# DISSERTATION

## ASSESSMENT OF THE ABATEMENT OF PESTICIDE MUTAGENESIS IN SITU BY A CORN/SOYBEAN INTEGRATED PEST MANAGEMENT PROGRAM

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# ASSESSMENT OF THE ABATEMENT OF PESTICIDE MUTAGENESIS *IN SITU* BY A CORN/SOYBEAN INTEGRATED PEST MANAGEMENT PROGRAM



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# 1. Pesticides as Environmental Genotoxicants - Some Health and Ecological Implications

Due to their very role as bioactive chemicals, pesticides tend to form electrophilic metabolites capable of alkylating biologic macromolecules. Preferred sites of action include nucleophilic oxygen as well as nitrogen atoms, both of which are abundant in DNA, predisposing the genetic material to mutagenic covalent binding (Hodgson and Levi, 1987, p.151). The electrophilic nature of numerous pesticides enhances the rate of their removal from the environment by degradation and dispersion processes. Consequently, exposure of the general public to agricultural pesticides is normally low (Crosby, 1982; Teshke et al., 1993).

The main deleterious effect resulting from exposure of human populations to environmental mutagens, such as pesticides, is the possible initiation of cancer. Mortality from all causes among farmers tends to be lower than that of the general population, a trend that might be linked to selective bias in sampling (healthy worker effect and life style differences). Epidemiological studies, however, provide evidence that several types of tumors and other carcinogenic manifestations are in excess among farmers and other occupational groups associated with pesticide handling (Blair, 1982; Brown et al., 1990; Godon et al., 1991; Hansen et al., 1992). Specifically, cancers of the lymphatic and hematopoietic systems, lip, kidney, colon, skin, lungs, brain, stomach and prostate, as well as leukemia, Hodgkin's disease, non-Hodgkin's lymphoma, and multiple myeloma, among others, have been shown to be significantly higher among farmers and orchardists. In addition, immunologic deficiencies, deterioration of neurologic functions, Parkinson's disease, and various reproductive and developmental anomalies have also been associated with

pesticide exposure (Blair, 1982; Sharpe et al., 1995; Weisenburger, 1993). Many of these relationships, however, are somewhat inconsistent, with some studies even describing decreased incidence in some of these effects on certain occasions (Blair, 1982; Weisenburger, 1993).

An extensive review of the health effects in humans from long-term exposure to pesticides was published recently (Maroni and Fait, 1993). From examination of the epidemiologic data (animal and acute effect studies were excluded) available in the literature between 1975 and 1991, the authors concluded that there was no consistent evidence of a difference in global cancer mortality for pesticide-exposed groups in relation to the general population. Increases in chromosome aberrations and sister-chromatid exchanges, however, were reported for pesticide-exposed groups, pointing out a generic genetic activity of pesticides in humans (Maroni and Fait, 1993).

A few have argued that the vast bulk of the potentially carcinogenic chemicals to which the general population is exposed through the diet is of natural origin (Ames et al., 1990a). It has been reasoned also that the inducible metabolic defense mechanisms responsible for eliminating these natural chemicals would be effective in handling the small doses of synthetic pesticides (Ames et al., 1990b). Several authors, however, dispute these assertions on the basis that, in relation to genotoxicity and carcinogenesis, the real question is not one of dose, but rather of the increased risk of adding mutagens to the pool of potentially mutagenic chemicals already occurring naturally (Culliney et al., 1992; Maugh II, 1978). Explicitly, this argument means that the presently deficient knowledge about the relationships between dose and effect in the genotoxicity (and carcinogenicity) arena suggests that a safe dose for a mutagen may be an untenable concept (Culliney et al., 1992; Marx, 1990; Maugh II, 1978).

Another complicating issue regarding the safety, or lack thereof, of pesticides and other environmental mutagens is the poor understanding of the health hazards implicated with an increase in the rate of mutation in a large population such as of humans (Mohrenweiser, 1994; Sutton, 1975; Trimble and Smith, 1977). These uncertainties in the relationship between dose and effect, with the resultant difficulty in defining a safe dose, coupled with the incertitude regarding the extension of the consequences on the human population, make it very unlikely that generalized schemes can be devised to protect against mutagens. They must be avoided in the first place (Drake et al., 1975).

In order to pursue this goal, there is a need for screening systems that are sensitive and able to detect the whole mutagenic spectrum. Such systems are defined as type II mutagenesis tests (Ennever et al., 1988), or those presenting high sensitivity and low specificity. Even though such systems may yield false positive results, they are the most appropriate for risk averse screening programs, because false negative results are unlikely. Most plant bioassays are considered to be type II tests, with special reference to the *Tradescantia* micronucleus (cytogenetic) and stamen hair mutation, and the corn waxy pollen assays (Ennever et al., 1988).

In addition to the risks to human health posed by environmental mutagens, there are important ecological risks as well. Sharma (1987) denounced the threat posed by pesticides to the stability of the ecosystems through the cumulative introduction of deleterious mutations into the genetic pool. Indeed, it has been demonstrated that pesticides and other environmental genotoxicants are capable of altering the genetic makeup of some natural populations. For instance, a six-fold increase in mutation frequency was observed in a population of ferns growing on the flood plains of a heavily PCB-contaminated river (Housatonic River) when

compared to populations from uncontaminated control areas (Klekowski, 1982). Even though the ecologic fitness of the affected populations was not evaluated the plants were morphologically aberrant.

The frequency of mitotic chromosome aberrations has been shown to increase in several weed species growing in herbicide-sprayed fields (Tomkins and Grant, 1976). Auxin-type phenoxyacetic herbicides induced a large proportion of lagging chromosomes, and a triazine (simazine) produced multipolar spindles, which may result in polyploidy (Grant, 1982). The genotoxic events observed in this study were unevenly distributed throughout the year, with increases in the frequency of chromosome aberrations following the spraying season. Such a pattern was noted as coincident with the occurrence of chromosome aberrations in occupationally exposed agricultural workers (Rupa et al., 1991a, b; See et al., 1990; Yoder et al., 1973).

