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I am submitting herewith a thesis written by Ouida Kimbrough Miller entitled "Effects of the Herbicide Paraquat on Nicotiana tabacum and Isolation of Paraquat Resistant Mutants." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Karen W. Hughes, Major Professor

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EFFECTS OF THE HERBICIDE PARAQUAT ON *NICOTIANA TABACUM* AND ISOLATION OF PARAQUAT RESISTANT MUTANTS

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

The purpose of this study was to determine the effects of the herbicide paraquat (1, 1'-dimethyl-4, 4'-dipyridylium salt) on Nicotiana tabacum var. Wisconsin 38 in culture and to isolate paraquat-resistant mutants. Tobacco cells were grown in modified Linsmaier and Skoog media as callus and liquid suspension cultures. Paraquat caused a marked decrease in growth of tobacco callus at 10^{-5}M and total inhibition of growth at 10^{-4}M. Four methods were used for the isolation of resistant cell lines: 1) X-irradiation of callus tissue at 10,000R prior to selection at 10^{-5}M paraquat, 2) selection of callus tissue at 10^{-5}M paraquat, 3) selection of callus tissue at 5×10^{-6}M paraquat prior to selection at 10^{-5}M, and 4) plating of liquid cell cultures on agarized medium containing paraquat at 10^{-5}M. Actively growing resistant tissues were selected, transferred to non-selective medium for a period of non-selective growth, and retested on selective medium. Cell lines which retained resistance to paraquat at 10^{-4}M (lethal level) were transferred to regeneration medium. Plantlets from regenerating cell lines were tested for resistance by floating leaves on a paraquat solution at 10^{-6}M, 10^{-5}M, and 10^{-4}M. Additionally, leaf derived callus from some cell lines was tested for resistance at 10^{-5}M and 10^{-4}M paraquat.

A higher percentage of cell lines retaining the resistant phenotype was obtained using X-irradiation as a mutagenic agent as compared to no mutagen treatment. Sub-lethal selection at 5 x 10^{-6}M paraquat prior to selection at 10^{-5}M paraquat did not enhance recovery of resistant mutants as compared to selection only at 10^{-5}M. Selection from plated cell suspension cultures did increase the percentage of resistant cell lines obtained as compared to selection using callus tissue. Some resistant cell lines were not capable of being regenerated. Approximately 35 percent of the regenerated plants retained resistance. Leaf derived callus from resistant plants of two cell lines retained resistance at 10^{-4}M paraquat. Leaf derived callus from non-resistant plants of five cell lines retained resistance at 10^{-4}M paraquat.

Possible protection mechanisms against paraquat toxicity were considered. Increased copper, a component of superoxide dismutase, in the growth medium enhanced growth of tobacco callus over that expected for paraquat and copper. Increased iron, a component of catalases and peroxidases, in the growth medium did not enhance growth of tobacco callus.

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LIST OF ABBREVIATIONS

IAA	3-Indole acetic acid
NAA	Napthalene acetic acid
2iP	6 (Gamma, gamma-dimethylallyamino) purine
2,4-D	2,4--Dichlorophenoxyacetic acid

CHAPTER I

INTRODUCTION

Plant cell and tissue culture is increasingly being used in higher plant genetics as a means of obtaining new varieties of plants. Traditionally crop improvement has been brought about by introducing desired genotypes from wild populations, but increasing development of land for agricultural purposes has decreased the availability of natural gene resources (Konzak, Nilan, and Kleinhofs, 1977). Furthermore, there is considerable time and expense involved in growing and screening large numbers of plants for a desired genotype by conventional means of propagation such as cuttings or seeds (Nabors, 1976).

Plant cell and tissue culture techniques overcome many of the disadvantages of traditional methods for genetic manipulations in higher plants. Some of these advantages are: 1) Large numbers of individuals can be handled in a small space (Smith, 1974), thus limitations of visual selection among small numbers of whole plants can be overcome through chemical selection from populations of cells (Bottino, 1975), 2) Cells in culture are genetically unstable which allows selection of variant phenotypes (Bottino, 1975). The large number of cells in a typical tissue culture (callus or suspension) makes it very likely that a culture would

contain at least one cell possessing a given mutation (Nabors, 1976). Furthermore, mutant characteristics at the cellular level are usually immediately expressed (Smith, 1974), 3) Recovery of a desired mutant phenotype may be increased by exposure to chemical or physical mutagens prior to selection, 4) Recessive mutations may be obtained by the use of haploid cell cultures (Nabors, 1976), 5) Selection procedures based on biochemical requirements can be carried out at the cellular level using techniques developed for microbial genetics (Smith, 1974). Plant cells evolve, under selection pressure, in the same manner that traditional plant populations evolve toward adaptation to natural or altered environmental conditions (Smith, 1974). The result is increased recovery of mutant phenotypes, 6) For many species, plants which retain the mutant phenotype may be regenerated from mutant cells. Such mutants may be of agricultural or genetic use (Nabors, 1976).

Problems do exist with using somatic cell culture for genetic manipulation of plants. Cultures are not easily produced in many species (Nabors, 1976), and with many economic plants, successful recovery of organized plants from cell culture has not been accomplished (Day, Carlson, Gamborg, Jaworski, Maretzki, Nelson, Sussex, and Torrey, 1977). Difficulties still exist in successful isolation of protoplasts for hybridization experiments and in techniques for haploid production (Day et al., 1977).

Previous Studies

The production of mutant plants by tissue culture techniques can be divided into four steps: 1) production of callus or suspension cultures, 2) mutation induction, 3) selection of desired mutants, and 4) regeneration of mutant plants from mutant cell lines (Nabors, 1976). The genus, Nicotiana, has been shown to be a successful organism for somatic cell genetics. It has been readily grown in culture, can be subjected to mutation induction and selection pressures, and can be regenerated into whole plants (Aviv and Galun, 1977; Barg and Umiel, 1977; Carlson, 1970, 1973; Chaleff and Parsons, 1978; Dix, 1974; Dix and Street, 1975; Dix, Joó and Maliga, 1977; Heimer and Filner, 1970; Lescure, 1973; Maliga, Sz Breznovitis and Márton, 1973b; Maliga, Lázár, Sváb and Nagy, 1976; Márton and Maliga, 1975; Nabors, Daniels, Nadolny and Brown, 1975; Palmer and Widholm, 1975; Polacco and Polacco, 1977; Radin and Carlson, 1978; Umiel and Goldner, 1976; Vasil and Vasil, 1972; Widholm, 1972a; Widholm, 1976b; Zenk, 1974).

Mutations in higher plants are of two types, auxotrophic and resistance. An auxotrophic mutant is one which has lost its ability to produce certain nutritional substances such as vitamins or amino acids (Strickberger, 1976). Compared to the isolation of resistant mutants, the isolation of auxotrophic mutants has been rare in higher plants. Li, Rédei and Shields-Gowans (1967) have suggested

that this may be because many higher plants have extensive duplication of genetic material, co-existence of alternative metabolic pathways, or coupling of basic biochemical reactions to photosynthesis. Rédei (1975) obtained approximately 200 mutants in Arabidopsis, using mutagen-treated seeds which were auxotrophic for thiamine. Carlson (1970) isolated six auxotrophs using Nicotiana tabacum L. var. Wisconsin 38. All of Carlson's mutants were leaky (growth on minimal medium was severely but not totally restricted). It is suggested that this may be due to a lack of functional diploidization of the N. tabacum genome (Chaleff and Carlson, 1974b). Table 1 summarizes known auxotrophic mutants in higher plants.

Resistant mutants are those cell lines which are able to grow on a normally toxic medium or under normally detrimental conditions. Any cell which proliferates under these conditions is a putative mutant. Table 2 summarizes the resistant mutants which have been isolated from higher plants. Resistant cell lines have been obtained for numerous substances including a folic acid analog, base analogs, amino acid analogs, increased amino acid levels, antibiotics, metals, fungal toxins, fungicides, and herbicides. Herbicide resistance has successfully been obtained in tissue culture with tobacco by Aviv and Galun (1977), Barg and Umiel (1977), Chaleff and Parsons (1978), Radin and Carlson (1978), and Zenk (1974). Resistance to the herbicide paraquat has only

TABLE 1. Auxotrophic Mutants in Higher Plants.

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent	Reference
Thiamine (pyrimidine)	*Lycopersicon esculentum* Mill. (2N)	Plants	-[a]	Langridge and Brock (1961)
Aspartic Acid	*Hordeum vulgare* L. (2N)	Seedlings	-	Walles (1963)
Leucine	"	"	-	"
Hypoxanthine	*Nicotiana tabacum* L. var. *Wis. 38* (N)	Plated Cells	EMS[b]	Carlson (1970)
Biotin	"	"	"	"
Para-amino Benzoic Acid	"	"	"	"
Arginine	"	"	"	"
Lysine	"	"	"	"
Proline	"	"	"	"
"	*Zea mays* L. (2N)	Embryos	-	Gavazzi, Nava-Racchi, and Tonelli (1975)

TABLE 1. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent	Reference
Thiamine	Arabidopsis sp. Heynhold (2N)	Seedlings	X-rays	Rédei (1975)
"	"	"	EMS	"

[a]Mutagenic agent not used.

[b]EMS = Ethylmethane sulfonate.

TABLE 2. Resistant Mutants in Higher Plants.

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
Analogs					
Aminopterin (Folic Acid) Analog)	Datura innoxia Miller (N)	Plated Cells	-	+/+	Mastrangelo and Smith (1977)
Azaguanine	Acer pseudo-platanus L. (2N)	Callus	NG	-	Bright and Northcote (1975)
"	Nicotiana tabacum L. var. Wis. 38 (2N)	Plated Cells	-	-	Lescure (1973)
5-Fluoro-uracil	Daucus carota L. (2N)	Liquid Suspension of Cells	NG	NI[c]	Sung (1976)
5-Bromo-deoxyuridine	Acer pseudo-platanus L. (2N)	Plated Cells	-	-	Zryd (1976)
"	Glycine max (L.) Merrill (2N)	Liquid Suspen-sion of Protoplasts	NG	-	Ohyama (1974)
"	Nicotiana tabacum L. cv. Petit Havana (N)	Callus	-	+/0	Maliga, Márton and Sz Breznovitis (1973a)

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
"	Nicotiana tabacum L. var. Wis. 38 (2N)	Plated Cells	-	-	Lescure (1973)
"	Nicotiana tabacum L. (N)	Callus	-	+/+	Márton and Maliga (1975)
S-(β-amino-ethyl)-cysteine	Oryza sativa L. (2N)	Plated Cells	EMS	-	Chaleff and Carlson (1974a)
"	Nicotiana tabacum L. cv. Xanthi (2N)	Liquid Suspension of Cells	EMS	-	Widholm (1976b)
Delta-hydroxy lysine	"	"	EMS	-	"
Ethionine	Daucus carota L. (2N)	"	EMS	-	"
"	Nicotiana sylvestris Speg. and Comes (N)	NI	NI	NI	Zenk (1974)
Hydroxy-proline	Daucus carota L. (2N)	Liquid Suspension of Cells	EMS	-	Widholm (1976b)

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
P-fluorophenyl-alanine	"	"	-	-	Palmer and Widholm (1975)
"	Nicotiana tabacum L. cv. Xanthi (2N)	"	-	-	"
5-Methyl-tryptophan	Solanum tuberosum L. (2N)	NI	NI	NI	Widholm (1976a)
"	Solanum stenotomum Juz. and Buk. (2N)	Liquid Suspension of Cells	-	+/+	Isaacs and Fisher (1977)
"	Daucus carota L. (2N)	"	-	-	Widholm (1972b)
"	"	"	-	+/+	Widholm (1974)
"	"	NI	-	-	Sung (1975)
"	Nicotiana tabacum L. cv. Xanthi (2N)	Liquid Suspension of Cells	-	-	Widholm (1972a)
Seleno amino acids	Nicotiana tabacum L. (2N)	NI	-	-	Flashman and Filner (1977)

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
Methionine Sulfoximine	Nicotiana tabacum L. cv. Havana Wis. 38 (N)	Plated Cells and Protoplasts	EMS	+/+	Carlson (1973)
Increased Amino Acids					
L-threonine	Nicotiana tabacum L. cv. Xanthi (2N)	Liquid Suspension of Cells	NG	-	Heimer and Filner (1970)
Antibiotics					
Cycloheximide	Daucus carota L. (2N)	Liquid Suspension of Cells	NG	NI	Sung (1976)
"	"	"	EMS	NI	"
"	Oryza sativa L. (N) and (2N)	Callus	NI	-	Sung, Pratt and Signer (1974)
"	Nicotiana tabacum L. cv. Samsun EN (N)	Liquid Suspension of Cells	EMS	+/+	Maliga et al. (1976)

