.2% EMS 4 h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X(4)-3</td>
<td>29.4 (17)</td>
<td>2.4 (5)</td>
<td>0.25 ns</td>
</tr>
<tr>
<td>(0.2% EMS 4 h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X(4)-4</td>
<td>46.1 (26)</td>
<td>16.9 (12)</td>
<td>0.70 ns</td>
</tr>
<tr>
<td>(0.2% EMS 6 h)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a X(4) = control explants from plants regenerated from non-mutagenized petiole tissues of monoploid plant isolate X(4)

^b ( ) = total number of explants cultured/plant

^c ( ) = total number of explants cultured/plant that produced shoots

^d monoploid plants regenerated from petioles of indicated monoploid isolate plant exposed to the indicated mutagenic treatment; each regenerated plant was of different petiole explant origin as indicated by the plant number following monoploid plant isolate designation.
to those surviving which increased the morphogenetic response of the latter. To test this possibility, petiole segments of monoploid and diploid plants were treated with $10^{-5}$ M KCN and cultured on shoot medium. After 4 weeks culture, the % explants surviving, and the mean number of shoots/surviving explant were determined (Figure 4.14). Unlike the difference observed between monoploid and diploid explant survival with EMS treatment, the kinetics of monoploid and diploid survival frequency with respect to KCN treatment time were observed to be essentially the same. However, there appeared to be a reversal in the relationship of ploidy level to shoots/surviving explant with respect to long EMS or KCN treatment time (Figure 4.13 and Figure 4.14). While the shoots/surviving diploid explant increased with EMS treatment time, an increase in this function with long KCN treatment time was not observed ($F=0.39$ ns). Conversely, the shoots/surviving monoploid explant increased over controls with a 2 h EMS treatment, and exhibited a plateau response at longer EMS treatment times and were significantly different ($F=3.42^*$).

It is possible to define a shoot production monitoring function which accommodates both the number of shoots formed and the % survival of the explants. This function, efficiency of shoot production, is the product of the mean number of shoots/explant and the % explant survival at any specific EMS or KCN treatment time. Figure 4.15 and Figure 4.16 show this function for monoploid and diploid tissues treated with EMS and KCN respectively. This data manipulation indicates that KCN treatment of monoploid and diploid tissues leads to a gradual decrease in the efficiency of shoot production. In contrast, monoploid tissue is stimulated to heightened shoot production with moderate EMS dose, while shoot pro-
Figure 4.14  Effect of KCN treatment on % explants forming shoots (—○—), and mean number of shoots/surviving explant (—●—), using petiole explants of (a) monoploid, and (b) diploid D. innoxia. Measurements were made 4 weeks post-treatment. Explants were cultured on shoot initiation medium in the light.
SHOOTS / SURVIVING EXPLANT

HOURS

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

KCN

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28

% EXPLANTS FORMING SHOOTS
Figure 4.15 Efficiency of shoot production from (a) monoploid, and (b) diploid petiole explants after EMS treatment.
EFFICIENCY OF SHOOT PRODUCTION

HOURS EMS

(b)

(a)
Figure 4.16 Efficiency of shoot production from (a) monoploid, and (b) diploid petiole explants after KCN treatment.
EFFICIENCY OF SHOOT PRODUCTION

HOURS KCN

1 3 5 7 6 8 10

0 2 4 6 8 10 12 14 16

(a) (b)
duction is decreased with a large EMS dose. Diploid tissue exhibited an ever increasing propensity for shoot production over the entire EMS dose range.

To serve as a further control, petiole segments of monoploid and diploid plants were incubated for 2 to 8 h in support medium only, and cultured on shoot medium. It was reasoned that the incubation with EMS or KCN perhaps caused leaching of morphogenesis inhibitory substances, thereby enhancing morphogenesis. As Table 4.5 indicates, this incubation does not affect the petiole explant survival or the shoots/surviving explant.

When petiole explants were treated with EMS or KCN for long periods, the explants blanched - a usual indication of death. Occasionally, a small sector of the explant became green, and all the shoots from this explant originated from this region (Figure 4.17).

Survival of EMS treated explants was greater when the tissues were cultured on callus medium than when cultured on shoot medium (Figure 4.11 and Figure 4.13). Using the criterion of shoot production, tissue growth from EMS treated explants was enhanced when cultured on shoot medium; EMS did not enhance the growth of explants cultured on callus medium.

The morphogenetic capacity of monoploid tissue was greater than diploid tissue in the initiation passage. Combined data from EMS and KCN controls are shown in Table 4.6.

To further characterize the effects of mutagens on the morphogenetic response of petiole explants in the initiation passage, whole petioles were exposed to γ-irradiation, surface sterilized, and cultured on shoot medium. After 4 weeks culture, the frequency of explants exhibiting
Table 4.5 Effect of washing on the shoot proliferation and survival frequency of monoploid and diploid explants of *D. innoxia*.

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Hours washing</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2X shoots/surviving explant</td>
<td>14.8</td>
<td>14.5</td>
<td>13.8</td>
<td>14</td>
<td>14.8</td>
<td>4</td>
<td>22 F=1.27 ns</td>
</tr>
<tr>
<td>% survival</td>
<td>50</td>
<td>16.7</td>
<td>50</td>
<td>57</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X(4)\textsuperscript{a}</td>
<td>22.3</td>
<td>22</td>
<td>17.8</td>
<td>25</td>
<td>37</td>
<td>4</td>
<td>21 F=1.45 ns</td>
</tr>
<tr>
<td>shoots/surviving explant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% survival</td>
<td>58</td>
<td>50</td>
<td>42</td>
<td>50</td>
<td>67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} X(4) = explants from monoploid plant X(4) were used in this experiment
Table 4.6 Comparison of morphogenetic competency of monoploid and diploid petiole explants of *D. innoxia* in the initiation passage.

<table>
<thead>
<tr>
<th>Tissue source</th>
<th>Shoots/surviving explant</th>
<th>Shoot production efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>X*</td>
<td>15.8</td>
<td>84</td>
</tr>
</tbody>
</table>

\[ t = 2.13^*; 86 \text{ df} \]

\[ X = \text{combined data from a number of different monoploid isolates} \]
growth was determined. The results of this experiment are shown in Table 4.7. The effect of \( \gamma \)-irradiation on the survival of explants in the initiation passage was similar to the effect of EMS, as monoploid tissue was more sensitive. Unlike untreated explants, or those exposed to EMS or KCN, which either produced shoots or died on shoot medium, many \( \gamma \)-irradiated explants only proliferated as brown callus in the initiation passage. Shoots/explant were not determined in this experiment as this procedure requires destruction of the tissue. Instead, the responding explants were scored on the basis of proliferating as brown callus or producing shoots,
Table 4.7  Effect of $\gamma$-irradiation on shoot initiation by petiole explants of monoploid and diploid
D. innoxia. Petiole explants were cultured on shoot initiation medium in the light.

<table>
<thead>
<tr>
<th>Dose (R)</th>
<th>% Response $^a$</th>
<th>% Callus $^b$</th>
<th>% Shoots $^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^d$</td>
<td>$^d$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$X$</td>
<td>$2X$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$X$</td>
<td>$2X$</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>71 (120)$^{e,f}$</td>
<td>45 (80)$^{e,f}$</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>57 (7)</td>
<td>nd</td>
<td>100</td>
</tr>
<tr>
<td>500</td>
<td>100 (7)</td>
<td>nd</td>
<td>0</td>
</tr>
<tr>
<td>750</td>
<td>nd</td>
<td>100 (6)</td>
<td>nd</td>
</tr>
<tr>
<td>1000</td>
<td>86 (14)</td>
<td>100 (11)</td>
<td>33</td>
</tr>
<tr>
<td>2000</td>
<td>100 (16)</td>
<td>100 (13)</td>
<td>67</td>
</tr>
<tr>
<td>4000</td>
<td>86 (17)</td>
<td>100 (14)</td>
<td>36</td>
</tr>
<tr>
<td>6000</td>
<td>29 (56)</td>
<td>nd</td>
<td>46</td>
</tr>
<tr>
<td>8000</td>
<td>0 (41)</td>
<td>11 (29)</td>
<td>54</td>
</tr>
</tbody>
</table>

$^a$ Measured as the percentage of explants showing shoot initiation
$^b$ Measured as the percentage of explants showing callus formation
$^c$ Measured as the percentage of explants showing shoot formation

$^d$ Data are mean values
$^e$ Values in parentheses indicate the number of explants
$^f$ Values in parentheses indicate the number of replicates
% response = percent of the explants treated with \( \gamma \)-irradiation which exhibited growth in the initiation passage

% callus = percent of those explants surviving \( \gamma \)-irradiation which produced only brown callus during the initiation and first passage

% shoots = percent of those explants surviving \( \gamma \)-irradiation which produced only shoots and green organogenetic callus during the initiation and first passage

\( X, 2X \) = source of petiole tissue in this experiment was \( X(4) \) and \( 2X \)

( ) = numbers in parentheses indicate the total number of explants which exhibited growth after \( \gamma \)-irradiation

data for control treatment is an average from EMS (Figure 4.13), and KCN (Figure 4.15) treatment controls

nd = not determined, cultures lost to contamination
and subcultured to fresh shoot medium. After an additional 4 weeks culture, the explants were again scored on the same basis. Compared with the initiation passage, no difference in the type of proliferation in the first passage was observed. The tissues were repeatedly subcultured to shoot medium. In the fifth passage, brown callus was observed to organize green shoots (Figure 4.18). The callus, however, remained brown.
An analysis of the data of Table 4.7 indicates a trend toward the proliferation of monoploid and diploid tissues as brown callus on exposure to low $\gamma$-dose. With higher $\gamma$-dose, however, the frequency of proliferation as brown callus apparently decreased, while the frequency of shoot production increased.