Grant (1972) speculated that, as strong mutagens, some pesticides could interfere with the evolutionary trends of natural fauna and flora. Three main mechanisms could come into play: (1) an increased resistance in certain species (due to selection), (2) the elimination of certain susceptible species (influencing the course of selection), and (3) the origination of morphological differences of a nature sufficient for taxonomic recognition (for example, through polyploidy). The emergence (through selection) of differentiated populations under situations of environmental stress, such as under pesticide pressure, is primarily dependent on the existing genetic variation of that population (Bradshaw and McNeilly, 1991). The pace of change, for instance development of resistance to pesticides, is directly proportional to the severity of the selection pressure, given that the appropriate adaptive character is present.

It has been hypothesized, however, that the adaptive challenges posed by environmental stresses dramatically enhance the mutation rate, by inducing *de novo* adaptive variation (McDonald, 1983). The molecular basis for such shifts in mutation rate is not clearly understood at present, but some effective mechanisms were described as early as 1944 with the discovery of transposable elements in corn (McClintock, 1984).

Actually, the utilization of chemical and physical mutagens to cause an increase in rate of mutation for the induction and subsequent selection of desirable traits is not a new concept in plant breeding, especially in relation to development of resistance to herbicides. In an early attempt, wheat and tomato seeds were soaked in the alkylating agent ethyl methanesulfonate (EMS) and the subsequent progeny were grown under herbicide pressure (Pinthus et al., 1972). The mutagen-treated progeny lines provided many resistant seedlings in both plant species, while the progeny of untreated plants provided none. It was concluded that increased resistance of crops to herbicides could be obtained by selection from mutagenically-treated populations (Pinthus et al., 1972).

Soybean varieties resistant to a wide range of sulfonylurea herbicides have been selected following seed mutagenesis with ethylnitrosourea (Sebastian and Chaleff, 1987), N-nitroso-N-methylurea, and EMS (Sebastian et al., 1989). Biochemical studies indicated that resistance was afforded by a reduction in the sensitivity of a single enzyme, and that the mutation was monogenic and semidominant. It should be emphasized that mutants were selected from the $F_1$ generation, after one single treatment of the $M_1$ seeds with the mutagens (Sebastian et al., 1989). A partially dominant mutation for herbicide tolerance was isolated also in corn, following exposure of tissue cultures to inhibitory concentrations of herbicide. Selected mutants were more than 40-fold more

tolerant to one herbicide than the wild-type cultures (Parker et al., 1990). Similar results are commonly found in the literature (Estelle and Somerville, 1987; Hattori et al., 1992; Heim et al., 1989; Marshall et al., 1992; Mourad et al., 1993).

It has been shown that a single nuclear mutation in *Arabidopsis thaliana* was responsible for a 300-fold increase in resistance to a herbicide (Haughn and Somerville, 1986). The mutant-isolated enzyme (acetohydroxy acid synthase), effected by an imidazolinone herbicide in this particular case, was 1000-fold more resistant to inhibition than that of the wild-type plants (Haughn and Somerville, 1990). Through DNA sequence analysis, it was shown that the herbicide-resistant mutants differed from the wild-type plants by only a single base pair, a G-to-A transition, predicting a proline-to-serine substitution in the amino acid sequence (Haughn and Somerville, 1987). This same transition was responsible also for resistance in yeasts.

The potential increase in mutation rate and selection pressure to which plants are subjected in pesticide-sprayed fields, coupled with the possibility that single mutations confer resistance to herbicides in plants (and possibly also in other organisms), may have direct implications to the very effectiveness of pesticides (Jasieniuk and Maxwell, 1994). Even though resistance development in plants tends to be a slower process than in microbes and insects, due to populational processes (Gressel, 1978; Jasieniuk and Maxwell, 1994), the introduction of adaptive variation into the genetic pool of weeds may have meaningful consequences. In other words, exposure to mutagenic pesticides may cause weeds growing in agricultural fields to accumulate mutations, hence genetic variability. In such a situation, an increased rate of resistance development could occur as a result of the mutagenic action of the same herbicides exerting selection

pressure in the weed populations of agricultural fields (Fuerst et al., 1986; Jasieniuk and Maxwell, 1994; Whitham and Slobodchikoff, 1981).

The subject of the present study originates from a large proportion of the pesticides presently used in agriculture being plant mutagens in laboratory tests (Sharma and Panneerselvan, 1990). The fundamental question concerns the possible occurrence of pesticide genotoxic activity under field conditions consequent to recommended usage rates and patterns. If mutagenesis can occur under these conditions, an increased mutation rate could be induced in the plant populations of agricultural fields, potentially interfering with the pace of genetic change. The central hypothesis may be enunciated as: Plants exposed *in situ* to pesticides and pesticide residues are subjected to a higher rate of mutation than non-exposed plants. This hypothesis was tested by comparing the genotoxic outcome of four mutagen-sensitive plant bioindicators exposed *in situ* to some commonly used pesticides.

A second inquiry concerns the possibility of lessening the induction of mutations by restricting the exposure rate of plants to pesticides. Even though apparently simple, this question collides with complex dose-response relationships observed in genotoxicity studies. This was investigated through a comparison of the genotoxic outcome of the same four plant bioindicators after *in situ* exposure in fields that received three different pest management programs. An hypothesis may be enunciated as follows: A reduction in pesticide dose will result in a proportional abatement in mutagenesis.

In summary, natural and anthropogenic stresses have been demonstrated to alter the extent and incidence of mutations in crops. These mutations can be of ecological, agronomic, and economic importance. In the present research the induction of mutations and other genotoxic events were assessed with four plant

bioassays *in situ* in a pesticide-managed corn/soybean agricultural field. Also, an integrated pest management program (IPM) utilizing a reduced pesticide application rate was evaluated in terms of abatement of genotoxicity. Finally, the research contributes an evaluation of the genotoxicity occurring in an agricultural environment that has been exposed to recommended pesticide application, utilizing four very sensitive test systems.