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
Kanamycin	Nicotiana sylvestris Speg. and Comes (N)	"	-	-/	Dix et al. (1977)
Streptomycin	Petunia hybrida Vilm. (N)	NI	NI	NI	Binding, Binding and Straub (1970) In Zenk (1974)
"	Nicotiana tabacum L. cv. Petit Havana (N)	Callus	-	+/+	Maliga et al. (1973b)
"	Nicotiana tabacum L. cv. Xanthi (N)	"	-	-	"
"	Nicotiana sylvestris Speg. and Comes (N)	"	-	-	"
"	Nicotiana tabacum L. (2N)	"	-	-	Umiel and Goldner (1976)
Temperature Tolerance					
0 to 3 degrees centigrade	Nicotiana sylvestris Speg. and Comes (N)	Callus	-	-	Dix (1974)

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
Salt Tolerance					
0.16% NaCl	Nicotiana tabacum L. cv. Samsun (2N)	Liquid Suspension of Cells	EMS	-	Nabors et al. (1975)
"	"	"	-	-	"
1% NaCl	Capsicum annuum L. var. Canape (2N)	Plated Cells	-	-	Dix and Street (1975)
"	Nicotiana sylvestris Speg. and Comes (N) and (2N)	"	-	-	"
"	Nicotiana sylvestris Speg. and Comes (N)	NI	NI	-	Zenk (1974)
2% NaCl	Capsicum annuum L. var. Canape (2N)	Liquid Suspension of Cells	-	-	Dix and Street (1975)
"	Nicotiana sylvestris Speg. and Comes (N) and (2N)	"	-	-	"

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
Metal Tolerance					
Aluminum	Lycopersicon esculentum Mill. (2N)	Callus	-	-	Meredith (1978)
"	"	Liquid Suspension of Cells	-	-	"
Pathotoxin	Zea mays L. (2N)	Callus	-	+/+	Gengenbach, Green and Donovan (1977)
Fungicide					
Carboxin	Nicotiana tabacum L. var. Wis. 38 (N)	Plated Cells	UV	+/+	Polacco and Polacco (1977)
Herbicides					
Terbutryn	Triticum aestivum L. (2N)	Seedlings	EMS	-	Pinthus, Eshel and Shchori (1972)
Diphenamid	Lycopersicon esculentum Mill. (2N)	"	EMS	-	"

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
Asulam	Brassica sp. L. (2N)	Seeds	-	-	Flack, Collin and Putwain (1978)
"	"	Seedlings	-	-	"
"	"	Callus	-	+/0	"
2,4,5-T	Trifolium repens L. (2N)	Liquid Suspension of Cells	-	-	Oswald, Smith and Phillips (1977)
2,4-DB	"	"	-	-	"
2,4-D	"	"	-	-	"
"	Daucus carota L. (2N)	NI	NI	+/+	Gressel In Zenk (1974)
"	Nicotiana sylvestris Speg. and Comes (N)	Liquid Suspension of Cells	NI	-	Zenk (1974)
Isopropyl N-phenyl Carbamate	Nicotiana tabacum L. cv. Xanthi (2N)	Plated Protoplasts	EMS	+/+	Aviv and Galun (1977)
Amitrole	Nicotiana tabacum L. (2N)	Callus	-	+/0	Barg and Umiel (1977)

TABLE 2. (Continued)

Mutant	Organism (Ploidy)	Tissue Type for Selection	Mutagenic Agent[a]	Regeneration[b]	Reference
"	"	Seedlings	-	-	"
Bentazone	Nicotiana tabacum L. var. Wis. 38 (N)	Leaves	γ-irradiation	+/+	Radin and Carlson (1977)
Phenmedipharm	"	"	"	+/+	"
Picloram	Nicotiana tabacum L. cv. Xanthi (2N)	Plated Cells	-	+/+	Chaleff and Parsons (1978)
Diquat	Zea mays L. (2N)	Seedlings	-	-	Miles (1976)
Paraquat	Lolium perenne L. (2N)	Plants	-	-	Faulkner (1974)
"	Glycine max var. (L.) Merrill var. SRF 425 (2N)	Callus	-	-	Hughes et al. (1979)

[a]NG N-methyl, N'-nitrosoguanidine.
EMS Ethylmethane sulfonate.
UV Ultraviolet light.
- Mutagenic agent not used.

TABLE 2. (Continued)

[b]- Regeneration not attempted.
+/ Successful regeneration.
-/ Unsuccessful regeneration.
+/+ Regenerated plants exhibiting resistance.
+/- Regenerated plants not exhibiting resistance.
+/0 Regenerated plants not tested for resistance.

[c]NI No information in the reference.

been obtained in tissue culture with soybean callus (Hughes, Schwarz and Bell, 1979). Cold-tolerant and salt-tolerant cell lines have also been isolated using tissue culture techniques. In only 14 cases have plants been regenerated which retain the resistance trait. Resistance was retained in varying degrees in the regenerated plants (Table 2). Genetic analysis of resistance has been carried out only for methionine sulfoximine-resistant mutants of tobacco (Carlson, 1973), streptomycin-resistant mutants of tobacco (Maliga et al., 1973b), 5-bromodeoxyuridine-resistant mutants of tobacco (Márton and Maliga, 1975), a pathotoxin resistant mutant in corn (Gengenbach et al., 1977), bentazone and phenmedipharm resistant mutants of tobacco (Radin and Carlson, 1978), and picloram resistant mutants of tobacco (Chaleff and Parsons, 1978).

Street (1974) has stated that when a mutagen is used, the objective should be to obtain as high a population as possible of mutated cells in a drastically reduced but still numerous population. He added that selection criteria should be that the mutation is stable in the absence of the selection agent and that the change is not lost during regeneration. A wide array of mutational techniques has been employed for resistant mutant isolation. Mutagens such as N-methyl, N'-nitrosoguanidine (NG), ethylmethane sulfonate (EMS), ultraviolet light (UV) and γ-irradiation have been used (Table 2). Tissue types for selection have

been liquid suspension cultures, plated cells from liquid suspension cultures, plated protoplasts, callus tissue, or in some cases seeds or seedlings (Table 2).

X-rays as a Mutagenic Agent

There are two possible ways in which X-irradiation can cause a mutational event: A direct "hit" by an X-ray on the DNA molecule or DNA modification resulting from ionized molecules. The relationship between mutation and X-irradiation has been interpreted as the gene being a "target" and mutation being caused by a single "hit" of radiation. The high energy ray impinges as a "hit" upon the gene with a high probability of causing a distinct mutation. One or several "hits" could cause point mutations, deletions, or chromosome aberrations. Not only can X-irradiation have a direct physical impact on the gene or chromosome but its ionizing effect may indirectly cause mutations by changing the internal environment of the cell. X-irradiation ionizes water molecules yielding hydrogen and hydroxyl radicals. Hydrogen atoms may easily be converted to hydrogen peroxide. Peroxides are highly reactive molecules and may be responsible for some mutations (Strickberger, 1976).

Nature of the Herbicide Paraquat

The herbicide paraquat (1, 1'-dimethyl-4, 4'-bipyridylium salt) is a non-selective, quick-acting herbicide

of importance to agriculture. It has a wide spectrum of activity against grasses as well as most broad leaf weed species; however, its use in growing crops is limited unless herbicide contact with the crop plants can be avoided. Paraquat is non-residual in soil because of inactivation by irreversible adsorption to soil particles and is thus often used as a defoliant for clearing fields before planting crops without leaving a residue in the soil which would affect new seedlings. The lack of residual activity in soils does limit its use for weed control over long periods (Calderbank and Slade, 1976).

Paraquat is extremely soluble in water, is very stable in acid or neutral solutions, withstands boiling in strong sulfuric acid, and may be autoclaved (Calderbank and Slade, 1976). Light has been shown to break down paraquat, but photochemical decomposition does not occur in solution (Slade, 1966). There are no indications that paraquat is metabolically degraded in plants (Calderbank and Slade, 1976; Funderburk, 1969). This is perhaps due to rapid destruction of plant tissues which occurs before metabolic degradation can take place (Calderbank and Slade, 1976).

The high herbicidal activity of paraquat lies in the fact that the molecules are flat, assuming a coplanar configuration of the two pyridine rings. When reduced, such compounds form stable free radicals by the addition

of one electron. The electron is able to occupy any one of the nuclear positions in the paraquat molecule, thus producing 18 possible resonance forms. The requirement of coplanarity is a necessary condition for the stability of the free radical. The stability depends on the delocalization of the odd electron over the whole molecule. The greater the number of possible resonance forms, the more stable the free radical, and the more active the molecule (Homer, Mees and Tomlinson, 1960).

Evidence indicates that the herbicidal action of paraquat is associated with photosynthesis, particularly with the light reaction. The reduction of paraquat to a free radical has been demonstrated in suspensions of illuminated chloroplasts. It is conceivable that paraquat diverts energy away from NADP of photosynthetic electron transport to itself, thus inhibiting NADP reduction. The result is loss of photosynthetic capacities and nutrient losses. If this were the only factor involved in the mode of action of paraquat, slow death of plant tissues would occur. Paraquat generally causes rapid and dramatic death of plants. Evidence suggests that plant or tissue destruction is due to the rapid reoxidation of paraquat in the presence of oxygen creating free radicals (Calderbank and Slade, 1976). Oxygen can be reduced to water via toxic intermediates such as the superoxide radical, O_2^-, and hydrogen peroxide, H_2O_2 (Fridovich, 1977). Farrington,

Ebert, Land and Fletcher (1973) indicate that both O_2^- and H_2O_2 are likely candidates for paraquat phytotoxicity. Giannapolitis and Ries (1977) report evidence for paraquat producing the superoxide radical. Accumulated by-products could cause an increase in cell permeability by lipid peroxidation and subsequent breakdown of cell membranes (Calderbank and Slade, 1976).

Three groups of enzymes, superoxide dismutases, catalases, and peroxidases protect living systems against the toxic effects of oxygen by-products. Superoxide dismutases (SOD) catalyze the reaction: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$; catalases and peroxidases catalyze the reaction: $H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$ (Fridovich, 1977). An increase in levels of any of the three enzyme systems may allow eukaryotic tissue to survive on a paraquat medium. Hasson and Fridovich (1977) report the rapid increase of SOD following the addition of paraquat to the growth medium of *E. coli*.

Purpose of This Study

The purpose of this study was to determine the effect of the herbicide paraquat on tobacco, *Nicotiana tabacum* var. *Wisconsin 38*, in culture and to isolate paraquat resistant cell lines by using somatic cell culture techniques. Additionally, the study is designed to examine the use of X-rays as a mutagenic agent for mutation induction versus

the occurrence of spontaneous mutations to paraquat resistance and to determine the effectiveness of sublethal selection procedures on mutant isolation. Further, possible protection mechanisms against paraquat toxicity were examined.

Paraquat resistant cell lines will add to the stock of available genetic markers in higher plants. Resistant cell lines should be potentially useful mutants for genetic studies to compare gene expression in undifferentiated cell cultures to that of organized plants and regenerated plants from resistant cell lines may be used in studies of the metabolic basis for the resistance trait. Paraquat resistant plants may also have agricultural value and may expand the range of species on which the herbicide can be used.

CHAPTER II

METHODS AND MATERIALS

All procedures involving plant tissues were carried out in a laminar flow hood using sterile technique. Paper towels soaked in 70 percent ethanol served as a surface for dissection. Cultures were incubated at 25 degrees centigrade and maintained on a 16 hour light photoperiod. Light intensity was 2.2×10^4 ergs cm^{-2} sec^{-1}. Test tubes were plugged with cotton and capped with Bellco KAPUTS. Flasks were plugged with cotton and capped with aluminum foil.

Establishment of Callus Tissue

Plant callus tissue is defined by Dougall (1972) as cells grown as a coherent mass on the surface of a solidified medium. The procedure for the establishment of callus tissue of tobacco, Nicotiana tabacum L. var. Wisconsin 38, was similar to that outlined by Skoog and Armstrong (1974). The upper portion of the plant stem of greenhouse grown adult tobacco plants was excised and the leaves were removed. The stem was cut into sections 5 to 6 centimeters (cm) long and surface sterilized in a 10 percent chlorox solution for 10 minutes. Following rinsing in sterile double-distilled water the stem sections were placed in 70 percent ethanol for 5 minutes. The sections were then returned to sterile

double distilled water and removed one at the time for pith excision.