In ten separate experiments, $\gamma$-irradiated (500 R to 8000 R) monoploid and diploid explants were cultured on callus medium (DM-1, 1 g/liter casein hydrolyzate, $10^{-6}$ M 2,4-D). The resulting calli were used to initiate suspension cultures (medium as above but with $2.5 \times 10^{-6}$ M 2,4-D). The suspensions were plated on shoot initiation medium, and cultured in the light or dark. Dark cultured platings remained a tan color. Plated suspensions grown in the light always proliferated as brown callus without recognizable regions of differentiation (Figure 4.19). Plated suspensions from untreated, or EMS treated tissues were always observed to become green and organogenetic when cultured in the light.

Figure 4.19  Morphology of plated suspensions derived from EMS treated explants (left), and $\gamma$-irradiated explants (right). Plates were cultured in the light.
4. Selection of variants

Table 4.8 lists the attempted number of trials and different mutagenic treatments used to isolate variants by plating of suspensions derived from mutagenized petiole explants. Although a wide variety of different mutagenic treatments were used on explants from different monoploid plant isolates, no variants were selected by plating on callus or shoot medium in the light or dark. No morphological variants were obtained when suspensions initiated from mutagenized petiole tissues were plated on medium without anti-metabolites and cultured in the light or dark. Pre-culture of the explants before mutagenic treatment had no effect on the recovery of variants.

Because of the space required for variant selection at the shoot level of organization, fewer trials were possible (Table 4.9); however, 5 cycloheximide-resistant lines were obtained by this method. Nine cycloheximide-resistant lines were originally selected, but 4 died on subsequent subculture to cycloheximide containing medium. The five lines which survived originated from different explants indicating they were derived from different "mutagenic" events. All 5 resistant lines were less green than normal shoot cultures (Figure 4.20), but upon subculture to medium without cycloheximide, were indistinguishable from the normal. The resistant lines were subcultured to non-selective medium for 3 passages, then transferred back to cycloheximide-containing medium. In all cases, after an initial browning of the resistant tissues, they proliferated as green shoots. Sensitive tissues subcultured to cycloheximide-containing medium became permanently brown (Figure 4.21). The resistance of all 5 cycloheximide-resistant lines was also tested in callus culture.
Table 4.8  Variants isolated by plating of suspension cultures derived from mutagenized petiole explants of *D. innoxia* on selective medium.

<table>
<thead>
<tr>
<th>Source of explant&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Initiation period (days)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mutagen</th>
<th>Dose</th>
<th>Duplicates&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Variants&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>X(1)</td>
<td>9</td>
<td>EMS</td>
<td>0.2%, 1 h</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>X(1)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 1 h</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>X(1)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>X(1)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>X(4)</td>
<td>19</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>X(4)</td>
<td>24</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>9</td>
<td>EMS</td>
<td>0.2%, 1 h</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>11</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 1 h</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>19</td>
<td>EMS</td>
<td>0.2%, 1 h</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 6 h</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 8 h</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>X(5)</td>
<td>0</td>
<td>EMS</td>
<td>1%, 1 h</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>9</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>11</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 1 h</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>17</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> source of explant = origin of tissue, either diploid (2X), or monoploid (X), where (n) is the monoploid isolate designation

<sup>b</sup> initiation period = period of time explants were cultured on callus initiation medium before they were exposed to mutagenic treatment; 0 denotes no pre-culture

<sup>c</sup> duplicates = number of petiole explants exposed to the same mutagenic treatment, produced callus, suspensions, and subjected to selection procedures

<sup>d</sup> variants = number, and type of variant isolated
<table>
<thead>
<tr>
<th>Source of explant</th>
<th>Initiation period (days)</th>
<th>Mutagen</th>
<th>Dose</th>
<th>Duplicates</th>
<th>Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 5 h</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 6 h</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>1%, 2 h</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>γ-ray</td>
<td>4000 R</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>γ-ray</td>
<td>6000 R</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>γ-ray</td>
<td>8000 R</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>none</td>
<td>-</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>10</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 1 h</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>12</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>24</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 5 h</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
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<td>EMS</td>
<td>0.2%, 6 h</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
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<td>EMS</td>
<td>1%, 1 h</td>
<td>12</td>
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</tr>
<tr>
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<td>γ-ray</td>
<td>1000 R</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>0</td>
<td>γ-ray</td>
<td>2000 R</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>0</td>
<td>γ-ray</td>
<td>6000 R</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>X(9)</td>
<td>0</td>
<td>none</td>
<td>-</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>X(10)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>X(10)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 6 h</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>X(10)</td>
<td>0</td>
<td>none</td>
<td>-</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>2X</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>2X</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 6 h</td>
<td>16</td>
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</tr>
<tr>
<td>2X</td>
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<td>EMS</td>
<td>0.2%, 8 h</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>2X</td>
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<td>none</td>
<td>-</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4.9 Variants isolated by subculture of shoot cultures derived from mutagenized petiole explants of *D. innoxia* to selective medium.

<table>
<thead>
<tr>
<th>Source of explant</th>
<th>Initiation period (days)</th>
<th>Mutagen</th>
<th>Dose</th>
<th>Duplicates</th>
<th>Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>X(4)</td>
<td>20</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>X(4)</td>
<td>20</td>
<td>EMS</td>
<td>1%, 1 h</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>X(4)</td>
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<td>EMS</td>
<td>0.2%, 4 h</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>X(4)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 6 h</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
<td>none</td>
<td>-</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>14</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>14</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>14</td>
<td>EMS</td>
<td>0.2%, 6 h</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 2 h</td>
<td>4</td>
<td>2 cyclo-heximide-resistant(^e)</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 3 h</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>EMS</td>
<td>0.2%, 4 h</td>
<td>3</td>
<td>2 cyclo-heximide-resistant(^f)</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>γ-ray</td>
<td>2000 R</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) source of explant = origin of tissue, either diploid (2X), or monoploid (X), where (n) is the monoploid isolate designation

\(^b\) initiation period = period of time explants were cultured on shoot initiation medium before they were exposed to mutagenic treatment; 0 denotes no pre-culture

\(^c\) duplicates = number of petiole explants exposed to the same mutagenic treatment, produced shoots, and subcultured to selective medium

\(^d\) variants = number, and type of variants isolated

\(^e\) cycloheximide-resistant variants = termed 1S and 3S, respectively

\(^f\) cycloheximide-resistant variants = termed 5S and 7S, respectively
Table 4.9 (continued)

<table>
<thead>
<tr>
<th>Source of explant</th>
<th>Initiation period (days)</th>
<th>Mutagen</th>
<th>Dose</th>
<th>Duplicates</th>
<th>Variants</th>
</tr>
</thead>
<tbody>
<tr>
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<td>γ-ray</td>
<td>4000 R</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>X(8)</td>
<td>0</td>
<td>none</td>
<td>-</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>2X</td>
<td>0</td>
<td>EMS 0.2%, 6 h</td>
<td>6</td>
<td>1 cycloheximide-resistant®</td>
<td></td>
</tr>
<tr>
<td>2X</td>
<td>0</td>
<td>none</td>
<td>-</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

® cycloheximide-resistant variant = termed 15S
Figure 4.20 Culture phenotype of normal shoot cultures (left), and of cycloheximide-resistant variant 15S on medium containing 10 μg/ml cycloheximide (right).

Figure 4.21 Retesting of cycloheximide-resistant isolate 3S on cycloheximide-containing medium after 3 subcultures on shoot medium lacking cycloheximide (left). Normal tissue never exposed to cycloheximide served as the control (right).
Petioles with leaves attached were removed from sterile shoot cultures of
the resistant lines and transferred to cycloheximide-containing callus
medium and cultured in the dark. Tissue from non-variant shoot cultures
served as controls. After 4 weeks, tissue of both cycloheximide-resistant
and control lines produced callus on medium without cycloheximide.
Neither of the lines formed callus on medium containing cycloheximide,
but the resistant lines remained green for approximately 1 to 2 weeks
longer (Figure 4.22).

All five cycloheximide-resistant lines have been rooted, and prop-
agated as whole plants under greenhouse conditions. The whole-plant
morphological phenotype of the cycloheximide-resistant lines was observed
to be the same as normal plants.