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## 2. Assessment of Genotoxicity in an IPM Program for Corn/Soybean with the *Tradescantia* Micronucleus Assay

### 2.1. *Tradescantia* and the Trad-MCN Assay

Since early studies on the genetic activity of chemical and physical agents, several species and clones of the genus *Tradescantia* have been used as experimental organisms, by virtue of a series of favorable genetic characteristics. Bearing just six pairs of large, easily observable chromosomes, cells from almost every part of the plant, from the root tips to the developing pollen tube, yield excellent material for cytogenetic studies (Ma and Grant, 1982).

As a consequence of the intensive use of *Tradescantia* in genetic studies, a series of genetic characteristics have been found that offer opportunities for the detection of agents affecting the stability of the genome. At least four such characteristics have been selected as endpoints for the establishment of assays to evaluate genotoxicity. Two of these, root-tip mitosis and pollen-tube mitosis, are chromosome aberration assays wherein one observes and evaluates the visible deformities in the chromosomes (Ma, 1982). A third, the stamen-hair mutation assay (Underbrink et al., 1973) is a point mutation mitotic assay based on the expression of a recessive gene for flower color in heterozygous plants and will be discussed in a later chapter. The fourth assay is a cytogenetic test based on the formation of micronuclei (Trad-MCN) which result from chromosome breakage in the meiotic pollen mother cells (Ma, 1979b). This assay has been chosen here for the complete (*in situ*, soil extract, and commercial formulation analyses) assessment of the genotoxicity of pesticides applied in a corn/soybean integrated pest management (IPM) program. The choice of this system for this detailed analysis of pesticide genotoxicity was due not only to its versatile amenability to

*in situ* as well as *in vivo* laboratory exposures, but also to its extraordinary sensitivity (Ma et al., 1982).

The present chapter examines the characteristics and fundamentals of the Trad-MCN assay and reviews the results obtained to date with this system in the assessment of environmental genotoxicants. Additionally, this chapter presents an assessment of the experiments conducted to evaluate the potetialeffects that an IPM program could provide for the abatement of the genotoxicity of pesticides.

## 2.2. Trad-MCN: Fundamentals and Development of the System

Studies of the *Tradescantia* genome began with the pioneering work of Sax and Edmonds (1933) on the development of the male gametophyte of *T. reflexa* Raf. The thorough description of the several developmental phases of the microspore and the determination of the length and timing of the meiotic events during such development were responsible for the initiation of research on chromosomal aberration.

Important observations were made in studies of the effects of X-rays on microspores of that species (Sax, 1938). Firstly, it was determined that meiotic chromosomes were more susceptible to breakage than mitotic chromosomes; more importantly, dividing chromosomes were at least ten times more susceptible than resting ones. Secondly, breaks were not randomly distributed along the chromosomes. Loci positioned closer to the centromeres were more likely to suffer the splitting effects of radiation. These observations led to the conclusion that coiling of the chromosomes during replication, with the consequent mechanical strain involved, would strongly influence susceptibility to mutational events. These inferences were later confirmed in a study of the effects of $^{60}$Co-gamma radiation on *T. paludosa* And. and Woods.(Sparrow and Singleton, 1953).

The concepts of temporality and sensitivity are important for the selection of bioindicators of mutagenicity, for synchrony in cell development and timely recovery periods are two decisive factors in the performance of bioassays.

The greater susceptibility of meiotic as compared to mitotic chromosomes was confirmed in a study of the influence of lack of oxygen on meiosis of *T. paludosa* (Steinitz, 1944). This research represented the first attempt to use micronuclei of the pollen mother cells as a direct indicator of chromosome fragmentation. A spontaneous level of 0.87% cells with micronucleus was defined for *T. paludosa*, a rate that rose to 8.0% for cells under anaerobic conditions at the early stages of prophase. The results corroborated the earlier findings of Sax (1938), indicating that prophase, especially pachytene and diplotene, was the most susceptible stage of meiosis.

The pace of the meiotic stages was characterized further in a study of the differentiation of excised anthers of *T. paludosa* (Taylor, 1950). Approximately 24 h elapsed during the development of the meiotic nucleus from pachytene to early tetrad. This result proved valuable for the definition of the appropriate recovery period required between exposure of the prophase nucleus in the developing inflorescence of *Tradescantia* and fixation of the material for tetrad analysis.

The growing interest during the 1950's in the radiomimetic (principally genotoxic) capabilities of chemical substances suggested the utilization of *Tradescantia* as a bioindicator. A pollen tube mitosis assay was first used in a comparative study of simple chemical agents in *T. paludosa* (Smith and Lofty, 1954). Ethylene oxide (a known effective mutagen), ketene (a compound giving conflicting results), and methyl chloride (an alkylating agent of low potency) were compared for inducing chromatid breaks and chromosome erosions and

contractions. The pollen tube assay proved effective in detecting genotoxicity, as the results revealed that the more active compounds, ethylene oxide and ketene, caused more extensive and numerous chromosome aberrations of all types. Possibly due to its postulated faster penetration into the cells, methyl chloride was effective in causing chromosome breaks. The propitious selection of chemicals in this early research proved instrumental in demonstrating the sensitivity of *Tradescantia* and showing its capacity for precisely differentiating nearly comparable effects.

In a series of papers concerning the role of selected nutrients on meiosis, the production of micronuclei in the microspores of *T. paludosa* was cited as indicative of chromosome breakage (Steffensen, 1953, 1954, 1955). When studying the effects of magnesium (Mg) deficiency on meiosis, the author noted the greater sensitivity of the microspores as compared to root tips, supporting previous evidence of greater susceptibility of meiotic than mitotic cells. Micronuclei were more numerous in the microspores of plants deficient in Mg, calcium (Ca), and sulfur (S). It was pointed out that the first two nutrients were responsible for bonding with macromolecules in the nucleus, contributing to the stability of proteins and DNA. This was demonstrated further by increased susceptibility to X-rays of plants grown on Ca-deficient media, because of the relationship of Ca with sulfhydryl groups in nuclear division, particularly in spindle formation. A spontaneous micronuclei frequency of 0.84% was recorded and increased to 3.89% in plants grown under suboptimal Ca supply (Steffensen, 1955). This corroborated earlier observations on micronuclei production in *T. paludosa* (Steinitz, 1944).