Cross sections approximately 2 to 3 millimeters wide were cut away and discarded from each end of a stem section to assure that any cells damaged during decontamination procedures were not utilized for further growth. The stem was then sliced into cross sections approximately 2 millimeters wide. The undifferentiated pith was removed from these cross sections with either the use of a cork borer or, if the cross sections were fairly small in diameter, by cutting away the outer vascular tissue. Pith pieces approximately 2 millimeters square were placed in 25 x 150 mm test tubes containing 25 milliliters (ml) of agarized modified Linsmaier and Skoog medium (Table 3) for cell proliferation. Four to five weeks were necessary for good callus growth. Callus tissue of this type was used for paraquat and X-irradiation series as well as for selection experiments.

Table 3 contains the components of media used for callus growth. Media 1, 2, and 3 produced callus but medium 2 showed faster callus proliferation than medium 1, perhaps due to additional organic nutrients. Due to the problems with contamination from filter sterilization of medium 2, medium 3 was primarily used for callus growth. Both medium 1 and medium 3 were autoclaved.

TABLE 3. Media for Growth of Nicotiana tabacum var. Wisconsin 38.

	Medium (mg/l)				
	1[a]	2[b]	3	4	5[c]
Linsmaier and Skoog Salts[d]	+	+	+	+	+
Sucrose	30,000	30,000	30,000	30,000	30,000
Inositol	100	100	100	100	100
Glycine	-	2	2	-	2
NAA	-	1	2	-	2
IAA	2	-	-	-	-
Kinetin	0.3	0.2	0.3	0.3	0.65
Nicotinic Acid	-	0.5	1	-	0.5
Pyridoxine	-	0.5	1	-	0.5
Thiamine	0.4	0.1	0.2	-	0.1
2,4-D	-	-	-	-	0.5
2iP	-	-	-	2.5	-
Agar	8000	8000	8000	8000	-
pH	5.6-5.8	5.6-5.8	5.6-5.8	5.6-5.8	5.5
Autoclaved	+	-	+	+	+

[a]Skoog and Armstrong, 1974.

[b]Henke, verbal communication, 1977.

[c]Bottino, verbal communication, 1977.

[d]Linsmaier and Skoog, 1965.

Establishment of Liquid Suspension Cultures

When callus tissue is grown in an agitated liquid medium, it may form a suspension composed of free cells and cellular aggregates ranging from a few to several hundred cells, depending on the friability of the callus (Narayanaswamy, 1977). The establishment of liquid suspension cultures from tobacco callus proved somewhat difficult to achieve. Several methods were undertaken to achieve healthy cultures that could later be plated for selection experiments. Evidence for healthy cultures was the ability to see cytoplasmic streaming under microscopic examination of cells. All liquid cultures were kept on a gyratory shaker at 70 rpm at room temperature using 125 ml Fernbach, triple baffled flasks containing 50 ml of medium.

Two methods were successfully used for obtaining liquid suspension cultures: Callus tissue was broken into smaller pieces and transferred to medium 5 containing 0.5 mg/l 2,4-D. Within two to three weeks these cultures proliferated to produce a cell suspension which could be plated. A second method which proved more efficient for obtaining a good cell suspension culture was to obtain friable callus prior to transferring to liquid medium. Callus grown on medium 3 modified with 1 mg/l 2,4-D produced a very friable callus within two weeks. This callus was then transferred to medium 5 without 2,4-D. After two to three weeks of growth, these cultures were plated. Further

subculturing in liquid medium was possible and yielded cultures thick with healthy cellular clumps. These cultures could also be plated. In any case, exposure to 2,4-D was for no more than four weeks. Exposure to 2,4-D for longer periods was avoided due to the possibility of inhibiting plantlet regeneration.

Establishment of X-ray Mutational Levels

To establish a radiation level to be used for mutagenesis, the effects of X-irradiation on tobacco callus was determined. Callus tissue was exposed to X-irradiation at five levels: 0, 2500R, 5000R, 10,000R, and 15,000R. The irradiated callus for each level was transferred to ten 25 x 150 mm test tubes, each containing 25 ml of medium 3 to protect against the possible inactivation of auxin in the X-irradiated medium (Skoog, 1934). After four weeks tissues were sacrificed and dried for 24 hours at 60 degrees centigrade, after which dry weights were taken. Additional dry weights were taken for a control size of callus pieces prior to the four week growth period. Means and standard deviations of the dry weights were calculated. Mean dry weights were graphed against radiation level (Figure 1).

There was a sharp decrease in callus growth (increase in dry weight over initial explant dry weight of 0.004 gram) between 0 X-irradiation exposure and 2500R, but growth was not completely inhibited even at the highest exposure.

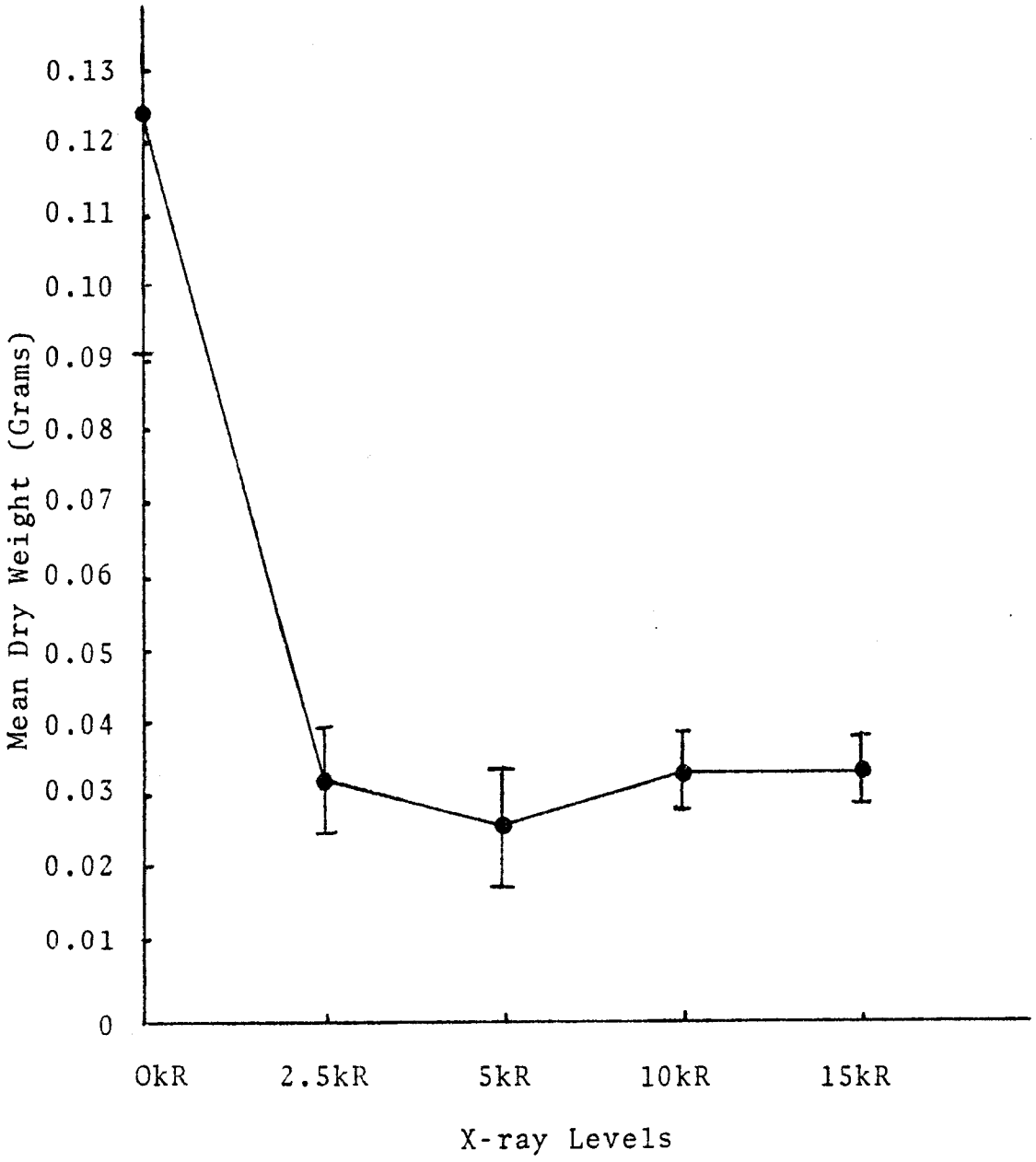

Figure 1. Response of tobacco callus explants to X-ray exposure. Dry weight of initial inoculum was 0.004 gram. Growth period was four weeks.

An X-irradiation exposure of 10,000R was chosen as a mutagenesis level for the selection experiment using a mutagenic agent. Exposure at 10,000R showed 78 percent growth inhibition with a mean dry weight of 0.03 gram as compared to a 0 X-irradiation mean dry weight of 0.124 gram.

Determination of Selection Levels for the Herbicide Paraquat

Experiments determining the effects of various concentrations of the herbicide paraquat on tobacco callus tissue were carried out to establish a lethality level and to establish a selection level. Paraquat dichloride, 29.1 percent, was obtained from Chevron Chemical Company, Ortho Division.

Pith explants were transferred to 25 ml of medium 1 in 25 x 150 mm test tubes modified with paraquat levels of 0, 10^{-8}M, 10^{-7}M, 10^{-6}M, 10^{-5}M, 10^{-4}M, and 10^{-3}M with eight replicates per level. After five weeks pith derived calli were sacrificed, dried for 24 hours at 60 degrees centigrade, and weighed. A control dry weight for the initial pith explant size was also determined. Means and standard deviations for each paraquat level as well as for pith explant size were determined. Mean dry weights were graphed against paraquat levels (Figure 2).

A second paraquat sensitivity experiment was carried out using medium 3 modified with paraquat levels as were used in the prior experiment. Callus pieces were used

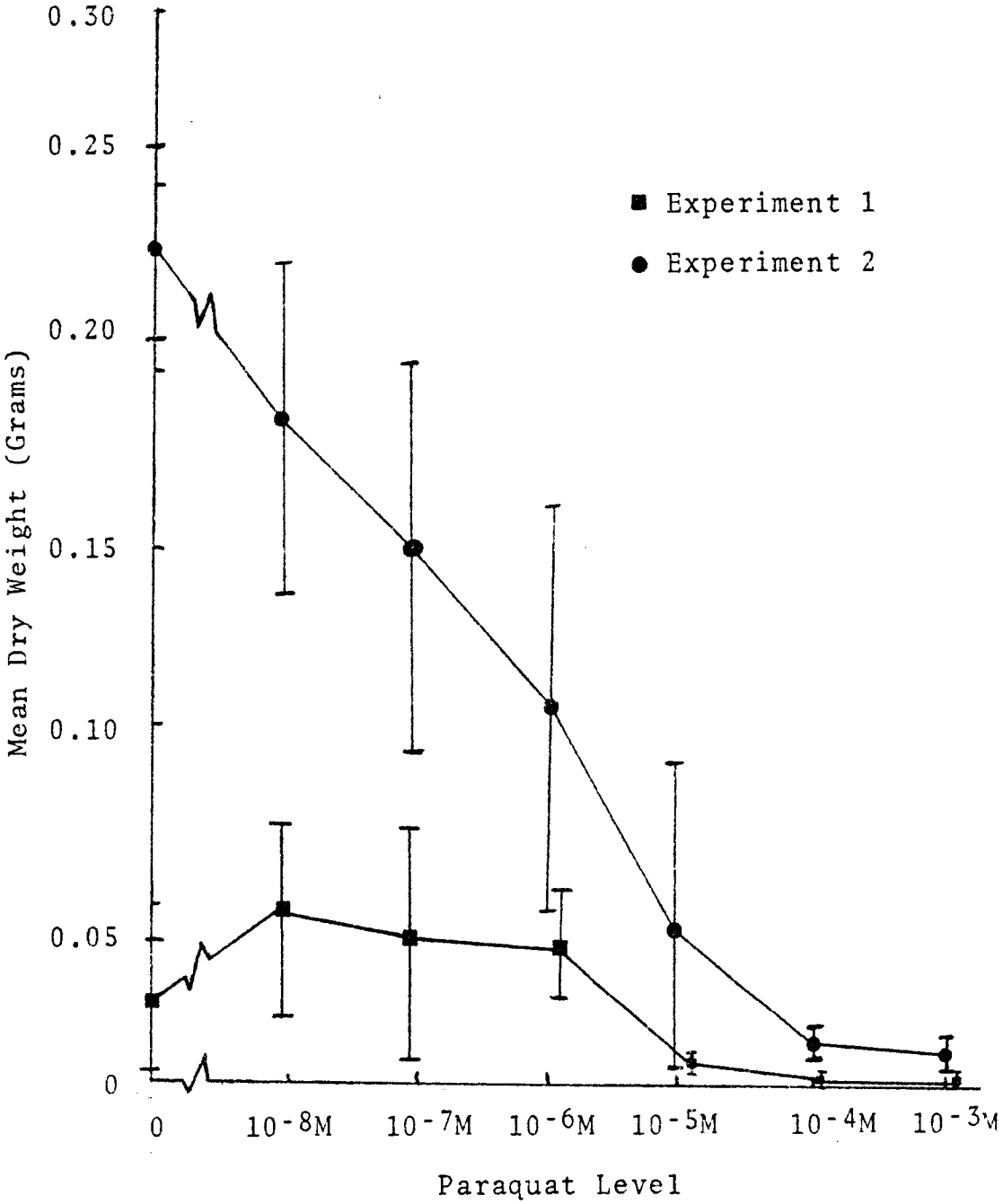

Figure 2. Response of tobacco pith and callus explants to paraquat levels. Experiment 1: pith explants grown on medium 1 (Table 3, page 25). Dry weight of initial inoculum was 0.0017 gram. Growth period was five weeks. Experiment 2: callus explants grown on medium 3 (Table 3). Dry weight of initial inoculum was 0.009 gram. Growth period was four weeks.

instead of pith explants. There were 10 replicates per paraquat level, using 25 x 150 mm test tubes containing 25 ml of medium. After four weeks dry weights and statistical data were calculated. Average dry weight for callus inoculum size was also determined. Mean dry weights were graphed against paraquat levels (Figure 2).