During testing of the effects of EMS and γ-irradiation on shoot
production by petiole explants (Section IV. B. 3.), different morpho-
logical variants, particularly pigment-deficient phenotypes, were screened
for. It was reasoned that the frequency of pigment-deficient phenotypes
could be used as a means of quantifying mutagen efficiency. However, no
morphological variants of any type were observed.
Figure 4.22  Culture of separated leaves from cycloheximide-resistant isolate 78 (top) and normal tissue (bottom) on callus medium with (left), or without (right) 10 μg/ml cycloheximide.
V. DISCUSSION

A. Turbidimetric Methods in Growth Determination

The use of turbidimetry to determine suspension culture growth kinetics under differing cultural regimes and pressures seemed to be an invaluable technique. The reproducibility of doubling times using this monitoring function was excellent (Table 4.1), thus illustrating the non-destructive merit of the technique. Turbidimetry permits simple, rapid, and reproducible determinations of growth kinetics. This method has been used in bacterial systems for many years.

Klett units were calibrated to mg dry weight, fresh weight, and packed cell volume (per unit volume) for cells in DM-1 with $2.5 \times 10^{-6}$ M 2,4-D (Figure 4.3). In this calibration experiment, the doubling time of the cultures as determined by turbidimetry, dry weight, fresh weight, and packed cell volume were 2.98 days, 2.21 days, 2.64 days, and 2.06 days respectively, and were not significantly different. The dry weight doubling time of cells in the same medium, but of an independent experiment, was 2.36 days (Figure 4.4). In these particular experiments, for some reason, an inordinate amount of error in the determinations of doubling times of the different growth parameters was encountered. Thus, these calibrations should be repeated, for when developing a new parameter to follow cell growth, calibrations to accepted growth monitoring functions should leave no doubt as to the relationship between different growth parameters. There is no reason to expect that doubling times of plant suspension cultures measured with different parameters should reflect the same value. During exponential growth, different parameters may display exponential increases at different rates. For example, King, Mans-
field, and Street (1973) have observed that batch suspension cultures of
A. pseudoplatanus displayed different doubling times depending on the
growth parameter measured. Cell number, total protein, and dry weight
(all expressed per unit volume) reflected doubling times of 2.36 days,
3.21 days, and 7.14 days, respectively. This phenomenon is termed
unbalanced growth. They also reported that the cell number doubling time
increased over the 2,4-D concentration range $4.5 \times 10^{-6}$ M to $9 \times 10^{-6}$ M,
but the dry weight doubling time was constant. With D. innoxia batch
suspensions grown in DM-1 + 1 g/liter casein hydrolyzate, no difference
in the slope of mg dry weight/ml vs. Klett was observed in the 2,4-D
concentration range $10^{-6}$ M to $10^{-5}$ M - the correlation coefficient of
mg dry weight/ml/Klett and 2,4-D concentration was 0.109. The slopes
of mg dry weight/ml vs. Klett, although appearing different for varying
2,4-D concentrations (Figure 4.5), were thus, in fact, not different.
Because Klett units are so much larger than mg dry weight/ml, even
relatively large deviations in the dry weight would result in only minor
variation in the slope. Thus, it is possible to infer that the dry
weight doubling time in these experiments increased equivalently with
turbidimetrically-determined doubling times from $10^{-6}$ M 2,4-D to $10^{-5}$ M
2,4-D in DM-1 with 1 g/liter casein hydrolyzate. It remains to be seen if
this relationship is maintained in media of different composition,
subculturing regime, or if other growth parameters respond similarly.

Turbidity is a function of light absorption and scattering. It
seems that absorption plays the dominant role, as the aggregation observed
with low 2,4-D concentrations did not influence the slope of mg dry
weight/ml vs. Klett (see above). Because light absorption is by the
Figure 5.1 Typical growth kinetics of D. innoxia in DM-1 + 2.5 X 10^{-6} M 2,4-D monitored by turbidimetry (—○—), and conversion of these data into mg dry weight/ml (—■—) by extrapolation from the calibration curve of Figure 4.3.
solid material of cells, it could be reasoned that the relationship of Klett to mg dry weight might exhibit less deviations due to unbalanced growth as they measure the same thing. The deviation observed in the calibration of Klett to other growth parameters at low Klett values might be due to factors influencing Beer's law. Calibration of Klett to other parameters at a different wavelength (420 nm used here) might prove to be useful.

To serve as a check on the relationship of turbidimetric and dry weight doubling times, a dry weight/ml vs. time plot was reconstructed (Figure 5.1) from a Klett vs. time plot using the calibration curve. Although the turbidimetric doubling time was 3.01 days in this experiment, the dry weight doubling time was computed to be 2.01 days, and was significantly different ($t=9.04**$; 12 df).

The initial lag phase observed turbidimetrically with *D. innoxia* suspensions (Figure 5.1) was associated with the initiation of a suspension culture with a small inoculum. If larger inocula were used, the lag period became shorter, but a decrease in the doubling time was observed, and the relationship of Klett to time became less log-linear. The cause for this is unknown. The minimum dilution permitting immediate exponential growth was 1:6 for cells grown in DM-1 with $2.5 \times 10^{-6}$ M 2,4-D. The lag phase observed turbidimetrically was also observed with dry weight (Figure 4.4), and the turbidimetric lag was also translated into dry weight lag by extrapolation using the calibration curve (Figure 5.1). Exponential growth was initiated at approximately 0.5 mg dry weight/ml (Figure 4.4), which corresponds well with the calibration value of 0.3 to 0.6 (Figure 4.3).
A drawback to the use of turbidimetry is the relatively narrow range in which growth can be monitored. Typically, *D. innoxia* suspensions could be monitored for only 2 logs of growth, while most other methods permit monitoring through 3 to 4 logs of growth (Figure 4.4). However, when this technique was used for the determination of anti-metabolite toxicity, response time was rapid (Figure 4.10). Compared to tissue cultures, suspension cultures generally grow more rapidly, consist of well-separated cells, and because of their submerged nature, are in more intimate contact with the culture medium. It was not unexpected, then, to find that the lethal concentration of an anti-metabolite might be less in suspension cultures than in tissue cultures (Table 4.2). Not only is transport in tissue cultures more restricted because the cells lie on the surface of a medium, but based on previous evidence, gene expression might not be similar at different organizational levels. These two factors probably account for most of the differences in sensitivity to anti-metabolites between suspension and tissue cultured cells.

B. Growth, and Dissociation of Aggregates in Batch Suspension Cultures

Engvild (1974) reported rapid proliferation of well-dispersed batch cultured *D. innoxia* suspensions, initiated from stem pith, in Murashige and Skoog medium with 1 g/liter casein hydrolysate, 0.1 X micronutrients, and $10^{-6}$ M 2,4-D. Growth was monitored by dry weight. Although $10^{-6}$ M 2,4-D was found to be near the 2,4-D concentration for optimal turbidimetrically-determined growth, the high degree of cell separation at this hormone concentration was not observed. Good cell separation occurred only with $2.5 \times 10^{-6}$ M 2,4-D and higher. The doubling time of *D. innoxia* suspensions in DM-1 with 1 g/liter casein hydrolysate varied linearly over
the 2,4-D concentration range, \(10^{-6}\) M to \(10^{-5}\) M. Bayliss (1977c) reported that the mean generation time of carrot suspension cultures varied linearly from \(4.5 \times 10^{-6}\) M 2,4-D to \(1.3 \times 10^{-4}\) M 2,4-D. The frequency of cells in M and S phase decreased with increased 2,4-D concentration, thus the increase in generation time was attributed to an increase in \(G_1\) or \(G_2\), or an increasing proportion of the cells failing to complete the cell cycle. Despite the increase in mean generation time with increased 2,4-D concentration, S phase had a constant duration. Leguay and Guern (1975), on the other hand, observed the cell number doubling time of *A. pseudoplatanus* batch suspension cultures was constant from \(4 \times 10^{-8}\) M 2,4-D to \(4 \times 10^{-6}\) M 2,4-D.

The reported toxic effect of full-strength micronutrients on *D. innoxia* suspension in the absence of casein hydrolyzate (Engvild, 1974), was not observed in the presence of casein hydrolyzate, either in doubling time (Figure 4.1), or lag phase (Figure 4.2). Reasons to be considered in accounting for the discrepancies between Engvild's observations and those reported here are the source of the original explant (stem pith vs. petiole), cultural conditions (light grown vs. dark grown), and genetic variability of the donor plants. In these experiments, even at low light intensities (50 lux), *D. innoxia* suspensions were green and non-friable.

Sopory and Maheshwari (1976) reported significant differences in shoot initiation between monoploid and diploid *D. innoxia*, with monoploid tissue differentiating more readily. Matthews and Vasil (1976) observed pith explants of haploid (allodihaploid) *N. tabacum* cv. 'Xanthi' proliferate in callus more rapidly than tetraploid, an observation also
reported by Kerbauy, Hell, and Hanbro (1976) with *N. tabacum* cv. 'Wisconsin 38' and 'IAG-70'. In experiments with *D. innoxia* suspension cultures, no significant difference was observed in the growth rate of monoploid or diploid-derived suspensions.

The data of Figure 4.5 suggests that the major component of total dry weight accumulation in batch culture varied with the 2,4-D concentration. At $10^{-6}$ M 2,4-D, the 30 mesh residue dry weight generally increased coordinately with total dry weight. At $2.5 \times 10^{-6}$ M to $10^{-5}$ M 2,4-D, dry weight preferentially accumulated with smaller aggregates.