More than 30 years after Steinitz's observation of micronuclei for the detection of chromosome damage in meiosis, Ma and coworkers (Ma et al., 1978)

at Brookhaven National Laboratory devised the Micronucleus-in-Tetrad Assay for Environmental Mutagenesis (later referred to as the Trad-MCN assay). Employing the hybrid clone 4430 (*T. hirsutiflora* Bush x *T. subacaulis* Bush) they compared the production of micronuclei in the pollen mother cells with the mutation for pink cells in the stamen hairs of *Tradescantia* exposed to the known mutagen 1,2-dibromoethane (DBE).

By that time, the stamen hair mutation assay (Underbrink et al., 1973) had been extensively applied and was a well-recognized test for radiobiological and chemical mutagenesis. The micronucleus assay, however, exhibited an efficiency approximately 36 times as great. This extraordinary sensitivity was credited to the much smaller specificity of the damage needed to produce a micronucleus as compared to a pink mutation. Indeed, it could be assumed that numerous sites in any of the 12 chromosomes of *Tradescantia* were subjected to damages that would result in a chromosome breakage, hence, in micronuclei. By contrast, only one locus in one chromosome could bear the mutation for pink pigmentation in the cells of the stamen hair (Ma et al., 1978).

The great sensitivity and simplicity of the Trad-MCN assay was demonstrated further in experiments in which X-rays at a low dose were compared with two well-known chemical mutagens, EMS and sodium azide ($NaN_3$) in both liquid and gaseous forms (Ma, 1979a). A low dose of X-rays induced high frequencies of micronuclei, i.e., 23 MCN/100 tetrads at a 20-rad X-ray exposure level, as compared with 1.8 MCN/100 cells of human lymphocytes at 50-rad of X-rays (Countryman and Heddle, 1976), or 2.5 MCN/100 cells of mouse erythroblasts of bone-marrow culture at 35-rad of X-rays (Janssen and Ramel, 1976). Whereas there were 0.2% pink mutations per rad in the stamen hair assay (Trad-STH), there were 1.6% MCN per rad for the Trad-MCN assay. The dose-

response relationship of the Trad-MCN assay to X-rays gave a correlation coefficient of 0.99. The results obtained for the chemical agents verified these findings, both in relation to sensitivity and the dose-response relationships (Ma, 1979a).

One additional advantage of the Trad-MCN assay was the short-term exposure needed for completion of a test - only 6 hrs, followed by a 24-hr recovery period to allow the cells treated at prophase to reach the scorable tetrad stage. This meiotic timetable was tested in a study of the stage of sensitivity using X-ray exposure of *T. paludosa* (Ma et al., 1980). Groups of plant cuttings received a single 35-rad X-ray exposure, after which inflorescences were removed and fixed at 3-hr intervals for 48 hrs post-exposure. A peak of sensitivity occurred after about 24 hrs post-irradiation, agreeing with the observations of Taylor (1950). A second peak appeared at about 39 hrs post-irradiation, suggesting that the earlier prophase I and/or pre-meiotic stages are also very sensitive.

The utilization of the Trad-MCN assay for *in situ* monitoring of environmental clastogens was proposed after studies involving promutagens (benzo-α-pyrene) and polluted sites (Ma, 1979b; Ma, 1981). No external enzymatic activation was needed, because the enzymatic apparatus was fully functional in the exposed plant cuttings.

A series of limitations of the Trad-MCN assay has been presented. The test obviously provides only a relative index of genetic damage. Translocations, inversions, and other types of chromosome and chromatid rearrangements and exchanges are not revealed as micronuclei. No carcinogenicity information may be easily extrapolated from the frequencies of micronuclei, and the metabolic pathways of mutagenic and promutagenic agents may be quite different in *Tradescantia* and other subjects (specially mammalian species). Also, the high

sensitivity of the system results in day-to-day variation in spontaneous micronuclei frequencies, requiring careful control of experimental conditions and simultaneous control samples (Ma, 1981).

One additional disadvantage of the Trad-MCN test is the labor-intensive and time-consuming procedure for micronucleus scoring in the tetrads (Ma, 1990). In order to overcome this limitation and to facilitate and standardize the scoring process, a micronucleus image analysis system was devised (Ma et al., 1992b). The computerized system scoring speed was 3.5 times faster than manual observation, with a 90% congruity in the frequencies scored.

A relatively recent review of the *in situ* monitoring of environmental clastogens (Ma, 1990) revealed that through 1990 about 300 tests had been conducted with the Trad-MCN assay in a variety of categories. About 50% of these tests exhibited genotoxicity. Contaminated water and soil samples resulted in about 60% positive tests. In the next section, some of these results are presented and discussed, so that the results of the experiments to be described later can be put into perspective.

## 2.3. The Trad-MCN Assay as a Monitoring System for Environmental Genotoxicity - A Review of the Literature

### *2.3.1. Air Pollution*

*T. paludosa* was exposed at several polluted sites in Illinois, and to gaseous agents commonly found in polluted atmospheres in a combination of *in situ* and *in vivo* laboratory tests (Ma et al., 1982). *In situ* assays carried out in public parking garages revealed a correlation between the rate of micronuclei production and the volume of traffic, with a positive dose-response (time of exposure, from 2 to

6 hrs) relationship ($\alpha$=0.01). A series of *in situ* exposures of *T. paludosa* and of clone 4430 to industrial sites, farms, and laboratory atmospheres resulted in positive results ($\alpha$=0.01) in most categories. Atmospheres of an office, a livestock farm, and a residential area gave negative results. A high frequency of micronuclei occurred consistently at sites exposed to agricultural chemical emissions (Ma et al., 1982).

*Tradescantia* plants were fumigated with the atmospheric pollutants $NO_2$, $SO_2$, and $O_3$, as well as gaseous hydrazoic acid ($HN_3$) and EMS (Ma et al., 1982). With the exception of $O_3$, all agents proved clastogenic to *Tradescantia*. Both $NO_2$ and $SO_2$ required long exposure times (24 and 22 hrs, respectively), while $HN_3$ and EMS gave positive results after 6-hr exposures.