Results indicate (Figure 2, experiment 1) that there was an increase in callus growth at 10^{-8}M, 10^{-7}M, and 10^{-6}M paraquat over that exhibited by the control (0 paraquat). The explanation for this peak is undetermined. There is a marked decrease in growth between 10^{-6}M and 10^{-5}M with essentially no growth at 10^{-4}M and 10^{-3}M. Mean dry weight of pith explant size was 0.0017 gram with a standard deviation of 0.0004 gram. Experiment 2 (Figure 2) shows a similar lethality level but does not show an increase in growth at 10^{-8}M, 10^{-7}M, or 10^{-6}M over the control. Growth at 10^{-5}M was severely restricted but not completely inhibited. Mean dry weight of callus explant size for experiment 2 was 0.009 gram with a standard deviation of 0.0016 gram. A 10^{-5}M paraquat concentration was chosen as a level for mutant isolation in selection experiments 1, 2, and 4. A 5 x 10^{-6}M paraquat concentration was chosen for selection experiment 3. This level is sublethal and is based on one half of 10^{-5}M.

Selection

Selection procedures involved exposing tissue to medium containing paraquat and observing growth over a selection period. Callus condition for experiments 1, 2 and 3 was visually determined and recorded as outlined below according to two conditions: 1) height of callus piece in the test tube correlated with growth over the inoculum size and (2) color of the tissue.

1) Callus Growth

(+) Callus filling less than 1 centimeter of the test tube.

\+ Callus filling 1 cm to 2 cm of the test tube.

++ Callus filling 2 cm to 3 cm of the test tube.

+++ Callus filling 3 cm to 4 cm of the test tube.

++++ Callus filling more than 4 cm of the test tube.

2) Callus Color

1- Callus piece brown or with very little greening of tissue.

2- Callus piece brown to green in color.

3- Callus piece entirely green.

Recovery of cell lines from plating liquid cultures was recorded as appearance of cell colonies on the plates.

Experiment 1. Callus tissue was subjected to X-irradiation as a mutagenic agent at an exposure of 10,000R. The callus was immediately transferred to 25 x 150 mm test tubes each containing 25 ml of medium 3 for a period of recovery from X-ray exposure. After two weeks callus pieces were transferred to 40 25 x 150 mm test tubes containing 25 ml of medium 3 modified with paraquat at 10^{-5}M. Weekly observations were made for five weeks to determine if new growth representing resistant cells had occurred.

Experiment 2. Callus tissue was transferred to 40 25 x 150 mm test tubes each containing 25 ml of medium 3 modified with paraquat at 10^{-5}M. Weekly observations were recorded for eight weeks on the condition of the calli to determine if new growth representing resistant cells had occurred.

Experiment 3. Callus tissue was transferred to 40 25 x 150 mm test tubes each containing 25 ml of medium 3 modified with paraquat at 5×10^{-6}M, a sublethal level. One tube was lost due to contamination. Following six weeks of growth calli from 39 tubes were transferred to 25 x 150 mm test tubes each containing 25 ml of medium 3 modified with paraquat at 10^{-5}M, twice the sublethal level. Weekly observations were made for six weeks for new cell growth.

Experiment 4. Plated liquid cultures were used for selection at 10^{-5}M paraquat. The liquid fraction of a cell

culture was mixed in a 1 to 1 ratio with sterile double distilled water containing agar at a double strength of 16 percent. This medium also contained paraquat at 2×10^{-5}M. Normal strength of agar and paraquat was restored following mixing with the liquid culture. The medium was cooled to 40 degrees centigrade prior to mixing with the cell culture. Ten milliliter aliquots of the mixture were poured into 9 cm petri plates containing 25 ml of medium 3 modified with paraquat at 10^{-5}M. Plates were sealed with parafilm and placed inside sterile clear bags to avoid contamination. Weekly observations were made for growth of cell colonies. Cell colonies which grew were circled on the bottom of the plates and observations were made for increased growth.

Period of Non-selective Growth

All callus tissues from experiments 1, 2, and 3 which survived the selection period were transferred to 25 x 150 mm test tubes containing 25 ml of medium 3 without paraquat for a period of non-selective growth. For experiment 1 this period was 8 weeks, and for experiments 2 and 3 this period was 4 to 5 weeks. For experiment 4 cell colonies which grew on selective medium were also transferred to 25 x 150 mm test tubes containing 25 ml of medium 3 without paraquat for a period of callus proliferation and non-selective growth. With colonies, the time factor varied with the particular growth rate of each cell line. Usually this

period was 4 to 5 weeks. In all experiments, weekly observations were made of callus condition as previously outlined.

Retesting of Selected Tissues

Those cell lines which proliferated during the non-selective period were retested for survival on selective medium. Due to the abundance of surviving cell lines from non-selective growth in experiments 1, 2, and 3, retesting was only carried out at 10^{-4}M paraquat. Survival at 10^{-4}M paraquat would indicate resistance above the selection level of 10^{-5}M. For cell colonies recovered from plates which survived the non-selective period, retesting was carried out at paraquat levels of 10^{-5}M and 10^{-4}M. The retesting period for all experiments was at least 4 weeks but continued recovery of calli was monitored beyond the 4-week period. If growth did not occur on retesting medium within 8 weeks, the cell line was considered not resistant and was discarded. All callus conditions were recorded as previously outlined. Test tubes (25 x 150 mm) containing 25 ml of medium 3 modified with the appropriate paraquat level were used for all retestings.

Regeneration of Plantlets

An experiment was carried out using undifferentiated tobacco callus to determine appropriate levels of auxin and cytokinin for organ formation. Two pieces of green callus

tissue were transferred to 9 cm petri plates containing 50 ml of medium 1 with varying concentrations of IAA, an auxin, and 2iP, a cytokinin. Sixteen combinations of the two hormones were used with two plates per combination. Plates were sealed in parafilm and incubated for 4 weeks. Weekly observations were made for plantlet formation. Table 4 summarizes hormone levels used and occurrence of regeneration at each combination of hormones. The most efficient regeneration of plantlets occurred at 0 mg/l IAA and 2.5 mg/l 2iP (Table 4). These levels were chosen for regeneration of selected tissues (medium 4, Table 3, page 25).

Cell lines from experiments 1, 2 and 3 which continued to grow at the 10^{-4}M paraquat retesting level were transferred to medium 4 for regeneration of plantlets. For experiment 4 only those cell lines showing continued growth at 10^{-4}M paraquat were transferred to regeneration medium. Flasks (125 ml) containing 50 ml of medium 4 were used for all plantlet regenerations. At the time that a cell line was transferred to regeneration medium, a stock of the cell line was maintained on medium 3 without paraquat.

Testing Plantlets for Paraquat Resistance

The effect of paraquat on leaves of small regenerated plantlets was carried out by floating leaves on solutions of double distilled water containing paraquat at 0, 10^{-6}M, 10^{-5}M, and 10^{-4}M. Petri plates (5 cm) containing 5 ml of

TABLE 4. Regeneration of Plantlets from Callus Tissue of Nicotiana tabacum var. Wisconsin 38 Following Exposure to Hormone Levels for Four Weeks.

2iP(mg/l)	Callus Number	IAA(mg/l) 0	1.0	2.5	5.0
0	1	-	-	-	0
	2	-	-	0	0
	3	0	0	0	0
	4	+	0	0	0
1.0	1	0	+	-	+
	2	0	-	+	+
	3	C	+	-	+
	4	C	-	+	-
2.5	1	0	-	+	+
	2	0	+	+	+
	3	++	-	+	+
	4	++	+	-	0
5.0	1	0	0	0	-
	2	+	0	-	-
	3	-	+	-	-
	4	-	+	-	0

0 = Callus brown; no growth.
- = No regeneration.
+ = Regeneration occurring.
++ = Regeneration occurring; plantlets greater than 2 centimeters in height.
C = Callus lost due to contamination.

the appropriate solution were used for this assay. The color of the leaf was noted after 1 week and if a color change took place, the number of days required for that change was recorded. A value of 1 was assigned to a bleached white leaf, a value of 2 was assigned to a pale green leaf, and a value of 3 was assigned to a leaf which remained green (no change in color) throughout the 1 week test period. A numerical value for a leaf was obtained by multiplying the color value by the number of days required for the color change. If no color change occurred (leaf remained green), a value of seven was given to the leaf for the number of days.

Control leaves from plants regenerated from non-resistant tobacco callus were tested with seven replicates per paraquat level. All leaves at 0 paraquat remained green for 7 days. Leaves at 10^{-4}M and 10^{-5}M paraquat all turned white but the number of days required for the change varied. Leaves tested at 10^{-6}M paraquat varied from white to pale to green after 7 days. Averaging the values of the seven replicates at each paraquat level resulted in a value of 3 for 10^{-4}M paraquat, a value of 3.4 for 10^{-5}M paraquat, and a value of 8.7 for 10^{-6}M paraquat. A total value of 15.1 was calculated for non-resistant control leaves by adding the three paraquat values together.

To determine if resistance was carried through to regenerated plants, leaves from those cell lines which

regenerated were assayed for paraquat resistance by the floating technique described above. Leaf size often varied between cell lines but was consistent within a cell line. A numerical value was assigned to each cell line by averaging replicate values for individual leaves and by adding the values of the three paraquat levels together. If the total value for a particular cell line was greater than 15.1, it was considered to show resistance. If the value was less than 15.1, the regenerated cell line was considered to be non-resistant.

Testing Leaf Derived Callus for Paraquat Resistance

In some cell lines, callus was induced to form from the regenerated leaves by placing a single small leaf into a 25 x 150 mm test tube containing 25 ml of medium 3. If callus proliferated, it was subsequently tested for resistance at 10^{-4}M and 10^{-5}M paraquat using 25 ml of medium 3 in 25 x 150 mm test tubes modified with paraquat.

Testing for Protection Mechanisms against Paraquat Toxicity

Gregory, Goscin, and Fridovich (1974) showed that the addition of copper, a component of cytosol eukaryotic superoxide dismutase, lead to increased amounts of the enzyme in the yeast, Saccharomyces cerevisiae var. ellipsoideus. This could provide a defense against the reactivity of superoxide radicals in the cell. Addition of iron, a component of peroxidases and catalases, to E. coli medium

has been shown to increase both enzymes (Yost and Fridovich, 1976). These enzymes could also provide a defense against hydrogen peroxide toxicity.

To determine if the addition of copper to the growth medium affected paraquat toxicity, callus pieces were transferred to 15 x 150 mm test tubes containing 15 ml of medium 3 modified with paraquat levels of 0, 10^{-6}M, 10^{-5}M, and 10^{-4}M. Each paraquat level had been further modified with $CuSO_4$ concentrations of 4 x 10^{-4}M, 2 x 10^{-4}M, 0.2 x 10^{-4}M, 0.02 x 10^{-4}M, and 0.001 x 10^{-4}M (normal concentration of $CuSO_4$ in Linsmaier and Skoog salts). After 4 weeks of incubation, tissues were dried for 48 hours at 60 degrees centigrade. Dry weights were taken and analyzed by analysis of variance, randomized block design (Li, 1957). Dry weights for inoculum callus size prior to the growth period were also determined.

To determine if the addition of iron to the growth medium would protect the tissue against paraquat toxicity, callus pieces were transferred to 15 x 150 mm test tubes containing 15 ml of medium 3 modified with paraquat levels of 0, 10^{-5}M, and 10^{-4}M. Each paraquat level had been further modified with $FeSO_4$ concentrations of 0.25 x 10^{-4}M, 0.5 x 10^{-4}M, 1 x 10^{-4}M (normal $FeSO_4$ concentration of Linsmaier and Skoog salts), 2 x 10^{-4}M, and 3 x 10^{-4}M. After 4 weeks of incubation, tissues were dried at 60 degrees centigrade for 48 hours. Dry weights were taken and analyzed

by analysis of variance, randomized block design (Li, 1957). Dry weights for inoculum callus size prior to the incubation period were also determined.