The mitotic index studies further characterized these observations. At $10^{-5}$ M 2,4-D, smaller aggregates displayed an absolute increase in mitotic index over similar fractions at $10^{-6}$ M 2,4-D. The mitotic index of these smaller aggregates at elevated 2,4-D concentrations also increased relative to the larger-aggregate fraction. During the early stages of the $10^{-6}$ M batch cultures, caution should be exercised in interpreting the differences in mitotic indices of the aggregate fractions. When subculturing, a truly representative sample can not be obtained because of the large size of the aggregates. Even wide-tip pipets become easily clogged. If an equilibrium exists between aggregates of various sizes, the perturbation resulting from the subculture of an unrepresentative sample may influence the growth of the aggregate fractions.

The decrease in aggregation observed at high 2,4-D concentrations was shown not to be associated with selection favoring the proliferation of less aggregated cells. Subculture of cells conditioned to $10^{-5}$ M 2,4-D into medium containing $10^{-6}$ M 2,4-D displayed an immediate reversal
to a more aggregated state. The effective decrease in 2,4-D concentration was from $10^{-5}$ M to $1.5 \times 10^{-6}$ M. The kinetics of total dry weight accumulation was not linear during this subculturing regime with respect to Klett units. It is doubtful if selection of more aggregated cell populations upon subculture to a lower 2,4-D concentration would occur as rapidly as observed. Indeed, even when suspensions were initiated from callus, less aggregation was observed with high 2,4-D concentrations. King (1976), using turbidostat cultures of A. pseudoplatanus, stepped-down the 2,4-D concentration from $4.5 \times 10^{-6}$ M to $5 \times 10^{-7}$ M over a period of 200 h. A 2.9-fold increase in the dry weight of aggregates $>1000 \mu m$ occurred after 2,4-D withdrawal, and the accumulation of lignin as % total dry weight increased 5-fold. It is probable that the dramatic increase in dry weight accumulated by D. innoxia suspensions was an over-compensatory reaction to the withdrawal of 2,4-D, for the total dry weight/ml accumulated was approximately the same when cells were subcultured into medium of the same hormone concentration in the range $10^{-6}$ M 2,4-D to $10^{-5}$ M 2,4-D (Figure 4.5). The non-linearity of mg dry weight/ml vs. Klett observed upon 2,4-D withdrawal might be due to a transient episode of extreme, inconstant unbalanced growth. The increased aggregation also observed in this subculture is due to an increased failure of daughter cells to separate, and is probably due to enhanced synthesis of cell wall polymers such as lignin.

Disruption of aggregates was attempted with a wide variety of chemical and mechanical treatments but was unsuccessful. Estimates of aggregate concentration, and cells/aggregate were therefore inaccessible. It is unknown if the decrease in aggregation with high 2,4-D concentrations is
a direct adaptive response at the cellular level. Decreased aggregation with high 2,4-D concentrations has been reported to occur in other species (Torrey, Reinert, and Merkel, 1962; Wallner and Nevins, 1973; King, 1976), and cell expansion under these conditions has also been observed (Digby and Wareing, 1966). The physiological basis for these effects remains largely speculative.

These observations certainly bear strong implications regarding the effect of 2,4-D on growth, and the contribution of aggregates to the behavior of suspension cultures. Generally, the growth potential of larger aggregates, as determined by mitotic index, has been observed to be greater than that of smaller aggregates and single cells (Torrey et al., 1962; Henshaw, Jha, Mehta, Shakeshaft, and Street, 1966). Aggregates would thus contribute the largest portion of growth in suspension culture, with single cells being sloughed off and subsequently exhibiting a comparatively slower growth rate. Henshaw et al. (1966) reported that during the period of maximum exponential growth of A. pseudoplatanus batch cultures, the greatest contribution to packed cell volume and total cell number was from large aggregates. Only upon reaching stationary phase did the number of smaller aggregates and single cells increase. Negrutiu and Jacobs (1977) have made use of the phenomenon that aggregates tend to dissociate near the end of exponential phase. Fresh medium was added to late log batch suspension cultures of Arabidopsis thaliana at a defined biomass:volume ratio, thus maintaining the cells in a perpetual state of late log. This technique not only increased the number of cells in the single cell fraction, but enhanced their viability as well.

Aggregation seemed to be coupled to the total culture doubling
time in some circumstances. Increased 2,4-D concentrations not only resulted in decreased aggregation, but also increased the doubling time. Malic and succinic acids at 20 mM to 30 mM, although enhancing growth, stimulated aggregation (Table 4.1, and Plate 4.1). When these organic acids were supplied at 40 mM, a dramatic decrease in growth rate and aggregation was observed. Thus it would seem that a consequence of rapid growth is decreased aggregate dissociation, a circumstance which might be related to the large contribution to total culture growth by large aggregates during exponential phase. These observations suggest that it may be difficult to obtain rapidly growing, single-cell suspensions in higher plants. However, although growth was enhanced with the addition of casein hydrolyzate, aggregation was not visually observed to increase (Table 4.1, and Plate 4.1). Wallner and Nevins (1973) observed a decrease in aggregation when casein hydrolyzate was supplied to Paul's Scarlet rose cells, but the growth rate remained about the same. A recent proposal regarding the involvement of peroxidase in cell wall biosynthesis may contribute an explanation for the increased aggregation observed with the addition of malic or succinic acids (Lamport, in press). Essentially, NADH produced by cell wall-bound malate dehydrogenase is used in the peroxidase-mediated evolution of H₂O₂, which in turn drives the free radical polymerization of various cell wall constituents and depletion of endogenous auxin thus limiting cell expansion. Large concentrations of extracellular malate may thus result in enhanced H₂O₂-mediated reactions and cause increased aggregation due to the enhanced cross linking of cell wall polymers.

Good growth of *D. innoxia* suspensions with nitrate as the sole
nitrogen source without organic acids was not observed, although Engvild (1974) reported a dry weight doubling time of 2.3 days for D. innoxia cells grown in such a medium. These observations also indicate the inability of D. innoxia to proliferate with ammonium as the sole nitrogen source with the addition of organic acids. Similar findings were reported by Gamborg, Miller, and Ojima (1968) with soybean, and by Behrend and Mateles (1976) using tobacco. Martin, Rose, and Hui (1977) were able to grow Ipomoea purpurea and soybean with ammonium as the sole nitrogen source without the addition of organic acids by controlling the pH during cultivation. The dramatic growth enhancement of D. innoxia suspensions in ammonium-only medium with the addition of Krebs cycle organic acids has also been observed by Gamborg and Shyluk (1970) with soybean, and by Behrend and Mateles (1976) using tobacco. Fukunga, King, and Child (1978) examined the effect of citrate and α-ketoglutarate on the growth of alfalfa, tobacco, and wheat suspension cultures with ammonium or nitrate as the sole nitrogen source. A medium with glutamine as the sole nitrogen source was also tested. In general, there were species differences in the ability to proliferate in any specific medium. All the species grew in nitrate-only medium, but only wheat suspensions were capable of good growth in ammonium-only medium, and only during the initiation passage. The growth of all three species in ammonium-only medium was enhanced by the addition of α-ketoglutarate, but while citrate was effective in promoting rapid growth of alfalfa and wheat suspensions, tobacco cells were inhibited. This is in contrast to the findings of Behrend and Mateles (1976), for they reported that although tobacco cells did not proliferate in ammonium-only medium, addition of citrate
greatly enhanced growth. The medium of Behrend and Mateles contained 10 mM NH₄Cl (10 mM NH₄), while the medium of Fukunaga et al. was composed of 12.5 mM (NH₄)₂SO₄ (25 mM NH₄). The mM ratio of citrate:NH₄ in the former case was 1.0, while in the latter ranged from 0.2 to 0.6. This suggests that not only the relative concentrations of citrate and ammonium, but the absolute concentrations of the two components was an important factor in the ability of tobacco cells to proliferate. This also suggests that the proliferation of cell in ammonium-only medium containing other TCA acids may be modified by the relative and absolute concentrations of ammonium and organic acid. Behrend and Mateles (1976), for example, observed that depending on the succinate concentration in ammonium-only medium, the ammonium concentration promoting optimal growth of tobacco suspensions changed. With 5 mM succinate, 10 mM NH₄Cl permitted the most rapid growth (succinate:NH₄, 0.5). When the succinate concentration was raised to 10 mM, 15 mM NH₄Cl promoted the most rapid growth (succinate: NH₄, 0.67). In experiments with D. innoxia, DM-2 contained 20 mM NH₄, and the mM ratio of succinate to NH₄ ranged from 1 to 2. A maximum doubling time was observed with a succinate:NH₄ ratio of 1.5. Cells in DM-1 (ammonium+nitrate, 20 mM NH₄) were found to exhibit maximal growth rates with a succinate or malate mM ratio to ammonium of 1.5. In contrast, cells proliferated most rapidly with 20 mM succinate in DM-3 (40 mM NO₃). The mM ratio of succinate to nitrate in this case was 0.5. Conceivably, even lower succinate concentrations might enhance the growth of cells in DM-3 medium to a greater extent. Because the mM ratio of succinate to ammonium resulting in optimal growth in DM-1 and DM-2 was the same (1.5), it may be suggested that succinate, and perhaps other TCA acids, exert a
growth stimulatory effect through an interaction with ammonium rather than with nitrate.