As a consequence of its versatility, *Tradescantia* was proposed as a monitor of indoor pollution. Several studies have assessed the proficiency of the Trad-MCN assay for the low level of contaminants customarily present in home environments. Among the positive responses found are several common commercial air fresheners, tobacco smoke, *p*-dichlorobenzene (moth balls) and other insecticides listed for residential use, and diesel exhaust gases (Ma and Harris, 1987a, b).

A somewhat unusual group of atmospheric pollutants evaluated *in situ* for clastogenicity with the Trad-MCN assay were the chemical smokes employed by the U.S. Army. These tests also involved other assays including chromosome aberrations and sister chromatid exchanges in a native rodent (Schaeffer et al., 1987). The smokes were generated from fogoil, tank diesel, and hexachloroethane. All of them induced a higher rate of genotoxic events for at least one dose when compared to the controls in the Trad-MCN assay ($\alpha$=0.1). The production of chromosome aberrations in rodents was depressed. There was a higher degree of

variability (expressed as larger standard deviations from the mean micronuclei production) for all *in situ* treatments relative to controls. Even though this statistical effect was discussed as merely obscuring the dose-dependence of the results, it might be indicative of a characteristic of the *Tradescantia* system. The smaller buds of a young inflorescence are hidden beneath larger buds and leaves which may result in their being protected from unrestricted exposure, especially in atmospheric exposures, an effect anticipated by Ma (1979b).

In an *in situ* study with *Tradescantia* clone 4430 in Mexico (Ruiz et al., 1992), the micronuclei frequency peaks for a heavily industrialized, a residential, and a mixed occupation area were evaluated. Plants exposed to the industrial site always showed higher levels of micronuclei than the controls ($\alpha$=0.01) throughout the year, while the residential area samples tended to be higher than the controls only in specific months. The Trad-MCN assay gave positive results in assessing the genotoxic risks posed by gaseous emissions from a municipal waste incinerator (Ma et al., 1993b) and from a landfill vent pipe (Ma et al., 1993a).

From the studies referred to earlier, it may be concluded that the Trad-MCN is well-suited for assessing atmospheric contamination, whether from heavily polluted industrial or urban areas or under diurnal indoor environmental conditions. Weather conditions such as variations in wind speed and direction normally led to relatively high levels of statistical variability in the data.

A summary of the results obtained with *Tradescantia* in assessments of atmospheric pollution and gaseous agents is presented in Table 2.1.

Table 2.1- Summary of environmental genotoxicity results using chromosome aberrations in *Tradescantia*, with special reference to the Trad-MCN assay of polluted atmospheres and individual atmospheric pollutants.

| Agent | Dose range | Result | | | |
|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | Reference |
| ***In situ Monitoring*** | | | | | | |
| **Air Pollution** | 4 - 6 hrs | | - | $p < .01$ | Parking garage-Chicago (IL) Clear wind from lake. | Ma et al. (1982) |
| | 1-4.5 hrs | | - | $p < .01$ | Parking garage-Decatur (IL). | " |
| | 2 - 6 hrs | | + | $p < .01$ | Parking garage-Peoria (IL). | " |
| | 2 - 4 hrs | | - | $p < .01$ | Truck and bus stop. | " |
| | 2-3 hrs | | - | $p < .01$ | Truck and bus stop. | " |
| | 2.5-5 hrs | | + | $p < .01$ | Truck and bus stop. | " |
| | 3 mo | | + | $p < .01$ | Industrial site, Granite City (IL). | " |
| | 3.5 hrs | | + | $p < .01$ | Industrial site, Granite City (IL). | " |
| | 4.5 hrs | | - | $p < .01$ | Residential area, China. | " |
| | 4 - 6 hrs | | + | $p < .01$ | Agrochemical industry site, China. | " |
| | 5 hrs | | - | $p < .01$ | Bus station, China. | " |
| | 6 hrs | | + | $p < .01$ | Rubber company, China. | " |
| | 4 hrs | | - | $p < .01$ | Office environment, China. | " |
| | 3 - 6 hrs | | + | $p < .01$ | *P*-dichlorobenzene treated herbarium, China. | " |

Table 2.1 (Continued)

| Agent | Dose range | | Result | | | Reference |
|---|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | |
| | 6 hrs | | - | $p < .01$ | Livestock farm, swine house exhaust. | " |
| Diesel exhaust fumes | 23-70 min | .3-4.2 ppm | - | $p < .01$ | Concentration measured as hydrocarbons. Exhaust generated by running engine. | " |
| | 23-70 min | 6-13 ppm | + | $p < .01$ | Concentration measured as hydrocarbons. Exhaust generated by running engine. | " |
| *Gases in chambers* | | | | | | |
| $NO_2$ | 6-24 hrs | 5.0 ppm | + | $p < .01$ | Positive for longer exposure only. | " |
| $SO_2$ | 6-22 hrs | 1.0 ppm | + | $p < .01$ | Positive for longer exposure only. | " |
| $O_3$ | 5.5 hrs | 5.0 ppm | - | $p < .01$ | Longer exposure not attempted. | " |
| $HN_3$ | 6 hrs | 136-272 ppm | + | $p < .01$ | Single application of gas, without replenishment. | " |
| EMS | 6 hrs | 1000 ppm | + | $p < .01$ | Same as above. | " |
| Benzo[α] pyrene | 6 hrs | .05-.10 mM | + | $p < .01$ | | Ma (1981) |
| 1,2-dibromo ethane | 6 hrs | 5 - 80 ppm | + | | Dose response correl. coefficient = 0.99 | Ma et al. (1978) |
| Industrial district | 6-12 hrs | | + | $p < .01$ | Seasonal variability, Mexico. | Ruiz et al. (1992) |
| Residential district | 6-12 hrs | | + | $p < .01$ | Seasonal variability, Mexico. | " |
| Mixed district | 6-12 hrs | | + | $p < .01$ | Seasonal variability, Mexico. | " |