CHAPTER III

RESULTS

Resistant cell lines obtained from experiments 1, 2, 3, and 4 were characterized for each step of experimentation including: 1) retesting of surviving cell lines at 10^{-4}M paraquat following a period of non-selective growth, 2) regeneration of plantlets from tissues showing continued resistance, 3) testing of plantlets for resistance, and 4) testing of callus derived from regenerated leaves for resistance (Table 5). Thirty-six resistant cell lines from experiment 1, 34 resistant cell lines from experiment 2, 40 resistant cell lines from experiment 3, and 131 resistant cell colonies from experiment 4 were obtained and characterized for genetically stable phenotypes. Complete data are not available on some cell lines which were lost as a result of contamination (C) with fungi or bacteria (Table 5). Additionally, some cell lines were not tested (NT) at all steps of experimentation (Table 5).

Period of Non-selective Growth

Virtually all cell lines derived from experiments 1, 2, and 3 exhibited growth when transferred to medium without paraquat (Table 5), while 43.4 percent of the cell colonies from experiment 4 which were transferred to non-selective medium did not grow or failed to produce enough tissue for

TABLE 5. Characterization of Putative Mutants--Experiments 1, 2, 3 and 4.[a]

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
1:1	C				
1:2	+	C			
1:3	+	-			
1:4	+	+	+	+	NT
1:5	+	C			
1:6	+	+	+	+	NT
1:7	+	-			
1:8	+	C			
1:9	+	+	+	-	+
1:10	+	+	+	-	NT
1:11	+	+	+	-	NT
1:12	+	-			
1:13	+	+	C		
1:14	+	+	+	-	+
1:15	+	C			
1:17	+	-			
1:18	+	+	B		
1:19	+	+	+	+	+
1:20	+	+	P		
1:21	+	+	+	+	+
1:22	+	+	+	+	+
1:23	+	+	+	-	+
1:24	+	+	+	-	NT
1:25	+	C			
1:26	+	+	+	-	+
1:27	+	+	C		

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
1:28	+	-			
1:29	+	-			
1:30	+	+	+	+	NT
1:31	+	+	C		
1:32	+	-			
1:33	+	C			
1:34	+	-			
1:35	+	C			
1:36	+	-			
2:1	+	C			
2:2	+	-			
2:3	+	+	P		
2:4	+	+	P		
2:5	C				
2:6	+	-			
2:7	C				
2:8	+	+	+	-	+
2:9	+	-			
2:10	+	+	+	-	NT
2:11	+	-			
2:12	+	-			
2:13	+	+	C		
2:15	+	-			
2:17	+	+	+	-	NT
2:18	+	-			
2:19	+	+	+	+	NT

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
2:20	C				
2:21	+	-			
2:22	+	+	+	NT	
2:23	+	-			
2:24	+	-			
2:25	+	+	P		
2:26	+	-			
2:27	+	-			
2:28	+	+	P		
2:29	+	-			
2:30	+	-			
2:31	+	-			
2:32	+	-			
2:33	+	-			
2:34	+	-			
3:1	+	-			
3:2	+	+	+	-	+
3:3	+	+	+	-	NT
3:4	+	+	+	+	NT
3:5	+	+	+	NT	
3:6	+	+	+	+	NT
3:7	+	-			
3:8	+	-			
3:10	+	-			
3:11	+	-			
3:12	+	-			

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
3:13	+	-			
3:14	+	-			
3:15	+	-			
3:16	+	-			
3:17	+	C			
3:18	+	C			
3:19	+	-			
3:20	+	+	B		
3:21	+	-			
3:22	+	-			
3:23	+	-			
3:24	+	+	+	-	NT
3:25	C				
3:26	+	+	+	NT	
3:27	+	-			
3:28	+	+	P		
3:29	+	-			
3:30	+	+	P		
3:31	+	+	C		
3:32	+	-			
3:33	+	+	+	-	+
3:34	+	C			
3:35	+	C			
3:36	+	C			
3:37	+	C			
3:38	C				

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
3:39	+	-			
3:40	+	C			
4:1	-				
4:2	-				
4:3	+	+	+	+	NT
4:4	+	C			
4:5	-				
4:6	+	-			
4:7	-				
4:8	-				
4:9	+	+	+	+	NT
4:10	+	C			
4:11	-				
4:12	C				
4:13	-				
4:14	-				
4:15	-				
4:16	+	-			
4:17	-				
4:18	-				
4:19	-				
4:20	-				
4:21	+	+	+	-	NT
4:22	C				
4:23	-				

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
4:24	-				
4:25	-				
4:26	+	+	C		
4:27	-				
4:28	-				
4:29	+	+	+	-	NT
4:30	-				
4:31	-				
4:32	-				
4:33	-				
4:34	-				
4:35	-				
4:36	-				
4:37	+	+	+	-	NT
4:38	-				
4:39	+	+	+	-	NT
4:40	+	+	+	NT	
4:41	+	+	+	NT	
4:42	+	+	+	-	NT
4:43	+	+	+	-	NT
4:44	+	-			
4:45	-				
4:46	-				
4:47	C				
4:48	-				
4:49	-				

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
4:50	-				
4:51	-				
4:52	-				
4:53	-				
4:54	C				
4:55	C				
4:56	C				
4:57	C				
4:58	+	C			
4:59	C				
4:60	-				
4:61	C				
4:62	C				
4:63	C				
4:64	-				
4:65	+	-			
4:66	-				
4:67	+	+	P		
4:68	+	+	P		
4:69	+	+	B		
4:70	+	+	+	NT	
4:71	+	+	+	NT	
4:72	-				
4:73	+	+	+	+	NT
4:74	+	+	B		
4:75	C				

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
4:76	C				
4:77	C				
4:78	C				
4:79	+	+	+	NT	
4:80	+	+	+	+	NT
4:81	+	+	P		
4:82	+	+	P		
4:83	+	+	P		
4:84	+	+	+	-	+
4:85	+	+	+	NT	
4:86	+	+	P		
4:87	+	+	B		
4:88	+	+	+	-	NT
4:89	+	+	+	-	NT
4:90	C				
4:91	C				
4:92	C				
4:93	C				
4:94	C				
4:95	C				
4:96	C				
4:97	+	+	B		
4:98	-				
4:99	+	+	P		
4:100	+	+	B		
4:101	-				

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
4:102	+	+	P		
4:103	-				
4:104	+	+	+	-	NT
4:105	C				
4:106	+	+	P		
4:107	+	C			
4:108	+	-			
4:109	+	+	B		
4:110	+	-			
4:111	+	+	P		
4:112	+	+	B		
4:113	+	+	P		
4:114	+	+	B		
4:115	+	+	P		
4:116	-				
4:117	+	-			
4:118	+	+	P		
4:119	+	+	+	-	+
4:120	+	+	+	+	+
4:121	+	+	B		
4:122	+	+	P		
4:123	C				
4:124	-				
4:125	-				
4:126	-				
4:127	C				

TABLE 5. (Continued)

Experiment: Cell Line	Non-selective Growth[b]	Retesting Resistant Callus at 10^{-4}M Paraquat[b]	Regeneration[c]	Testing Leaves[d]	Retesting Callus Derived from Leaves[e]
4:128	-				
4:129	+	-			
4:130	+	+	+	+	NT
4:131	+	C			

[a]C = Contamination.
NT = Not tested.

[b]+ = Growth evident.
- = No growth.

[c]B = Formation of buds only from the callus.
P = Formation of small plantlets less than 1 centimeter in height.
+ = Positive regeneration of plantlets; leaves able to be tested.

[d]+ = Regenerated leaves showing resistance.
- = Regenerated leaves not showing resistance.

[e]+ = Growth evident on paraquat medium.
- = No growth.

retesting (Table 5). Cell lines surviving the period of non-selective growth (Table 5) are indicated by a + and those cell lines not surviving the period of non-selective growth are indicated by a -.

Retesting of Selected Tissues

Retesting of cell lines from experiments 1, 2, and 3 for continued growth on medium containing paraquat at 10^{-4}M after a non-selective period (Table 6) resulted in survival (+) of 65.4 percent of all cell lines tested in experiment 1 (involving X-ray exposure prior to selection at 10^{-5}M paraquat), 35.7 percent survival (+) for experiment 2 (no mutagen treatment and selection at 10^{-5}M paraquat), and 40 percent survival (+) for experiment 3 (selection a 5 x 10^{-6}M paraquat prior to selection at 10^{-5}M paraquat). Cell lines 1:7, 1:28, 2:2, 3:11, and 3:22 not only failed to grow (-) on paraquat retesting medium but also failed to show continued growth (-) on control non-selective medium during the retesting period (Table 6). Six controls using non-resistant callus tissue showed no growth (-) on the paraquat medium but 100 percent growth on normal medium (Table 6).

Sixty cell lines from experiment 4 which survived the period of non-selective growth were retested for paraquat resistance on medium 3 containing paraquat at 0, 10^{-5}M, and 10^{-4}M (Table 7). Cell lines 4:4, 4:10, 4:58, 4:107, and 4:131 became contaminated (Table 7) and were not

TABLE 6. Retesting Selected Tissues--Experiments 1, 2 and 3.

Experiment: Cell Line	Paraquat 10^{-4}M	Paraquat 0	Experiment: Cell Line	Paraquat 10^{-4}M	Paraquat 0
Control 1	-	+	2:15	-	+
Control 2	-	+	2:17	+	+
Control 3	-	+	2:18	-	+
Control 4	-	+	2:19	+	+
Control 5	-	+	2:21	-	+
Control 6	-	+	2:22	+	+
1:3	-	+	2:23	-	+
1:4	+	+	2:24	-	+
1:6	+	+	2:25	+	+
1:7	-	-	2:26	-	+
1:9	+	+	2:27	-	+
1:10	+	+	2:28	+	+
1:11	+	+	2:29	-	+
1:12	-	+	2:30	-	+
1:14	+	+	2:31	-	+
1:17	-	+	2:32	-	+
1:18	+	+	2:33	-	+
1:19	+	+	2:34	-	+
1:20	+	+	3:1	-	+
1:21	+	+	3:2	+	+
1:22	+	+	3:3	+	+
1:23	+	+	3:4	+	+
1:24	+	+	3:5	+	+
1:26	+	+	3:6	+	+
1:27	+	+	3:7	-	+
1:28	-	-	3:8	-	+
1:29	-	+	3:10	-	+
1:30	+	+	3:11	-	-
1:31	+	+	3:12	-	+
1:32	-	+	3:13	-	+
1:34	-	+	3:14	-	+
1:36	-	+	3:15	-	+
2:2	-	-	3:16	-	+
2:3	+	+	3:19	-	+
2:4	+	+	3:20	+	+
2:6	-	+	3:21	-	+
2:8	+	+	3:22	-	-
2:9	-	+	3:23	-	+
2:10	+	+	3:24	+	+
2:11	-	+	3:26	+	+
2:12	-	+	3:27	-	+
2:13	+	C	3:28	+	+

TABLE 6. (Continued)

Experiment: Cell Line	Paraquat 10^{-4}M	0	Experiment: Cell Line	Paraquat 10^{-4}M	0
3:29	-	+	3:32	-	+
3:30	+	+	3:33	+	+
3:31	+	+	3:39	-	+

+ = Growth evident.
- = No growth.
C = Contamination.

TABLE 7. Retesting Selected Tissues--Experiment 4.