Casein hydrolyzate, succinate, and malate were observed to be good media buffers, and generally allowed more rapid growth. Martin and Rose (1976) observed the rate of biomass accumulation and final dry weight of I. purpurea cultures under pH-stat conditions to be greatest at pH 5.6 to 6.3. It may be suggested, then, that the enhancement of growth by the addition of casein hydrolyzate, malic or succinic acids, may be closely related with the buffering capacity of these addenda, thus allowing better ammonium, nitrate, and sucrose utilization rather than by providing carbon skeletons. In DM-1, however, succinate permitted a relatively higher growth rate than equivalent concentrations of malate. In general, the growth rates of D. innoxia suspensions do not correlate with the maintenance of a stable pH. The results of Gamborg and Shyluk (1970), and Fukunaga et al. (1978) indicate a similar effect. In all cases, the pH was determined at the termination of a passage. Final pH does not reflect pH changes during the batch growth cycle, so it is unknown if the differences in supporting growth by these organic acids was due to more stringent pH control, or whether some secondary effects relating to utilization of these acids.

As demonstrated in this study, cultures supplied with nitrate-only became more alkaline, while cultures supplied with ammonium, or ammonium with nitrate, became more acidic (Gamborg, Miller, and Ojima, 1968). Addition of various organic acids do not alter trends in this regard (Gamborg and Shyluk, 1970; Fukunaga et al., 1978).
On the basis of these studies, DM-1 with \(2.5 \times 10^{-6}\) M 2,4-D is proposed as a basal medium to be used in the selection of amino acid auxotrophs or amino acid analog resistant lines from *D. innoxia* suspension cultures. Medium of this composition, although still permitting the formation of cellular aggregates, is the most conducive to aggregate dissociation of all media tested while still permitting relatively rapid growth rates. Casein hydrolyzate may be added to culture media at 1 g/liter with no apparent increase in aggregation of cells, and could be used in cases which will not affect the specificity of selection.

*D. innoxia* suspension cultures have also been subjected to a frequent subculturing regime (10 ml into 30 ml fresh medium at 4 d intervals), and the dry weight doubling time using the suggested medium was 0.61 days. Aggregation with this subculturing regime was not visibly increased over the level already described for cells grown in this medium.

C. Variant Selection

1. **Analysis of the method**

Variant selection in the initiation passage of adventitious shoot formation from explants as described here fulfills many of the recommendations for an efficient mutant isolation scheme from higher plant cell and tissue culture. Mutagenesis was directed at cells of known ploidy by determining the chromosome number of the donor plants, and the morphogenetic potential of initial explants was high. Chromosomal stability was high as all plants regenerated from initial explants possessed the same chromosome number as that of the explant from which they originated. Most importantly, selections were attempted at a highly organized ontogenetic level. Petioles were chosen as a source of tissue for initial explants
as they were available in large numbers, and petiole tissues respond, apparently similarly, at least with *D. innoxia*, to callus and shoot induction conditions as do stem pith explants (Engvild, 1973).

Selection of variants at the whole plant level after mutagenesis of cells has precedent. The "advantageous bud technique" makes use of the phenomenon that vegetative buds and roots form at the base of the petiole of leaves separated from plants (Broertjes, Haccius, and Werdlich, 1968). The origin of these plants apparently stems from a single cell as Broertjes (1969) reported that only 45 of 857 different γ-irradiation induced mutants isolated from *Streptocarpus* were chimera using this technique. In callus and cultured epidermal tissues, the primordia of organs may be formed from a single cell, but are more generally organized from a small group of cells (Reinert et al., 1977; Tran Thanh Van, Chlyah, and Chylah, 1974). Embryogenesis in suspension cultures, however, is generally observed to stem from a single cell (Reinert et al., 1977; Tran Thanh Van et al., 1974). Thus, direct controlled formation of organs (shoots, roots, floral organs) from single cells is not observed *in vitro*, but requires a more voluminous tissue.

In experiments with *D. innoxia*, after mutagenesis of petiole segments, the tissue was cultured on a non-selective medium, thus allowing proliferation of both mutant and non-mutant cells. If shoot organogenesis originated from more than one cell, it is possible that the low number of variants obtained was due to the chimera nature of the regenerated shoots, as a mixture of mutant and non-mutant cells might be normal in phenotype. It is unknown if shoot organogenesis from petiole explants of *D. innoxia* originates from a single cell or group of cells. During selections
using chemical selective agents, even if regenerated shoots were chimeral, wild type cells might die while the resistant cells proliferate to produce a shoot containing cells of the same genotype. However, perhaps the death of wild type cells in the shoot during selective conditions resulted in leaching of toxic substances thereby killing variant cells as well. One might term this latter possibility "cooperative death". It is important to note that throughout all the mutagenesis experiments, using EMS or \( \gamma \)-irradiation, no chlorophyll-deficient or morphological variants were recovered. With visually selected phenotypes, if indeed the regenerated plants were chimeral, the close association of normal and mutant cells may have permitted cross-feeding from the normal to the mutant cells, thus allowing mutant cells to appear normal.

It was hoped that the sectioning of petioles into small pieces prior to mutagenesis would limit the number of morphogenetically competent cells per tissue segment and so permit sectoring of mutant and non-mutant cells. Thus, in any one small area of the developing organogenetic callus, cells would be of the same genotype. To further aid in the localization of these sectors, the tissues, after mutation fixation, were divided into smaller pieces and transferred to selective medium.

In contrast to selection at the shoot level in which cells are closely associated, competition and cross-feeding between cells when suspensions are plated on selective medium should be minimal. All the cells of an aggregate are genetically the same, and the separation between aggregates should be sufficient to neutralize "cooperative death". However, no variants were selected at this level of organization either.

Conceivably, the mutation fixation period was of a duration not
conducive to mutant rescue. In *D. innoxia* suspension cultures, only the initial phases of growth were characterized by a high mitotic index (Figure 4.6). In latter stages of logarithmic growth, cell division decreased, and subsequent growth probably occurred by cell expansion. Since maximal cell division occurred between Klett 100 and 400, approximately 2 cell doubling occurred (assuming no unbalanced growth). In preliminary callus growth experiments, the fresh weight of callus in the initiation passage increased logarithmically from 0.2 g/callus to 10 g/callus after 35 days. If the kinetics of callus mitotic index is assumed to parallel that of suspension cultures, one-third of the callus exponential phase would be devoted to cell growth by cell division. From the time petiole explants were mutagenized, to the time suspensions from these explants were plated on selective medium, conservatively, a total of 5 cell doublings occurred. This would appear to be a suitable period for mutation fixation. The number of cell generations intervening between mutagenesis and selection at the shoot level are inestimable based on the observations reported here.

It is doubtful that the mutagens used in this study were inappropriately chosen, or used at a dose not conducive to efficient mutant isolation. As described previously, EMS has been shown to be an effective mutagen at the seed level, and as reviewed in Table 2.1, has been successfully used in plant cell and tissue culture. Even if EMS and γ-irradiation prove to be inefficient mutagens in this system, certainly spontaneous mutations would occur, and although occurring at low frequency, and depending upon when they occurred, a number of variants due to spontaneous mutation should have been rescued. Although accurate cell counts could
not be made, a conservative estimate would place the number of cells/ml at the time of suspension plating at $10^5$. Given that 594 total duplications were performed with various tissue sources and mutagen treatments, about $6 \times 10^7$ total cells were screened. If the spontaneous mutation rate were $10^{-6}$/cell/generation, about 60 spontaneously occurring mutations would have occurred. Assuming EMS and $\gamma$-irradiation were truly mutagenic in this system, this number could only increase. It might be argued that EMS could not efficiently penetrate the petiole segments, thus all the responding cells were not exposed to the mutagen. There is evidence that only the cells in the outer regions of explanted tissues are induced to divide (Aitchison, MacLeod, and Yeoman, 1977). It is possible that such a response was elicited from *D. innoxia* petiole explants, as no lateral growth of the segments was observed. Instead, callus proliferated from the cut ends of the petiole segments, providing a "dumbbell-like" appearance. One can infer, then, that callus from petiole explants originated from cells close to the cut surfaces of the segments (the wound response). It seems probable that all the responding cells of the explant were exposed to equivalent doses of EMS.

As cells in the petiole explant at the time of mutagen treatment were non-dividing, and were probably suspended in $G_i$ (Aitchison et al. 1977), mutagenic treatments might be more effective when applied during a time of cell division. However, pre-culture of explants prior to mutagenesis had no apparent influence on the frequency of variant recovery. Additionally, the observation that monoploid tissues were more sensitive to EMS and $\gamma$-irradiation than diploid explants would indicate that these mutagens were effecting cell death at the DNA level by the accumulation of
lethal mutations.