Table 2.1 (Continued)

| Agent | Dose range: Exposure time - max | Dose range: Concentration | Result: +/- | Result: Statistical Significance | Remarks | Reference |
|---|---|---|---|---|---|---|
| *Chemical Smokes* | | | | | | |
| Fogoil | 30 min | 15 - 100 m from smoke source | + | $p < 0.1$ | Gas concentrations reported in relative terms. All distances produced positive results. | Schaeffer et al. (1987) |
| Tank diesel | 30 min | 15-100 m | + | $p < 0.1$ | Same as above. | " |
| Hexachloro ethane | 30 min | 15 - 100 m | + | $p < 0.1$ | Same as above. | " |
| Landfill gaseous emissions | 4 - 6 hrs | | + | | Positive responses in 5 of 13 monitoring trips. Gases burned at emission source. | Ma et al. (1993a) |
| Municipal incinerator | 4 - 6 hrs | 50 - 500 m from source | + | | Positive results obtained with stagnant atmosphere. | Ma et al. (1993b) |
| *Indoor pollutants* | | | | | | |
| Dry cleaning | 15 hrs | | - | $p < .05$ | Night hours. | Ma and Harris (1987a, b) |
| House | 16 hrs | | + | $p < .05$ | After carpet shampooing. | " |
| House | 17 hrs | | - | $p < .05$ | Clean air. | " |
| Pipe smoke | 24 hrs | | + | $p < .05$ | Within office room. | " |
| Tobacco smoking room | 10 hrs | | + | $p < .05$ | In a public school. | " |
| Air fresheners | 1-6 hrs | | + | | Several brands. | " |
| Formaldehyde fumes | 1-6 hrs | | + | | Positive dose-response relationship. | " |

### *2.3.2. Water Pollution*

Nearly all studies that assess the presence of mutagenic agents in natural waters must incorporate a step to concentrate possible active agents to be evaluated by bioassay or chemical analysis. This happens because of the intrinsic low mutagenicity of the putative agents most frequently present in natural waters, because of the very low concentrations at which they are found, or both.

The necessity for sample concentration was clearly illustrated in an evaluation of the probability of detecting a mutagen in natural waters with the Ames assay (Johnston and Hopke, 1980). The analysis considered a weight-dependent variable taking into account the mutagenic potency and average concentration of organic mutagens commonly found in natural waters, and the amount of chemical needed to induce a doubling in revertants in the Ames assay. Considering that (a) generally only 1-ml aliquots are assayed in a test plate, (b) organic compounds typically occur in water at µg/L concentrations, and (c) 95% of the chemicals tested so far have a doubling dose of at least 1500 µg, the average environmental sample that would permit the detection of contaminants with 95% confidence should contain about 1500 L. This means that a concentration factor of six orders of magnitude is required to reduce the volume of such a sample for testing. It was concluded that the lifetime exposure of a human to mutagens present in drinking waters could be appreciable, despite the failure to detect them in environmental samples (Johnston and Hopke, 1980).

Perhaps the most important feature of the Trad-MCN assay, as well as of certain other plant assays, is its capability to detect low level genotoxicity in either short-term *in situ* exposures or *in vivo* tests with unconcentrated water samples. This was demonstrated in a 2-yr genotoxicity study of the surface waters of Spring Lake reservoir and of the municipal drinking water obtained from the reservoir in

Macomb, IL (Ma et al., 1985). Water samples were drawn from the reservoir weekly and tested for genotoxicity and for the presence of nutrient elements and metals. The most prominent result of this study was a recurring seasonality in the expression of micronuclei frequency peaks ($\alpha$=0.05) which coincided with periods of intense precipitation and runoff to the reservoir from corn and soybean fields. Micronuclei production for the drinking water tended to follow the patterns observed for the reservoir ($\alpha$=0.05), though the peaks were lower.

In a follow-up investigation, tests were conducted with a chronic mouse-erythrocyte-micronucleus test, and additional samples of a shallow- and a medium-depth well located in the same rural area as the reservoir (Ma et al., 1987). A similar pattern of micronucleus frequency ($\alpha$=0.01) following heavy precipitation or snow thaw was found for the Trad-MCN assay, with delays matching the times supposedly required for the arrival or accumulation of mutagens at the sampling sites. Analyses of water samples for organic compounds from the shallow well showed elevated levels of methylene chloride, dichlorobromoethane , trichloroethylene, and tetrachloroethylene. The mouse assays confirmed these results, although 6-mo exposures to the mutagenic samples (as compared to 30-hr exposures for *Tradescantia*) were required for a significant ($\alpha$=0.03) increase in micronuclei frequencies.

The experiments described for the detection of genotoxicity in natural waters cannot be qualified as *in situ*, for in all cases samples were brought to the laboratory and assayed under controlled conditions. Appropriate evaluation of *in situ* mutagenicity in aquatic environments became possible after the introduction of the "aquatoon," a floating device specifically designed to hold plant material for exposure to water bodies. The aquatoon was successfully employed in an *in situ* genotoxicity study of the effluents of a pulp and paper mill on the north shore

of Lake Superior (Grant et al., 1992). The *Tradescantia* (clone 4430) micronucleus and stamen-hair-mutation assays and the *Vicia faba* L. root tip chromosomal aberration assay were performed in the creek containing the raw effluents and in the bay into which the creek emptied. The Trad-MCN and *V. faba* assays showed positive responses after 24-hr exposures at both sites ($\alpha$=0.05). There was partial agreement between the genotoxic effects and the optical density of the samples. In addition to being more sensitive, the two tests that showed the best results were much better adapted to *in situ* studies. The material could be fixed immediately after exposure, while the stamen hair mutation assay requires a long recovery period under conditions that cannot be attained in the field or during transportation.

In a study of industrial wastewater in Mexico a higher level of micronuclei production was found relative to the tap water controls ($\alpha$=0.01), even after a dilution to 1/3 strength (Ruiz et al., 1992). Seasonal variations observed in the data could not be correlated to any environmental parameter measured. One interesting aspect of these experiments was the lower than normal spontaneous frequency of micronuclei found in *Tradescantia* (0.8 to 1.5 MCN/100 tetrads). It was postulated that the high elevation and subtropical climate typical of the region could be more favorable to growth of clone 4430, due to its genetic relationship to the alpine species *T. hirsutiflora*.