Cell Line	Paraquat 10^{-4}M	Paraquat 10^{-5}M	Paraquat 0	Cell Line	Paraquat 10^{-4}M	Paraquat 10^{-5}M	Paraquat 0
Control 1	-	+	+	4:82	+	+	+
Control 2	-	+	+	4:83	+	+	+
Control 3	-	-	+	4:84	+	+	+
Control 4	-	+	+	4:85	+	+	+
Control 5	-	+	+	4:86	+	+	+
4:3	+	+	+	4:87	+	+	+
4:4	-	-	C	4:88	+	+	+
4:6	-	+	+	4:89	+	+	+
4:9	+	+	+	4:97	+	+	+
4:10	C	C	C	4:99	+	+	+
4:16	-	+	+	4:100	+	+	+
4:21	+	+	+	4:102	+	+	+
4:26	+	+	+	4:104	+	+	+
4:29	+	+	+	4:106	+	+	+
4:37	+	+	+	4:107	C	C	C
4:39	+	+	+	4:108	-	+	+
4:40	+	+	+	4:109	+	+	+
4:41	+	+	+	4:110	-	-	+
4:42	+	+	+	4:111	+	+	+
4:43	+	+	+	4:112	+	+	+
4:44	-	-	+	4:113	+	+	+
4:58	C	+	+	4:114	+	+	+
4:65	-	+	+	4:115	+	+	+
4:67	+	+	+	4:117	-	+	+
4:68	+	+	+	4:118	+	+	+
4:69	+	+	+	4:119	+	+	+
4:70	+	+	+	4:120	+	+	+
4:71	+	+	+	4:121	+	+	+
4:73	+	+	+	4:122	+	+	+
4:74	+	+	+	4:129	-	-	+
4:79	+	+	+	4:130	+	+	+
4:80	+	+	+	4:131	-	C	-
4:81	+	+	+				

+ = Growth evident.
- = No growth.
C = Contamination.

included in the final results. Five controls were tested using non-resistant callus tissue, four of which did not grow (-) at 10^{-4}M paraquat but exhibited limited growth at 10^{-5}M paraquat (Table 7). One control did not grow (-) at both 10^{-4}M and 10^{-5}M paraquat (Table 7). Cell lines 4:44, 4:110, and 4:129 did not grow (-) at 10^{-4}M and 10^{-5}M paraquat but growth was evident (+) at 0 paraquat (Table 7). These cell lines were considered not resistant. Cell lines 4:6, 4:16, 4:65, 4:108, and 4:117 did not grow (-) at 10^{-4}M paraquat but growth was evident (+) at 10^{-5}M paraquat as well as 0 paraquat (Table 7). Cell lines 4:6, 4:108, and 4:117 were considered not resistant. Cell lines 4:16 and 4:65 exhibited a faster growth rate at 10^{-5}M paraquat than non-resistant control callus at 10^{-5}M paraquat but cell line 4:65 exhibited a slower growth rate than non-resistant control callus at 0 paraquat. Cell lines 4:16 and 4:65 were considered partially resistant. Forty-seven cell lines continued to grow (+) on both 10^{-5}M and 10^{-4}M paraquat as well as 0 paraquat, thus survival of retested cell lines in experiment 4 was higher than either experiments 1, 2, or 3 (Table 5, page 43; Table 7).

Regeneration of Plantlets

Cell lines which retained resistance after a period of non-selective growth were transferred to regeneration medium (Table 3, page 25, medium 4). Response of callus on regeneration medium was varied. Plantlets in some cases

did not form from the callus but the callus acquired a budding or bumpy appearance (Table 5, B, page 43). Cell lines which showed this characteristic and no further plantlet development were considered non-regenerating. In some cases the callus tissue would form buds and then small plantlets which failed to grow larger than 1 centimeter in height (Table 5, P). Small leaves from such plantlets could not be tested for continued resistance. Thirdly, if plantlets greater than 1 centimeter in height formed from the callus, the cell line was considered to show regeneration (Table 5, +). Leaves from such plantlets could be tested for resistance.

The results of the regeneration experiments are given in Table 5. Regeneration of plantlets (+) occurred in 86.6 percent of the cell line tested in experiment 1 with 6.7 percent forming small plantlets (P) and 6.7 percent demonstrating only the budding appearance (B). Regeneration of plantlets (+) occurred in 55.6 percent of the cell lines tested in experiment 2 with 44.4 percent forming small plantlets (P). Regeneration of plantlets (+) occurred in 72.7 percent of the cell lines tested in experiment 3 with 18.2 percent forming small plantlets (P) and 9.1 percent demonstrating only the budding appearance (B). Regeneration of plantlets (+) occurred in 50 percent of the cell lines tested in experiment 4 with 30.4 percent forming small plantlets (P) and 19.6 percent demonstrating only the budding appearance (B).

Testing Plantlets for Paraquat Resistance

The results of testing regenerated leaves at 10^{-4}M, 10^{-5}M, and 10^{-6}M paraquat for continued resistance as outlined in Chapter II are given in Table 8. For each leaf tested, the color of the leaf (W, white; P, pale; and G, green) following the seven day test period is given with the number of days required for a change in color preceding the color symbol. No change in color (leaf remained green) is indicated by a seven preceding the color symbol. Additionally Table 8 gives the average value for the retested leaves and the total numerical value for a cell line. Resistance of a cell line (Table 8) is indicated by a + if the total value was greater than 15.1, the total value for non-resistant control leaves. If the total value was less than 15.1 the cell line was considered to be non-resistant (-).

Results (Table 8) indicate that 46.2 percent of the tested regenerated cell lines from experiment 1 retained resistance at the plant level, 25 percent of the tested regenerated cell lines from experiment 2 retained resistance, 33.3 percent of the tested regenerated cell lines from experiment 3 retained resistance, and 35.3 percent of the tested regenerated cell lines from experiment 4 retained resistance. Leaves from all plants which retained resistance remained green (no change in color) throughout the testing period when floated on water without paraquat.

TABLE 8. Testing and Analysis of Leaves for Paraquat Resistance.

Experiment Cell Line	Paraquat 10^{-4}M Days, Leaf Color[a]	10^{-4}M Numerical Value[b]	10^{-5}M Days, Leaf Color[a]	10^{-5}M Numerical Value[b]	10^{-6}M Days, Leaf Color[a]	10^{-6}M Numerical Value[b]	Total Value[c]	Resistance[d]
Control	2W 3W 4W 3W 3W 3W 3W	3	3W 3W 3W 3W 4W 4W 4W	3.4	4W 7G 5W 6W 5W 4P 6P	8.7	15.1	
1:4	3W	3	7W	7	6P	12	22	+
1:6	2W 2W	2	6W 3W	4.5	7P 7G	17.5	24	+
1:9	3W 3W	3	3W 4W	3.5	4W	4	10.5	-
1:10	3W	3	3W	3	4W	4	10	-
1:11	2W 3W	2.5	3W 3W	3	3W 4W	3.5	9	-
1:14	2W	2	3W	3	3W	3	8	-
1:19	3W 3W 3W	3	3W 4W 5W	4	7W 6W 7G	11.3	18.3	+
1:21	3W 4W 2W	3	4W 3W 3W	3.3	6W 5P 6W	22	28.3	+
1:22	7W 2W 2W	3.7	4P 3W 4W	5	4P 5W 6P	8.3	17	+

TABLE 8. (Continued)

Experiment Cell Line	Paraquat 10^{-4}M Days, Leaf Color[a]	10^{-4}M Numerical Value[b]	10^{-5}M Days, Leaf Color[a]	10^{-5}M Numerical Value[b]	10^{-6}M Days, Leaf Color[a]	10^{-6}M Numerical Value[b]	Total Value[c]	Resistance[d]
1:23	2W 2W	2	2W 2W	2	3W 3W	3	7	-
1:24	3W 3W	3	4W 2W	3	6W 6W	6	12	-
1:26	2W 3W	2.5	3W 3W	3	3P 3W	4.5	10	-
1:30	7W 7W	7	7W 7W	7	7P 6P	13	27	+
2:8	3W	3	3W	3	3P	6	12	-
2:10	2W	2	2W 2W	2	5W 6W	5.5	9.5	-
2:17	2W 3W 2W 2W	2.25	3W 4W 2W 3W	3	5W 6W 5W 5W	5.25	10.5	-
2:19	4W 3W	3.5	6W 6W	6	4W 6P	8	17.5	+
3:2	4W	4	4W	4	5W	5	13	-
3:3	3W 4W 4W	3.7	5W 4W 4W	4.3	3P 5W 4W	5	13	-
3:4	7W 7W	7	7W 7W	7	7P 4P	11	25	+

TABLE 8. (Continued)

Experiment Cell Line	Paraquat 10^{-4}M Days, Leaf Color[a]	10^{-4}M Numerical Value[b]	10^{-5}M Days, Leaf Color[a]	10^{-5}M Numerical Value[b]	10^{-6}M Days, Leaf Color[a]	10^{-6}M Numerical Value[b]	Total Value[c]	Resistance[d]
3:6	3W 6W 3W 3W 5W	4	7G 2P 4W 6W 7P	4	4P 3W 7G 7G 7G	14.8	25.8	+
3:24	2W 2W	2	2W 3W	2.5	3W 3W	3	7.5	-
3:33	3W 3W	3	3W 3W	3	3W 7W	5	11	-
4:3	7W 7W	7	7W 7W	7	6P 7W	9.5	23.5	+
4:9	6W 3W	4.5	6W 3W	5	7P 7P	14	23.5	+
4:21	3W 3W	3	3W 3W	3	4W 4W	4	10	-
4:29	2W 2W	2	3W 3W	3	4W 4W	4	9	-
4:37	2W 2W	2	3W 3W	3	4W 4W	4	9	-
4:39	3W	3	5W	5	3P	6	14	-
4:42	3W	3	3W	3	6W 7W	6.5	12.5	-
4:43	2W	2	3W	3	4P	8	13	-
4:73	3W	3	4W	4	6P	12	19	+
4:80	1W 2W	1.5	5W 7W	6	7W 7P	10.5	18	+

TABLE 8. (Continued)

Experiment Cell Line	Paraquat 10^{-4}M Days, Leaf Color[a]	10^{-4}M Numerical Value[b]	10^{-5}M Days, Leaf Color[a]	10^{-5}M Numerical Value[b]	10^{-6}M Days, Leaf Color[a]	10^{-6}M Numerical Value[b]	Total Value[c]	Resistance[d]
4:84	3W	3	3W	3	7W	7	13	-
4:88	2W	2	2W	2	3W	3	7	-
4:89	2W	2	2W	2	5W	5	9	-
4:104	3W	3	3W	3	4W	4	10	-
4:119	3W	3	3W	3	4W	4	10	-
4:120	2W 3W 3W	2.7	3W 5W 3W	3.7	4P 4P 7G	12.3	18.6	+
4:130	3W	3	4W	4	5P 7G	10.3	17.3	-

[a]The color of a leaf (W = white, P = pale, G = green) after floating in a paraquat solution for 7 days is indicated preceded by the number of days required if a color change took place. A seven precedes G if no change in color took place.

[b]A value for a leaf was obtained by multiplying the number of days and a color value (W=1, P=2, G=3). The numerical value for the cell line was obtained by averaging leaf values.

[c]The total value was obtained by adding the numerical values for the three paraquat levels.

[d]- = Not resistant; the total value was equal to or less than 15.1, the total value for non-resistant control leaves. + = Resistant; the total value was greater than 15.1.

Testing Leaf Derived Callus for Paraquat Resistance

Resistance of callus derived from regenerated leaves of some cell lines is indicated in Table 5, page 43. Leaves were resistant and leaf derived callus was resistant at 10^{-5}M paraquat for cell lines 1:21 and 4:120. Leaves were resistant and leaf derived callus was resistant at 10^{-5}M and 10^{-4}M paraquat for cell lines 1:19 and 1:22. Leaves were not resistant but leaf derived callus was resistant at 10^{-5}M paraquat for cell lines 1:23, 1:26, 3:2, and 3:33. Leaves were not resistant and leaf derived callus was resistant at 10^{-5}M and 10^{-4}M paraquat for cell lines 1:9, 1:14, 2:8, 4:84, and 4:119.