Taking all these observations and manipulations into account, it seems that there are two major reasons why so few variants were selected. First, it is possible that any mutant cells were at a competitive disadvantage. If non-mutant cells proliferated at a much faster rate than the variant cells, it may be difficult to rescue the variant cells. In addition to a decrease in the growth rate, the application of a mutagen, either by causing lesions in the DNA or by reacting with other cellular constituents, may produce such changes that a lag in growth occurs. If the mutation fixation period was too short, variant cells may have never had the opportunity to proliferate.

Secondly, the method of exposure to the selective agent was probably the most important factor in causing the low frequency of recoverable variants. In both shoot level and suspension plating selections, the cells were exposed to the selective agent continuously (chronic selection). Cells resistant to the selective agent should proliferate under these circumstances. Perhaps due to the accumulation of toxic by-products from those normal cells not resistant to the selective conditions the mutant cells were killed. In future experiments, it may be advisable to add the selective agent directly to suspension cultures derived from mutagenized petiole explants during early exponential phase (Klett 100 to 400) when the mitotic index is highest. This exposure could be chronic - the selective agent added and the culture examined periodically for re-growth as proliferating cells may be resistant variants. Perhaps a better alternative would be to expose the suspensions to the selective agent for a short time (acute exposure; about 10 days, at least for the anti-metabo-
lites examined here), washing the suspensions, and resuspending them in non-selective medium to permit re-growth of the variant cells. To eliminate any "cooperative death", after selective treatment and washing of the suspensions, they might be plated on non-selective, conditioned medium at low densities to minimize any cross-feeding.

The 5 cycloheximide-resistant variants isolated need to undergo further genetic and biochemical testing to establish them as due to a mutation. Maliga et al. (1976) have isolated cycloheximide-resistant variants of *N. tabacum* from suspension cultures, but these variants were unstable in the absence of cycloheximide. The cycloheximide-resistant variants of *D. innoxia* described here, however, when cultured on non-selective medium for 3 passages, still retained their resistance to cycloheximide. All the cycloheximide-resistant variants have been regenerated to whole plants, and it would not only be necessary to test shoot cultures initiated from tissues of these plants for cycloheximide resistance, but sexual crosses must be made to determine if the trait is heritable.

2. **Tissue responses to mutagenic agents**

The increase in morphogenetic response by both monoploid and diploid tissues after EMS treatment of petiole explants prompted the impression that mutation was the immediate cause. There is rapidly accumulating evidence that genotype plays an important role in not only the capability of a species to be cultured *in vitro* (Evans and Cocking, 1977), but also in the expression of totipotency. For example, Green and Phillips (1975) showed that different lines of maize differed dramatically in their ability to organize shoots. Izhar and Power (1977) demonstrated that the
hormonal requirements for protoplast development from a wide variety of inbred lines of *Petunia hybrida* differed markedly. It was observed that the hormonal requirements for good protoplast growth from hybrids of these inbred lines were considerably broadened in range toward the parental hormonal requirements. In one case tested, a hybrid was back-crossed to both parental inbred lines, and the hormonal requirements of the progeny were examined. The data indicated that genetic control was probably the determining factor for hormonal requirements as these requirements had a high heritability. Another indication that genotype may be a factor in the response of plant cells and tissues in culture is seen in the relatively higher morphogenetic potential of monoploid *D. innoxia* petiole explants compared to diploid explants. It is unknown if the ultimate determinant of this effect is ploidy level *per se*, or whether allelic segregation occurred to yield androgenetic haploid plants with monoploid genotypes conducive to growth in tissue culture.

Maliga, Lazar, Joo, Nagy, and Menczel (1977) reported that protoplast fusion of cultured cells of *N. sylvestris* resistant to kanamycin (Dix et al., 1977) with wild type *N. knightiana* mesophyll protoplasts resulted in the isolation of kanamycin-resistant clones possessing isozyme patterns indicative of a hybrid between the two species. The kanamycin-resistant line of *N. sylvestris* was not capable of organogenesis, but the hybrid, like the *N. knightiana* parent, could be readily induced to organize shoots. The apparent hybrids could also initiate shoots on kanamycin-containing shoot medium. Thus, some genetic factor lost in the kanamycin-resistant line was restored by complementation with wild type cells.
This is not to suggest that a specific locus is responsible for the expression of totipotency - a mutation at this locus then allowing prolific shoot organogenesis. The enhanced morphogenetic response with EMS treatment of petiole explants, at least with D. innoxia, apparently occurred with such high frequency that a mutagenic event at a single locus can not be implicated. Rather, mutation at any one or more of a large number of structural or regulatory genes may sufficiently modify the biochemistry and physiology of the cells to increase the morphogenetic response.

Experiments measuring the morphogenetic response of petiole explants incubated only in a liquid support medium up to 8 h indicated that this treatment had no observable effect on shoot morphogenesis (Table 4.5). Leaching of morphogenesis inhibitory substances during EMS treatment thus cannot be invoked as a cause for the increase in morphogenetic response after EMS treatment. It was consequently necessary to test if EMS treatment, in causing cell death within the explant, effected the production of morphogenesis enhancing substances which cross-fed from those dead cells to surviving morphogenetically competent cells. Possibly, cell death due to EMS exposure resulted in the nuclease digestion of nucleic acids within dead cells. Since cytokinins are substituted purines, the evolution of cytokinin-like substances through nucleic acid degradation may enhance shoot organogenesis. There are precedents in support of this cross-feeding hypothesis as a cause for the increased shoot production of EMS-treated explants. X-irradiated cells have served as feeder layers for protoplast proliferation at low plating densities (Raveh, Huberman, and Galun, 1973; Street, 1977), and plating of cells on con-
ditioned medium has been shown to enhance the growth response of the plated cells (Bergmann, 1977; Street, 1977). If the increased morpho-
genetic response after EMS treatment of petiole explants was due to cross-
feeding, then KCN treatment of petiole explants should effect a similar response as KCN causes death, but not mutation. Because the kinetics of % explant survival was the same for both monoploid and diploid explants (Figure 4.14), this was a further indication that KCN at the concentration and times used was not mutagenic. In contrast to KCN-treated explants, the kinetics of % survival of monoploid and diploid explants treated with EMS were considerably different (Figure 4.13). The cause of this difference can be attributed to the reduced gene dosage of monoploid tissue thus allowing the immediate expression of lethal mutations. It is possible that EMS caused more non-mutagenic damage in monoploid tissue than in diploid tissues, thus resulting in a higher mortality rate in monoploid tissues, but there is no evidence to support this suggestion.

The interpretation of the effect of KCN on shoot morphogenesis from petiole explants is not straightforward. Although there was no significant effect on shoot production by KCN-treated diploid explants (Figure 4.14), the 16 h treatment of monoploid explants with KCN caused a dramatic increase in shoot production/explant. If an analysis of variance is performed on all the data from KCN-treated monoploid petiole explants with the exclusion of the 16 h treatment, the shoot production/surviving explant is found to be the same regardless of KCN treatment time ($\chi^2 = 0.55$ ns).

If cross-feeding is the cause for the increased shoot production with EMS treatment, an experiment can be designed to further test this
possibility. Explants might be treated with KCN or EMS, and after a period of culture on shoot medium, the explants could be homogenized and extracted with hot and/or cold water. These extracts could then be used at different concentrations as an addendum to shoot medium. Non-mutagenized explants would be cultured on these media, and the effect of these extracts on shoot morphogenesis could be monitored. Alternatively, mutagenized petiole segments could be used as a nurse tissue with un-mutagenized petiole explants, or callus fragments, cultured on the surface of these explants separated by a raft of filter paper (Street, 1977).

The observation that petiole explants from plants regenerated from mutagenized petiole tissue did not exhibit an enhanced morphogenetic response would argue against mutation as the inducement to enhanced shoot production. However, more regenerated plants should be tested to certify this observation, as it is impossible to determine if any specific shoot organized from these tissue explants originated from mutagenized tissue, or whether it developed due to normal morphogenetic responses.

In examining the efficiency of shoot production with EMS (Figure 4.15) or KCN (Figure 4.16) treatment, the interpretation of the increased morphogenetic response of petiole tissues with these substances might be more straightforward. The efficiency of shoot production from monoploid and diploid KCN-treated petiole explants gradually decreased with increasingly longer KCN-treatment times. Thus, KCN, in causing death of the cells, decreased the shoot forming capacity of these explants. Apparently, cross-feeding of morphogenesis enhancing substances did not occur with KCN treatment. EMS-treatment of monoploid tissue resulted in an increase in the shoot forming efficiency with low dose, but gradually
decreased with longer EMS treatment times. In contrast, diploid tissue exhibited an increasingly higher efficiency of shoot production with extended EMS treatment. These data foster a possible interpretation concerning the effect of EMS on morphogenesis from petiole explants. It is well-known that EMS does not solely alkylate DNA. Experiments with EMS have demonstrated that at mutagenically effective doses injected into mice, 82% of the alkylations in the spermatozoa occurred outside of the DNA (Sega, Cumming, and Walton, 1974). It is conceivable that alkylation of structural or regulatory proteins, or other receptor or messenger molecules, may profoundly influence the activity of the molecules and so modify developmental processes. Because monoploid tissue possesses, theoretically, most structural genes in single dosages, the decline in shoot production efficiency with large doses of EMS can be viewed as an accumulation of lethal lesions which result in cell death precluding the enhanced morphogenetic response due to EMS-mediated alkylation of cellular constituents. Diploid tissue, on the other hand, can tolerate more lethal lesions and so is favorably affected by these chemical modifications.