The leachates of an abandoned 20 yr-old landfill were tested for genotoxicity with the Trad-MCN assay (Ma et al., 1993a). The high toxicity of the samples precluded tests with solutions diluted less than ten-fold, while genotoxicity could still be detected in 20-fold dilutions of the leachate samples.

The genotoxicity of contaminated groundwaters treated in a purification plant designed to clean one of the most important aquifers in Austria was assessed

in a series of experiments with the Trad-MCN assay (Helma et al., 1993; Helma et al., 1994).The purification methods consisted of activated charcoal filtration and UV irradiation. Samples drawn before any treatment exhibited positive, dose-dependent clastogenic effects ($\alpha$=0.05) after a 24-hr exposure. When treated in the laboratory with increasing amounts of UV light (up to 1500 J/m$^2$), these samples induced micronuclei formation in a dose-dependent fashion relative to UV applied. Results for irradiated clean tap water were negative. Chemical parameters routinely measured at the purification plant indicated that the activated charcoal-filtered samples were of drinking water quality. In many cases, however, higher micronuclei frequencies were found for these samples, before or after UV irradiation. The mechanism responsible for the effects observed was postulated to be activation of water pollutants to genotoxic compounds by UV irradiation. The enhancement of clastogenicity by UV light decreased upon storage, with an estimated half-time of approximately 1 d. The authors concluded that similar UV light treatment of waters for drinking could produce hazardous compounds that might pass undetected in the treatment plants.

Studies on water pollution demonstrate the capabilities and advantages of the Trad-MCN assay for the *in situ* assessment of environmental genotoxicants. The ability to detect biological effects in samples considered clean by most chemical standards and the absence of any requirement for tedious concentration procedures which may result in loss and chemical alteration of the compounds are undoubtedly desirable characteristics of this system.

Table 2.2 presents a summary of results obtained with *Tradescantia* in the evaluation of water pollutants.

Table 2.2- Summary of environmental genotoxicity results using chromosome aberrations in *Tradescantia*, with special reference to the Trad-MCN assay of water pollutants.

| Agent | Dose range | | Result | | | |
|---|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | Reference |
| **Water Pollution** | | | | | | |
| Drinking water | 30 hrs | | + | $p < .05$ | Both lake and tap water produced peaks in MCN frequency following rainy season. | Ma et al. (1985) |
| Drinking water | 30 hrs | | + | | Lake, shallow- and medium-depth well waters analyzed. MCN frequency increased following rainy season. | Ma et al. (1987) |
| *In situ* exposure to Lake water | 24 hrs | | + | $p < .05$ | Lake Superior and inlet polluted by pulp and paper mill effluent. | Grant et al. (1992) |
| Wastewater | 30 hrs | 3-fold dilution | + | $p < .01$ | Industrial effluents present. Positive responses over entire year. | Ruiz et al. (1992) |
| Landfill leachates | | 20-fold dilution | + | | | Ma et al. (1993a) |
| Groundwater | 30 hrs | 3-fold dilution | + | $p < .05$ | PAH-contaminated groundwater. UV treatment increased clastogenicity. | Helma et al. (1993), Helma et al. (1994) |

### *2.3.3. Soil Contaminants and Soil Amendments*

Several studies have evaluated the mutagenicity of soils *in situ*, of extracts of soils from contaminated sites before and after the application of remediation measures, and of soil amendment materials themselves. Perhaps the most voluminous soil amendment used throughout the world is municipal sewage sludge. Generally, the impact of this material on the environment has been judged by its heavy metal contamination (L'Hermite and Dehandtschttler, 1980). However, complex organic compounds are sometimes introduced into the sewage treatment systems. The possible mutagenicity of such sludges was evaluated in Chicago, utilizing two higher plant assays (*Zea mays* L. and *Tradescantia*) and two strains of *Salmonella* in the histidine reversion test (Hopke et al., 1982). Laboratory tests with *T. paludosa*, showed that sludge dilutions above 1:4 showed increased micronuclei frequency. This result was in agreement with results obtained for other species, as will be discussed in more detail in a forthcoming section on *Z. mays* assays.

The clastogenicity of several chemicals commonly found in hazardous waste sites were evaluated in a series of experiments aimed at elucidating the possible synergistic or antagonistic behavior of chemical mixtures in the Trad-MCN assay (Sandhu et al., 1989). Initially, seven chemicals selected from the U.S. EPA Superfund Priority 1 chemical list (Waters et al., 1987) were tested to determine their minimum effective doses (MED). Five of seven chemicals tested produced positive results ($\alpha$=0.05) and could be ranked in descending order of potency according to their MED in the Trad-MCN as follows: lead tetraacetate in DMSO (0.4 ppm), heptachlor in DMSO (2.0 ppm), dieldrin in DMSO (3.8 ppm), arsenic trioxide in NaOH (4.0 ppm), and 1,2-benz[*a*,*h*]anthracene in ethanol (12.50 ppm). Both tetrachloroethylene (TCE) and aldrin were immiscible with

water, precluding adequate exposure in solution. When exposed in the gaseous state at 30 ppm for 2 hrs, TCE gave a positive response ($\alpha$=0.05) but aldrin did not.

Armed with these data, Ma et al. (1992a) assessed the clastogenicity of chemical mixtures. All mixtures of TCE (a non-clastogen) and dieldrin (at a concentration below the MED) gave positive results ($\alpha$=0.05), suggesting a synergistic relationship. Surprisingly, all mixtures of lead tetracetate and arsenic trioxide (both potent clastogens) proved negative, suggesting an antagonistic relationship. This effect was believed to result from a neutralization of the acid (from acetate) and the base (from sodium hydroxide) that dissolved arsenic trioxide. The other combinations were generally slightly antagonistic, especially for the combined action of three agents. Some mixtures were toxic, preventing the normal development of tetrads. This complex and frequently unpredictable response induced by chemical mixtures led the authors to conclude that *in situ* evaluations are warranted when multiple compounds interact, such as normally happens in hazardous waste sites.