Testing for Protection Mechanisms against Paraquat Toxicity

Results of the experiment to determine the effects of additional copper in the growth medium on paraquat toxicity are indicated in Figure 3. Mean dry weights are graphed against paraquat levels for each copper level. At normal copper levels, restriction in growth due to paraquat toxicity was similar to previous paraquat survival curves (Figure 2, page 30) in that growth was highly restricted at 10^{-5}M paraquat and virtually totally inhibited at 10^{-4}M paraquat. Differences in growth response on medium containing different paraquat levels were significant at the 0.01 probability level (Table 9). Figure 3 also indicates that at 0 paraquat there was a decrease in growth with each increase in copper concentration. Lethality level for

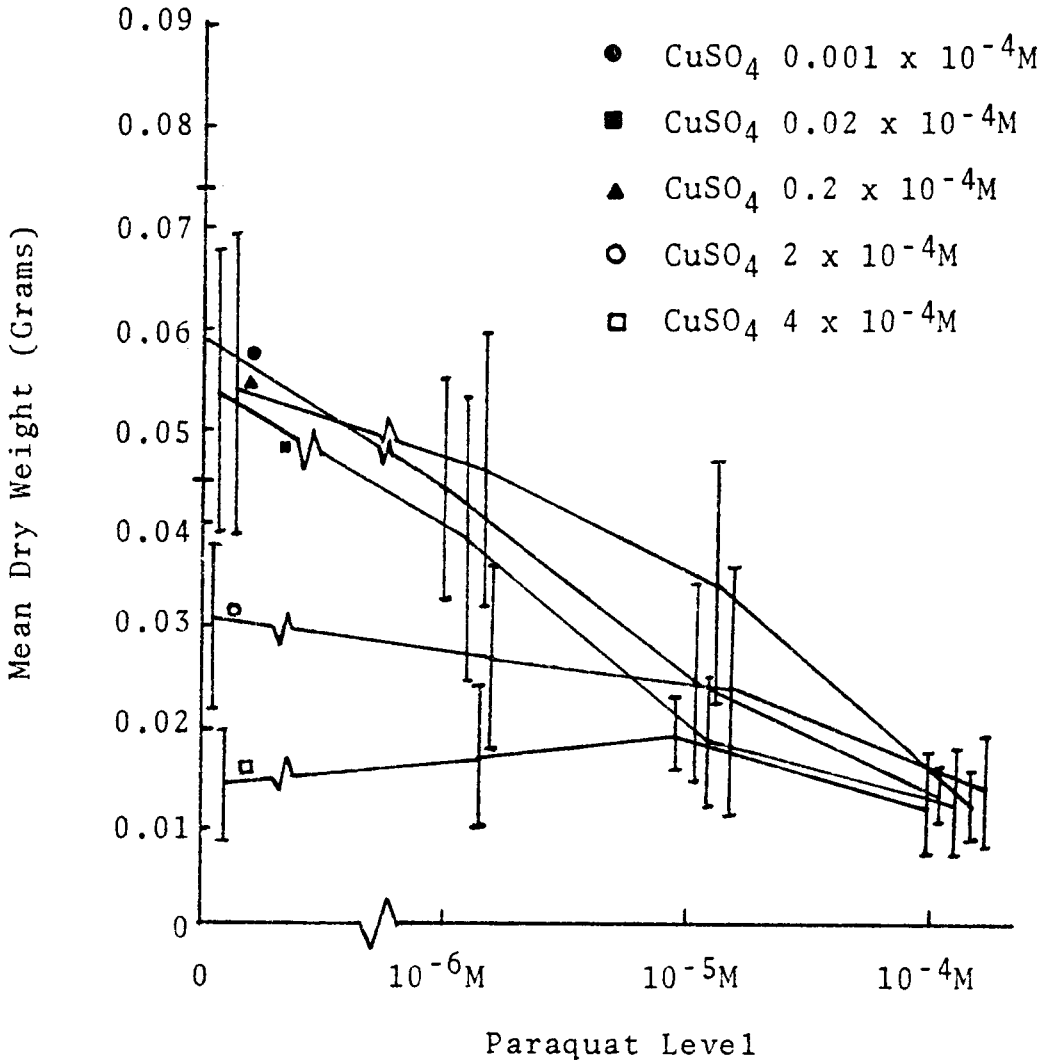

Figure 3. Effect of copper on paraquat toxicity in Nicotiana tabacum callus. Initial inoculum dry weight was 0.0085 gram. Growth period was four weeks.

TABLE 9. Statistical Analysis for Experiments Involving the Effect of Copper and Iron on Paraquat Toxicity in *Nicotiana tabacum* Callus.

Experiments	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Effect of copper on paraquat toxicity				
Total	240	.0917		
Treatments		.0684		
Paraquat	3	.0258	.0086	86.00**
Copper	4	.0338	.0085	85.00**
Interaction	12	.0088	.0007	7.00**
Error	221	.0233	.0001	
Effect of iron on paraquat toxicity				
Total	149	.0833		
Treatments		.0565		
Paraquat	2	.0513	.0257	128.25**
Iron	4	.0012	.0003	1.50
Interaction	8	.0400	.0005	2.50*
Error	135	.0268	.0002	

**Significant to the 0.01 level.
*Significant to the 0.05 level.

copper on tobacco callus appears to be 4 x 10^{-4}M. Differences in growth response on medium containing different levels of copper were also significant at the 0.01 probability level (Table 9). There was also a highly significant (p=0.01) interaction between paraquat and copper (Table 9). At 0.02 x 10^{-4}M copper and 10^{-6}M paraquat callus growth was enhanced over that expected for paraquat and copper. Increased growth at 0.2 x 10^{-4}M copper and 10^{-5}M paraquat was also observed. The mean dry weight for inoculum callus size was 0.0085 gram with a standard deviation of 0.003 gram.

Results of the experiment to determine if additional iron in the growth medium could protect tobacco callus against the toxic effects of paraquat are given in Figure 4. Dry weights are graphed against iron levels for each paraquat level. A marked decrease in growth at normal iron concentration (1 x 10^{-4}M) was seen between 0 paraquat and 10^{-5}M paraquat with almost total inhibition of growth at 10^{-4}M paraquat. This corresponds to previous paraquat lethality tests (Figure 2, page 30). Response of tobacco callus growth on medium containing different levels of paraquat was statistically significant at the 0.01 probability level (Table 9). A comparison of various iron levels at 0 paraquat indicates increased dry weight at all levels except two times the normal concentration (2 x 10^{-4}M) over the normal iron concentration (1 x 10^{-4}M). Iron treatment

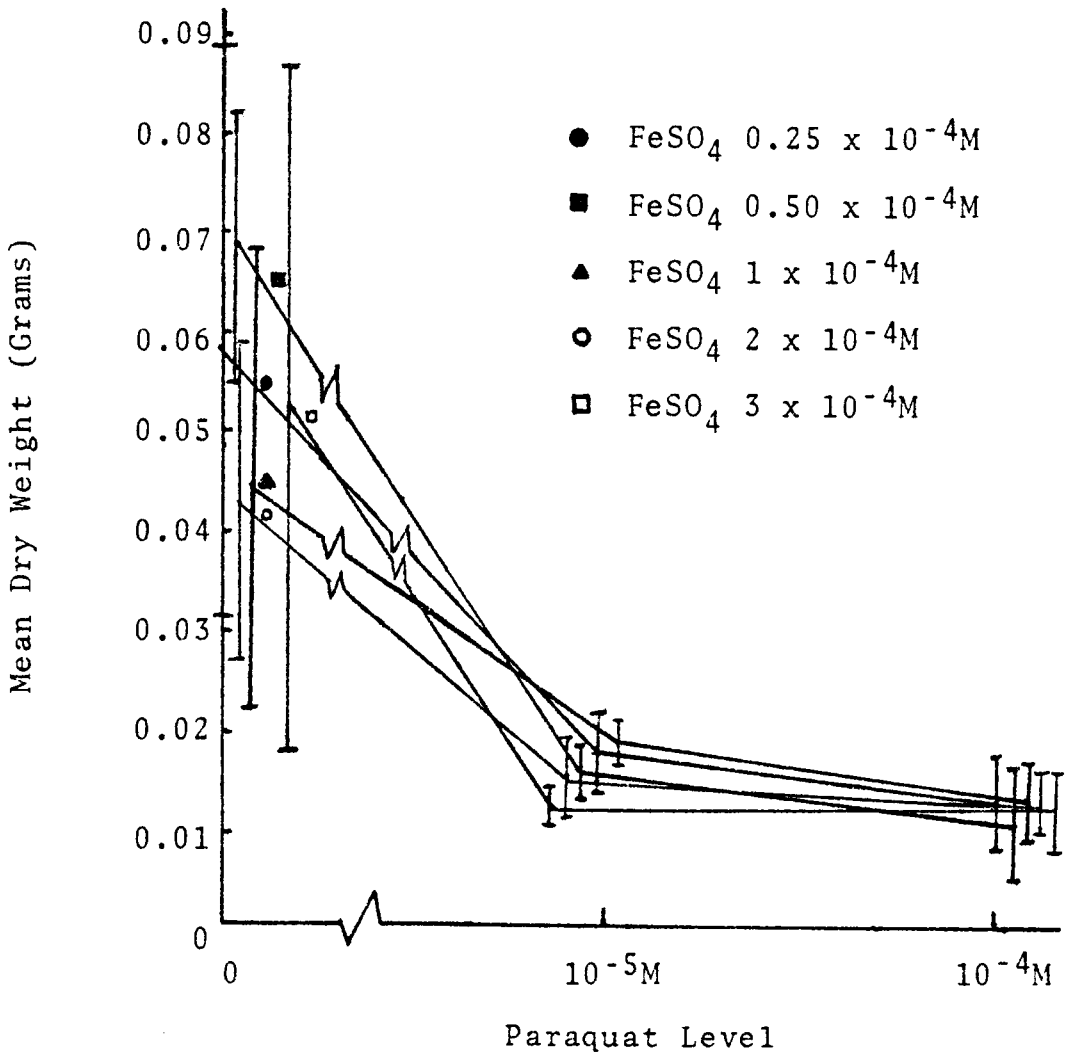

Figure 4. Effect of iron on paraquat toxicity in Nicotiana tabacum callus. Initial inoculum dry weight was 0.0092 gram. Growth period was four weeks.

was not significant (Table 9). The interaction of paraquat and iron was significant at the 0.05 probability level which probably reflects the non-linear response of callus to different iron levels (Table 9); however, there was no significant increase in growth at any iron concentration where callus was also treated with paraquat. Growth was similar for all iron concentrations at 10^{-5}M paraquat as well as at 10^{-4}M paraquat. The mean dry weight for inoculum callus size was 0.0092 gram with a standard deviation of 0.0031 gram.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The purpose of this study was to determine the effect of the herbicide paraquat on tobacco, Nicotiana tabacum L. var. Wisconsin 38, in culture, to isolate paraquat resistant cell lines by using somatic cell cultures (both callus tissue and liquid cell suspension cultures), to examine the use of X-rays as a mutagenic agent for mutation induction versus the occurrence of spontaneous mutations to paraquat resistance, and to determine the effectiveness of sublethal selection on mutant isolation. Additionally, possible protection mechanisms against paraquat toxicity were considered.

Paraquat Toxicity

The effect of the herbicide paraquat on tobacco callus as indicated in Figure 2, page 30, was a marked decrease in growth at 10^{-5}M paraquat and no growth at 10^{-4}M paraquat or higher (lethal level). The lethal level of paraquat for tobacco callus was higher than that observed for pine callus. Callus from five species of pine exhibited no growth at 10^{-5}M paraquat or higher (Hughes and Caponetti, 1976). The inhibitory levels of paraquat for soybean callus and tobacco callus were similar (Hughes et al., 1979). Jordan, Murashige, Mann, and Day (1966) reported that the

toxicity of paraquat to dark grown tobacco callus was dramatic with an abrupt decrease in growth between 10^{-6}M and 10^{-5}M paraquat with death occurring at 10^{-5}M paraquat, thus light grown tobacco callus in our experiments was somewhat less sensitive to paraquat than dark grown tobacco callus.

Variations were evident between the first paraquat series and the second paraquat series for growth of tissue from 0 to 10^{-6}M paraquat (Figure 2, page 30). The first series exhibited an increase in growth at 10^{-8}M, 10^{-7}M, and 10^{-6}M paraquat over the control (0 paraquat). This increase in growth has been seen in other cases such as with the response of pine callus to paraquat (Hughes and Caponetti, 1976). The explanation for this is unknown. It is possible that an overall increased growth was observed in the second paraquat series due to the use of callus explants rather than pith explants and due to the use of medium 3 with additional vitamins rather than medium 1.

Isolation of Paraquat Resistant Cell Lines and Plants

This study was successful in isolating paraquat resistant strains of tobacco using plant cell and tissue culture techniques. Several techniques were shown to be effective for isolating paraquat resistant cell lines and/or plants: 1) X-irradiation treatment of callus and selection at 10^{-5}M paraquat, 2) selection of resistant callus at 10^{-5}M paraquat without prior mutagenic treatment, 3) treatment

of callus at a sublethal level of paraquat (5×10^{-6}M) prior to selection at 10^{-5}M paraquat, and 4) plating of liquid suspension cultures on 10^{-5}M paraquat medium.

Selection of callus versus selection of plated suspension cultures. Putative mutants recovered from selection procedures in which small pieces of callus were placed on medium containing 10^{-5}M paraquat or in which cells from suspension cultures were plated on medium with 10^{-5}M paraquat were allowed a period of growth on medium without paraquat (non-selective growth). All of the cell lines derived from callus (experiments 1, 2, and 3) continued to grow (Table 10) but only 56.6 percent of the suspension culture derived cell lines (experiment 4) exhibited further growth. A factor which may have resulted in a lower survival percentage for non-selective growth in experiment 4 was the small size of cell colonies which were transferred from selection plates to non-selection medium. Twenty-eight out of the 46 cell colonies exhibiting no growth (-) on non-selection medium (Table 5, page 43) were 2 to 3 mm in diameter when transferred to non-selection medium. Only four small cell colonies (2 to 3 mm in diameter) exhibited continued growth on non-selection medium. These were cell lines 4:3, 4:26, 4:29, and 4:129 (Table 5). All other surviving cell colonies on non-selection medium were larger and perhaps had a survival advantage over smaller colonies.