It was expected that shoot production would be more affected by EMS treatments than would callus formation. It is certain that organogenesis requires the concerted and sequential action of more genes than callus initiation, but investigations centered on the relative amount of gene activity during callusing and organogenesis have not been reported. Isozyme patterns, however, do markedly differ between these two organizational levels (Scandalios and Sorenson, 1977). Callus formation by monoploid explants was terminated with a 15 h treatment with 0.2% EMS,
while diploid explants were still viable after the same treatment. Survival of monoploid and diploid explants cultured on shoot medium, in contrast, was nil with an 8 h treatment with 0.2% EMS.

Any manipulation of tissue explants prior to, or during culture, which enhances the morphogenetic response, would be of extreme importance to both plant propagation and developmental biology. If an enhanced morphogenetic response could be elicited with a non-mutagenic compound, the economic feasibility of tissue culture propagation of plants would be strengthened. This stimulation of organogenesis might also be studied with the aim of understanding the biochemical aspects of gene expression in developmental phenomena, particularly with respect to the effect of the nuclear environment. These results also suggest that EMS (or other "morphogenesis-enhancing" substance) pre-treatment of tissue explants from species which previously could not organize shoots in vitro might be influenced to elicit a morphogenetic response.

The qualitative differences in morphogenetic response of petiole explants after treatment with EMS or γ-irradiation are certainly interesting. While EMS-treated explants either produced shoots or died, γ-irradiated explants displayed preferential shoot production with low dose with a higher frequency of explants producing brown callus with higher doses (Table 4.7). Much work has been done on the effect of X- and γ-irradiation on morphogenesis in plant cell and tissue cultures. King (1949) cultured X-irradiated callus (6000 R) of tobacco next to un-irradiated callus on un-irradiated medium. Callus growth was inhibited in the un-irradiated explant, but meristem formation was increased. The tissues were cultured 1, 2, or 3 mm apart, and the rapidity of callus
inhibition was observed to increase when the tissues were cultured closer together. Similarly, Rao, Bapat, and Harada (1976) reported that low doses of γ-irradiation stimulated organogenesis from stem explants of *Antirrhinum majus*, while large doses inhibited organogenesis but enhanced callus formation. It is important to note that these experiments were conducted on un-irradiated medium, for Ammirato and Steward (1969) have shown that when low concentrations of irradiated sucrose (irradiated with $2 \times 10^6$ R) was added to culture media, rooting and callus production by carrot explants was enhanced. Embryogenesis in carrot and *Sium suave* suspension cultures, however, was abnormal with the addition of irradiated sucrose.

Spiegel-Roy and Kochba (1973a) reported that high doses of γ-irradiation (approximately 15 kR; 50 R/min) stimulated embryo formation in 'Shamouti' orange ovular callus, while low doses stimulated callus proliferation. If higher irradiation fluxes were used, the outcome was the same. When ovular callus was irradiated, the tissue was not removed from the medium upon which it was irradiated, so the observed effects could have been due to the presence of a radio-lysis product produced in the medium. To test this possibility, un-irradiated ovular callus was subcultured to irradiated medium. The response observed paralleled those experiments in which both callus and medium was irradiated simultaneously. The medium used in these experiments contained both kinetin and IAA in concentrations favorable to callus formation only. Since un-irradiated callus subcultured to irradiated medium resulted in an organogenetic response, it is possible that the cytokinin:auxin ratio was modified by γ-ray destruction of one or both of these hormones. This could result in
alteration of the relative or absolute hormone concentrations to produce a circumstance more conducive to organogenesis. Skoog (1935) had previously demonstrated that auxin was destroyed by X-irradiation.

Degani and Pickholz (1973) observed that N. tabacum callus cultures could differentiate shoots in the light but not in darkness. When callus cultures were γ-irradiated (0.5 kR; 5 kR/min), they were capable of differentiation in the dark. Un-irradiated tissue could also differentiate shoots in the dark if cultured on irradiated medium. Irradiated IAA added to the medium did not permit differentiation, but if IAA was omitted from the culture medium, differentiation occurred. Thus, irradiation-caused destruction of IAA, in these experiments, was apparently not the causal factor in permitting shoot differentiation in the dark.

Many of the effects described by the previous reports may have been caused by radio-lysis products produced in culture media. Vardi, Spiegel-Roy, and Galun (1975), however, reported that protoplasts from irradiated cultured ovules of Citrus sinensis, after 10 weeks in culture, produced many embryos. Un-irradiated protoplasts formed no embryos. There could be no effect of irradiated medium in these experiments, as after treatment with γ-irradiation, the protoplasts were washed, and cultured with fresh un-irradiated medium. Although X-irradiated cells served as good feeder layers allowing protoplast regeneration at low plating densities, EMS-treated cells were not a good source of feeder layer cells. Thus it appears that X- and γ-irradiation promote specific changes in cells not caused by EMS and perhaps other mutagens and killing agents.

The enhancement of growth observed after irradiation of cultured plant cells has also been reported to occur in the whole plant.
Spiegel-Roy and Kochba (1973b) observed that γ-irradiation of citrus seeds at low doses (1 to 2.5 kR; 166 R/min) enhanced seed germination. In addition, the number of seedlings per seed was increased with γ-irradiation.

To further illustrate the effects of different mutagens on the morphogenetic responses of cultured plant cells, Eapen (1976) observed that _N. tabacum_ suspension cultures treated with γ-irradiation or UV, washed, and plated on shoot induction medium, decreased in morphogenetic response with increasing irradiation doses. No increase in shoot formation was observed with low γ-irradiation doses, but UV-irradiated cells, after exposure to photoreactivating light, regained morphogenetic potential to nearly the control level.

The morphogenetic response observed upon γ-irradiation, as previously suggested, might be a result of the modification of the phytohormone concentration in irradiated medium, or for that matter, within an irradiated explant. Pandey, Sabharwal, and Kemp (1978) quantitatively measured cytokinins in callus cultures of _Haworthia mirabilis_ treated with γ-irradiation, and cultured on un-irradiated medium for 3 passages (3 to 10 weeks) without hormones or inositol. They observed a dramatic increase in the accumulation of cytokinin-like substances in these tissues relative to control, un-irradiated tissues. It is interesting to note that in this case, the irradiated tissue was capable of growth on hormone-free medium - un-irradiated tissue was incapable of growth on hormone-free medium. Thus, γ-irradiation may have caused an epigenetic alteration to hormone habituation, and all previous results must be reviewed with this in mind.
The hormone independence of callus and shoot cultures from γ-irradiated *D. innoxia* tissues is currently being tested. The shoot cultures transferred to hormone-free medium develop roots, but brown callus subcultured to hormone-free medium, after 4 weeks in darkness, produced tan protuberances on the surface of the callus. These cultures must be subcultured further, however, before attributing the appearance of this sectorial growth to hormone prototrophy. The effect of γ-irradiation on the expression of morphogenetic potential was observed to be extended over many subcultures, as shoots from irradiated petiole explants were not initiated, in some cases, until the fifth passage. Suspensions initiated from γ-irradiated tissues, when plated on shoot medium, produced only brown callus regardless of dose (Figure 4.18). Suspensions from EMS-treated explants, when plated on shoot medium, grew rapidly as green organogenetic callus. Any effect of γ-irradiation on morphogenesis in the initiation passage of shoot cultures might be explained by transient alterations in cell growth and developmental responses, but after recovery from γ-irradiation, the tissue would be expected to respond as do un-irradiated tissues. These results indicate that either γ-irradiation-induced damage is not repaired rapidly, or the change observed is permanent.

The literature allows only speculation concerning the effects of mutagens or other biocidal agents on differentiation from plant cell and tissue cultures. While in some instances, X- and γ-irradiation have been shown to modify morphogenetic responses by an apparent direct action on cells, in other cases the responses can be attributed to irradiation effects on culture media. It should be realized that a tissue, particu-
larly freshly excised organs or organ segments (as with the petiole explants used in this study) are composed of closely associated cells. The response of these cells upon exposure to a specific medium composition will necessarily be influenced by the ontogenetic status of neighboring cells, and their physiological and metabolic state. The surrounding cells compose a complex "medium" not well-understood. As development is generally accepted to be epigenetic (Markert and Ursprung, 1971), developmental phenomena do not occur de novo, but are influenced by the previous history of the reactive cell(s), and the immediate cellular environment. Treatment of these closely associated cells with mutagens could produce such changes (as with EMS-mediated alkylation of cellular constituents other than DNA) within, or between cells, to dramatically modify developmental signals. The unpredictability of morphogenetic responses to mutagenic agents, and the lack of knowledge concerning the mechanism in the expression of totipotency serve as evidence to this point. The development, and use of the variant selection techniques described here must therefore proceed at several investigational levels.
VI. SUMMARY

Plant cell and tissue culture has promised to be of great value in the selection of desirable phenotypes. If each cultured cell may be considered a complete organism, a mutant selected at the cellular level could be induced to organize into a whole plant with the same phenotype. This technique assumes that the expression of a phenotype is similar in different ontogenetic states. The lack of in vivo stabilization factors, however, precludes the use of cell cultures for the efficient isolation of desirable mutants, as maintenance of a stable karyotype and morphogenetic competency are not generally realized.