TABLE 10. Results for Non-selective Growth, Retesting Callus, Regeneration, and Testing Plantlets for Experiments 1, 2, 3 and 4.

Procedures	Percentage Response Experiment 1	2	3	4
Growth of putative mutants (on non-selective medium				
No growth	0	0	0	43.4
Growth evident	100	100	100	56.6
Retesting putative mutants on 10^{-4}M paraquat following non-selective growth				
No growth	34.6	64.3	60	14.5
Growth evident	65.4	35.7	40	85.5
Regeneration of cell lines exhibiting continued resistance on retesting medium				
Formation of buds only	6.7	0	9.1	19.6
Formation of small plantlets less than 1 centimeter in height	6.7	44.4	18.2	30.4
Formation of plantlets greater than 1 centimeter in height	86.6	55.6	72.7	50.0
Testing plantlets derived from resistant cell lines for paraquat resistance				
Plantlets non-resistant	53.8	75.0	66.7	64.7
Plantlets resistant	46.2	25.0	33.3	35.3

The use of callus tissue for mutant isolation (experiments 1, 2, and 3) was easier to handle and involved less time in terms of culturing and transferring tissue but cross feeding of paraquat sensitive cells by paraquat resistant cells was possible leading to chimeric callus. Plating of liquid cultures consisting of single cells or small clumps of cells for mutant isolation (experiment 4) probably enhanced the possibility of acquiring a mutant cell line from a single cell and prevented excessive cross feeding between cells. If these factors were operating, a higher survival of cell lines from plated liquid cultures when retested on paraquat medium following non-selective growth would be expected. This expected result was observed with 85.5 percent of the cell lines tested in experiment 4 surviving retesting on paraquat medium compared to 65.4 percent surviving in experiment 1, 35.7 percent surviving in experiment 2, and 40 percent surviving in experiment 3 (Table 10). There appears to be no correlation (Table 10) between regeneration of cell lines or resistance being maintained in regenerated plantlets with the tissue type used for selection (callus or plated liquid suspension cultures).

Selection of mutagenized callus versus non-mutagenized callus. A comparison of retested calli for experiment 1 (X-ray treatment) and experiment 2 (no mutagenic treatment)

indicated that a higher percentage of cell lines expressed the mutant phenotype in experiment 1 (65.4 percent) over experiment 2 (35.7 percent) (Table 10). X-ray treatment results in higher recovery of mutant cell lines. This is in agreement with X-ray induced mutants auxotrophic for thiamine in Arabidopsis (Rédei, 1975). Widholm (1977) also stated that treatment of carrot cells with the mutagens ethylmethane sulfonate or ultraviolet light increased the frequency of resistant cell line recovery at inhibitory concentrations of 5-methyl tryptophan.

Additionally, mutagen treated cell lines exhibited a higher regeneration rate, 86.6 percent compared to 55.6 percent for untreated cell lines (Table 10). Mutagen treated cells also exhibited a higher percentage of resistant plantlets, 46.2 percent compared to 25 percent for untreated cell lines (Table 10). It appears that mutagen treatment enhances resistant mutant recovery over that for spontaneous mutations and that mutagen treatment enhances persistence of resistance in the regenerated plantlets, but it is possible to isolate paraquat resistant cell lines of tobacco with or without mutagen treatment. This is in agreement with Nabors et al. (1975) who stated that it is possible for salt-tolerant cell lines of tobacco to be rapidly developed through the selection of either induced or spontaneous mutants.

Selection of resistant cell lines at 10^{-5}M paraquat versus selection at 2 x 10^{-6}M paraquat prior to selection at 10^{-5}M paraquat. A comparison of the selection of resistant calli at 10^{-5}M paraquat (experiment 2) and the treatment of calli at a sublethal paraquat level of 2 x 10^{-6}M prior to selection at 10^{-5}M paraquat (experiment 3) indicated that sublethal selection procedures did not increase the recovery of resistant phenotypes. Selection at 10^{-5}M paraquat gave 35.7 percent survival (experiment 2) and selection at 2 x 10^{-6}M paraquat prior to selection at 10^{-5}M paraquat gave 40 percent survival (experiment 3). Regeneration of selected tissues was somewhat higher in experiment 3 (selection at 2 x 10^{-6}M paraquat prior to selection at 10^{-5}M paraquat) with 72.7 percent of the tissues tested showing regeneration as compared to 55.6 percent showing regeneration in experiment 2 (selection at 10^{-5}M paraquat) but the results of testing regenerated plantlets for resistance was similar for the two experiments with 25 percent of the tested cell lines exhibiting resistance in experiment 2 and 33.3 percent of the tested cell lines exhibiting resistance in experiment 3 (Table 10).

Sublethal paraquat selection at 2 x 10^{-6}M prior to selection at 10^{-5}M did not enhance the production of resistant cell lines or plants of tobacco even though sublethal selection procedures have been successful in other cases. Experiments to isolate pathotoxin resistant cell

lines of Zea mays using callus tissue resulted in regenerated plants which did not exhibit toxin resistance after four cycles of selection but after five cycles of selection, the regenerated plants exhibited resistance (Gengenbach et al., 1977). Additionally, Mastrangelo and Smith (1977) reported that resistance to aminopterin was maintained in regenerated plants from Datura cells which were cultured at three increasing concentrations of the toxin; however, the process was very time consuming and may encourage selection of multiple site mutants or cell lines may gradually acclimate to the toxin so that physiological variants rather than true genetic mutants are produced. It is possible that increasing the number of levels of paraquat for selection or that increasing the number of cycles (subcultures) on one paraquat level would have resulted in higher percentages of plants expressing the resistant phenotype.

Genetic Versus Epigenetic Resistance

Maintenance of the resistant phenotype in the absence of selective pressure in a cell line is considered to be a strong indication that a true genetic change has occurred to confer the resistant phenotype (Mastrangelo and Smith, 1977). Retesting of tissues by exposure to selective pressures following non-selective growth should eliminate cell lines where epigenetic effects such as extensive

cross feeding between resistant and sensitive cells during selection, altered gene expression, or habituation to paraquat may have conferred temporary resistance. Only true nuclear mutants should survive retesting but it is possible that a few epigenetic variants may survive. Chaleff and Parsons (1978) demonstrated that stability of a variant phenotype in the absence of selection pressures is not always an adequate criterion for distinguishing between genetic and epigenetic events.

When resistance is transmitted from cell to cell in culture but abolished when plants are regenerated from the cells, it may be an example of a surviving epigenetic variant and not a true nuclear mutant (Márton and Maliga, 1975). This may have occurred in our experiments since percentages of resistance in retested plantlets were lower in all experiments (1, 2, 3, and 4; Table 10, page 73) as compared to percentages of resistance of retested callus tissue from which leaves were regenerated. Another possibility for lower percentages of mutant phenotype expression in regenerated plantlets versus callus tissue from which plantlets were derived was that genes conferring the resistant phenotype may be active in callus tissue but may not function in differentiated plantlet tissue. Additionally, metabolic pathways conferring resistance to paraquat may differ between callus and leaf tissue. Chaleff and Parsons (1978) state that it cannot be assumed that a trait

selected for in undifferentiated tissues will be expressed in differentiated cells that constitute the intact plant.

Variation in the response to paraquat between replicate leaf testings such as in cell lines 1:22, 3:6, and 4:120 (Table 8, page 60) may be explained by the formation of shoot initials from chimeric callus. Márton and Maliga (1975) suggest that mosaic tissue may explain the appearance of sensitive sublines from BUDR resistant cell lines of tobacco. If regeneration of plantlets had been carried out on medium containing paraquat, cell lines chimeric for sensitive and resistant cells may have been selected against; however, the total number of regenerating cell lines may have been less. Mastrangelo and Smith (1977) report regeneration of aminopterin resistant cell lines of Datura on medium containing aminopterin and not containing aminopterin. The frequency of regeneration was much lower on medium with aminopterin but the resistant phenotype was not retained unless aminopterin was present in the regeneration medium. Plantlets regenerated in medium containing paraquat should be true nuclear mutants expressing the mutant phenotype but this procedure would lead to the loss of other nuclear mutations expressed only in callus tissue.

If a resistant trait can be carried from cell culture to plants and back into culture, the indications are that the trait is stable (Widholm, 1974). Resistance was exhibited by callus cultures derived from regenerated plants

in cell lines 1:9, 1:14, 1:19, 1:21, 1:22, 1:23, 1:26, 2:8, 3:2, 3:33, 4:84, 4:119, and 4:120 (Table 5, page 43). All other cell lines were not tested. Similar responses have been observed for 5-methyl tryptophan resistant cell lines of carrot in petiole suspension cultures (Widholm, 1974), for a streptomycin resistant cell line of tobacco in leaf derived callus (Maliga et al., 1973b), for a carboxin resistant cell line of tobacco in plant derived callus (Polacco and Polacco, 1977), for the herbicide isopropyl N-phenyl carbamate resistant cell line of tobacco by mesophyll protoplasts (Aviv and Galun, 1977), and for the herbicide picloram resistant cell lines of tobacco by leaf derived callus and by germinating seeds (Chaleff and Parsons, 1978). Cell lines 1:9, 1:14, 1:23, 1:26, 2:8, 3:2, 3:33, 4:84, and 4:119 in which regenerated leaves did not exhibit resistance but leaf derived callus did exhibit growth on paraquat medium may be representative of alterations of gene expression between undifferentiated callus tissue and differentiated leaves.

Phenotypic Alterations in Resistant Cell Lines

Some resistant cell lines exhibit additional phenotypic characteristics such as slower callus growth rate, morphologically abnormal shoot regeneration (P; Table 5; Table 10, page 73), or no shoot development (B; Table 5, Table 10). Mastrangelo and Smith (1977) state that

independently isolated resistant cell lines of Datura to aminopterin differed with respect to growth and ability to differentiate. This may be explained by more than one mutational event having occurred which may especially be true of mutagenized tissues or by pleiotropic effects of a single mutation.

Possible Protection Mechanisms against Paraquat Toxicity

Growth of tobacco callus in the presence of paraquat as well as increasing copper levels to determine the effects of copper on paraquat toxicity (Figure 3, page 65) indicated a highly significant difference in growth between paraquat levels and between copper levels. The interaction of paraquat and copper was also highly significant. Copper and paraquat are toxic at 4×10^{-4}M and 10^{-5}M, respectively, but at 0.2×10^{-4}M copper and 10^{-6}M paraquat there is a synergistic effect resulting in enhanced growth over that expected for paraquat and copper. A similar effect was observed at 0.2×10^{-4}M copper and 10^{-5}M paraquat. It is possible that additional copper in the growth medium lead to increased amounts of cytosol eukaryotic superoxide dismutase which may have provided a defense against the reactivity of superoxide radicals created by paraquat treatment.

Growth of tobacco callus in the presence of paraquat as well as various iron levels to determine the effects of iron on paraquat toxicity (Figure 4, page 68) indicated a

highly significant difference in growth between paraquat levels but iron treatment was not significant. In no case did iron protect the cell against paraquat toxicity. The cell either did not increase levels of catalases or peroxidases or these enzymes are not especially active in protecting against paraquat toxicity.

Conclusions

Successful regeneration of intact plants from the herbicide resistant cell lines and expression of selected traits in the regenerated plant are essential for using tissue culture techniques for plant improvement. Successful regeneration and maintaining of resistance in the regenerated plant was evident in experiments 1, 2, 3, and 4 (Table 10, page 73).

The effect of the herbicide paraquat on tobacco callus was a marked decrease in growth at 10^{-5}M and total inhibition at 10^{-4}M. Both callus tissue and plating of cell suspension cultures were successful means of isolating paraquat resistant cell lines although a higher percentage of cell lines exhibiting continued resistance were isolated using plated liquid cultures (experiment 2 versus experiment 4, Table 10). The use of X-rays as a mutagenic agent was a successful means of acquiring resistant cell lines over spontaneous mutations conferring resistance (experiment 1 versus experiment 2, Table 10). Sublethal selection at 5×10^{-6}M paraquat prior to selection at 10^{-5}M paraquat

resulted in approximately the same percentage of resistant cell line recovery as selection only at 10^{-5}M paraquat (experiment 3 versus experiment 2, Table 10, page 73). All resistant cell lines recovered from experiments 1, 2, 3, and 4 were able to grow at 10^{-4}M paraquat, a level which kills non-resistant cell lines.

It was observed that increased copper in the growth medium could enhance growth of tobacco callus on paraquat medium over that expected for copper and paraquat possibly due to increased levels of the enzyme superoxide dismutase. Increased iron in the growth medium did not enhance growth of tobacco callus on paraquat medium.

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