Selection of mutants in shoot cultures after mutagenesis of tissue explants fulfills the most important biological aspects of an efficient mutant isolation scheme from cell and tissue cultures. Mutagenesis was directed at cells of known ploidy by determining the chromosome number of the donor plants, shoot cultures were demonstrated to possess a stable chromosome number, a high capacity for differentiation was evident, and selection was carried out at a high level of organization.

Resistance to a wide variety of anti-metabolites was selected for at the shoot level of organization, but only 5 cycloheximide-resistant lines were recovered. The cycloheximide-resistant trait was stable in the absence of the selective agent, and whole plants have been obtained. The cycloheximide-resistant trait was not expressed in callus cultures initiated from the resistant plants. The lack of variants obtained at the shoot level was probably due to competition between variant and non-variant cells, or insufficient mutation fixation time.

The responses of tissues to mutagenic agents were examined to esta-
lish the effect of mutagens on the survival and morphogenetic competency of fresh explants. If mutagenic treatment would cause a decrease in shoot formation, it was reasoned that this might be used as a measure of mortality. Monoploid tissues exhibited a higher sensitivity to EMS and \( \gamma \)-irradiation than diploid tissues. However, EMS-treated petiole tissue exhibited an increased morphogenetic competency. The increased morphogenetic response was shown not to be associated with a mutational event to enhanced morphogenetic competency, as shoot cultures from plants regenerated from mutagenized petiole explants did not display an increased morphogenetic response. KCN treatment of petiole explants did not increase the efficiency of shoot production, so cross-feeding of morphogenesis enhancement factors from dead cells to those surviving was not the immediate cause for the increased morphogenetic response. It was concluded that the increase in shoot production from EMS-treated petiole explants of \textit{D. innoxia} was due to a unique chemical action of the chemical.

EMS and \( \gamma \)-irradiation effected different qualitative responses from petiole explants. EMS-treated explants either produced shoots, or died. But \( \gamma \)-irradiation, at low doses, permitted shoot formation, while with high doses, only brown callus was initiated. Shoot formation from the callus was not observed until at least the fifth passage on shoot medium.

Mutant selection was also attempted, as a control, at the callus level of organization by plating suspensions derived from mutagenized petiole explants on selective medium. Suspensions were plated on callus or shoot medium, and cultured in the light or dark. No variants were selected using this technique.

As a necessary prerequisite to the selection of mutants by suspension
plating, a medium was developed which permitted rapid growth with maximal aggregate dissociation without the addition of organic nitrogen. Maximal aggregate dissociation is desirable in order to minimize cross-feeding during selection. The lack of organic nitrogen permits the unhindered selection of amino acid auxotrophs, and amino acid analog resistant mutants. Malic and succinic acids, at concentrations promoting optimal growth, significantly increased the formation of aggregates. The phytohormone, 2,4-D, was demonstrated to be the aggregate dissociating factor in the medium used. Although $10^{-6}$ M 2,4-D permitted optimal growth rate, aggregation was extreme. Concentrations of $2.5 \times 10^{-6}$ M to $10^{-5}$ M 2,4-D, although causing a decreased growth rate, permitted maximal aggregate dissociation. Increased aggregate dissociation with supraoptimal 2,4-D concentrations was not due to selection for less aggregated populations with higher 2,4-D concentrations. The enhanced proliferation of smaller aggregates with high 2,4-D concentrations was correlated with increased mitotic activity of smaller aggregates relative to larger aggregates. Growth in ammonium- or nitrate-only medium was inferior to growth in medium containing both nitrogen sources.

The use of turbidimetry as a growth monitoring function was examined. This technique proved to be valuable as determination of growth rates were reproducible, simple, and non-destructive.

Results from these experiments suggest that, with proper future development, the selection of mutants in shoot cultures might be an important advance toward more efficient mutant isolation in plant cell and tissue cultures. It would be advisable to quantitatively examine the effect of mutation fixation period on the recovery of mutants.
Additionally, the tissues should be frequently subdivided into smaller pieces to increase the probability of isolating a proliferating fragment of a mutant sector, and to minimize cross-feeding. Use of other mutagens should also be investigated.
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IX. APPENDIX A: COMPOSITION OF BASAL MEDIUM

With the exception of the source of inorganic nitrogen (Section III. A. 2.), the basal salt medium used in the cell and tissue culture of D. innoxia was that of Murashige and Skoog (1962) medium.

<table>
<thead>
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<th>Macronutrients</th>
<th>mg/liter</th>
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<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>170</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
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</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>370</td>
</tr>
</tbody>
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<table>
<thead>
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<th>Micronutrients</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_3$BO$_3$</td>
<td>6.2</td>
</tr>
<tr>
<td>MnSO$_4$·7H$_2$O</td>
<td>16.9</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>8.6</td>
</tr>
<tr>
<td>NaMoO$_4$·2H$_2$O</td>
<td>0.25</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl$_2$·6H$_2$O</td>
<td>0.025</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>EDTA·2H$_2$O</td>
<td>37.3</td>
</tr>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>27.8</td>
</tr>
</tbody>
</table>

Standard Murashige and Skoog medium contains as inorganic nitrogen sources in mg/liter, NH$_4$NO$_3$ (1650), and KNO$_3$ (1900).
Vitamins used to culture *D. innoxia* cells and tissues were those suggested by Engvild (1973)

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-inositol</td>
<td>100</td>
</tr>
<tr>
<td>nicotinic acid</td>
<td>5</td>
</tr>
<tr>
<td>thiamine</td>
<td>5</td>
</tr>
<tr>
<td>pyridoxine</td>
<td>5</td>
</tr>
</tbody>
</table>
X. APPENDIX B: DETERMINATION OF DOUBLING TIME

To determine the doubling time of a cell population, different growth monitoring functions may be used. These functions include turbidity, fresh or dry weight, packed cell volume, and cell number. Doubling times are usually determined during exponential (logarithmic) growth when the rate of increase in the monitoring function per unit of the monitoring function is constant.

Mathematically,

\[
\frac{1}{X} \frac{dx}{dt} = \frac{d(\ln x)}{dt} = \frac{\ln 2}{t_d} = \mu ,
\]

where \( x \) = value of monitoring function at time, \( t \),
\( \mu \) = specific growth rate \( (\text{time}^{-1}) \), and
\( t_d \) = doubling time, the time required for the value of the monitoring function to double.

Thus,

\[
\mu = \frac{d(\ln x)}{dt} = \frac{\ln x + \ln x_0}{t},
\]

where \( x_0 \) = initial value of the monitoring function, and
\( x \) = final value of the monitoring function at time, \( t \).

Rearranging (2),

\[
\ln x = t + \ln x_0 .
\]

Values of the monitoring function \( x \) are plotted against time \( t \) on semi-logarithmic graph paper, and a straight line is observed for the exponential growth phase. All data points included within the log-linear phase of growth are submitted to a linear regression. The slope of this regression line is \( \mu \).
Rearranging (1),

\[ t_d = \frac{\ln 2}{\mu} , \quad (4) \]

the slope of the regression line is used to compute the doubling time.

The number of monitoring function doublings between two values of the monitoring function can be determined by a substitution, and rearrangement of (2),

\[ \frac{\ln 2}{t_d} = \mu = \frac{\ln x + \ln x_0}{t} , \quad \text{thus} \]

number of doublings \[ = \frac{t}{t_d} = \frac{\ln x + \ln x_0}{\ln 2} . \quad (6) \]
XI. APPENDIX G: METHOD OF BACK-EXTRAPOLATION

The technique of back-extrapolation (Sung, 1976b) may be used to determine the lethality of a treatment to a population of cells, particularly when the response of single cells is difficult or impossible to measure. Essentially, the method is based on the quantitative determination of lag phase subsequent to a treatment.

Replicate cultures are subjected to the desired treatment, and removed to normal growth conditions. All cultures or tissues must initially reflect the same value of the monitoring function. Growth is monitored, and plotted on semi-logarithmic graph paper. The y-intercept (eq. (3), Section X) of the regression line fitted to the growth of untreated cells is assigned a 100% survival. If the treatments cause death, fewer cells survive the treatment compared to the control, and the treated cells will lag for a longer period before entering logarithmic growth. The y-intercepts of the regression lines fitted to the logarithmic growth of treated cells will thus be correspondingly less than the control regression y-intercept. The y-intercepts of regressions from treated cells are then divided by the y-intercept of the control regression with the quotient being a relative measure of survival.

Data acquired by this method must be interpreted cautiously. A lag phase subsequent to a treatment may not only be caused by death, but may also result as a consequence of some non-lethal, but physiologically detrimental event which results in a transitory cessation of growth, perhaps proportional to the harshness of the treatment. Thus,
lag need not be caused by death. Appropriate controls must be incorporated into the experimental design to establish the lethality of a treatment to correctly interpret data acquired by back-extrapolation.