Gill and Sandhu (1992) expanded on these findings by testing the same chemicals after soil incorporation, using rooted *Tradescantia* plants. Most of the results agreed with those previous, but in some instances interactions within soil altered the expression of clastogenicity. For example, arsenic trioxide and lead tetraacetate did not induce increased micronuclei production in solution (as in Ma et al., 1992a), but did so in soil. In general rooted plants showed higher micronuclei frequencies for the same chemical mixtures than cuttings treated in solution. A possible enhancement of metabolic activation of such mixtures in the root systems and/or by soil microorganisms was suggested as a likely reason. These results demonstrated once again that predicting the genotoxic effects of

chemical mixtures from their components may not be feasible and emphasized the importance of *in situ* assessments.

The significance of these conclusions was accentuated by the demonstration that tannic acids may act as synergists in the induction of clastogenicity in *Tradescantia* (Knasmuller et al., 1992). Exposure of *Tradescantia* for 24 hrs to increasing amounts of tannic acids caused a dramatic dose-dependent increase in the clastogenic effects of X-rays (35 rad), while tannic acids alone showed only moderate genotoxicity. Similar results were obtained with tannic acids in combination with other chemicals. This result may be important because tannic acids are present in many foods and beverages and in natural waters and soils. Therefore, practically all chemicals released into the environment may interact with, and be potentiated by, tannic acids.

The value of the Trad-MCN assay as a tool in environmental assessment was emphasized once again in an evaluation of bioremediation measures at a hazardous waste site (Baud-Grasset et al., 1993a, b). Heavily creosote-contaminated soils (more than 5000 ppm polyaromatic hydrocarbons) were incubated with the lignin-degrading fungus *Phanerochaete chrysosporium* Burdsall for 8 wks. Aqueous extracts of soils before and after incubation were evaluated for clastogenicity with the Trad-MCN. Extracts of the original soils were highly clastogenic, with the lowest effective concentration being 0.25% for a 30-hr exposure. *P. chrysosporium* caused a decrease in soil contamination, doubling the concentration of extract needed to induce a micronuclei frequency similar to that before incubation (from 1 to 2%). The Trad-MCN assay again proved to be extraordinarily sensitive, permitting the detection of differences between closely comparable samples.

The results obtained with *Tradescantia* in the assessment of soil contaminants are summarized in Table 2.3.

Table 2.3- Summary of environmental genotoxicity results using chromosome aberrations in *Tradescantia*, with special reference to the Trad-MCN assay of soil amendments and contaminants.

| Agent | Dose range | | Result | | | |
|---|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | Reference |
| **Soil contaminants** | | | | | | |
| Sewage sludge | 24 hrs | 4-fold dilution | + | | | Hopke et al. (1982) |
| Aldrin | 30 hrs | 2.0-36 ppm | - | $p < .05$ | | Sandhu et al. (1989) |
| Tetrachloro ethylene | 2 hrs | 30 ppm | + | $p < .05$ | Positive only when exposed in gaseous form. | " |
| Arsenic trioxide | 30 hrs | 3.96 ppm | + | $p < .05$ | Diluted in NaOH. | " |
| 1,2-benz[a,h] anthracene | 30 hrs | 12.5 ppm | + | $p < .05$ | Diluted in ethanol. | " |
| Dieldrin | 30 hrs | 3.81 ppm | + | $p < .05$ | Diluted in DMSO. | " |
| Heptachlor | 30 hrs | 1.88 ppm | + | $p < .05$ | Diluted in DMSO. | " |
| Lead tetraacetate | 30 hrs | 0.44 ppm | + | $p < .05$ | Diluted in DMSO. | " |
| Hazardous waste site soil | 30 hrs | 0.5% aqueous extract | + | $p < .05$ | Over 5000 ppm mixed PAHs. | Baud-Grasset et al. (1993a, b) |

### *2.3.4. Pesticides and Health-Related Agents*

Plants are the main biologic receptors of pesticides applied in the field. Thus, it is not surprising that a great deal of attention has been directed to the genotoxicity of pesticides to plants. An extensive review of the genetic toxicology of pesticides in higher plant systems (Sharma and Panneerselvan, 1990) listed a total of 178 active ingredients tested in at least one of 31 different plant species, utilizing a variety of organs and genetic endpoints. Approximately 30% of these compounds were found to be genotoxic, while only 6% were considered to be free of such genetic hazards.

*Tradescantia* appeared only once in that review, indicating that this assay has not been among the preferred systems for pesticide evaluation of genotoxicity, despite its sensitivity and amenability for field testing. The earliest references in which *Tradescantia* was employed to test pesticide genotoxicity involved the cytological activities of the insecticide mevinphos and the herbicide cyanazine (Ahmed and Grant, 1972b), and the methyl mercury-containing seed treatment fungicide Panogen 15® (methylmercury dicyandiamide) (Ahmed and Grant, 1972a). In these cases, chromosomal aberration in root tip mitosis was tested. Ahmed and Grant (1972b) reported that mevinphos and cyanazine induced augmented aberration frequencies, but exposures were very high (200 to 600 ppm), considered to be too extreme in terms of environmental contamination. They found that concentrations of Panogen 15® as low as 10 ppm caused cytotoxicity, while clear genotoxicity was noticed with just 1 ppm (Ahmed and Grant, 1972a). These results might have been significant for human exposure in mixing and spraying operations.

Acknowledging the special merit of *in situ* studies of agricultural chemicals Grant (1982) opined that no other organisms were as useful as *Tradescantia* nor

was any test as adequate as the Trad-MCN for the evaluation of genetic hazards *in situ*. These assertions were soon tested in a study of the genotoxicity of the insecticide malathion used for pest control in a greenhouse (Ma et al., 1983). In one treatment, intact potted plants were sprayed with malathion in the greenhouse, simulating conventional pest control treatment. Additional treatments involved absorption of malathion solutions through stems (with and without prior DMSO dissolution or treatment with S-9 microsome fraction of Aroclor™-induced mouse liver macerate), and exposure of intact plants to heat-generated malathion fumes in air-tight chambers. All exposures to malathion solutions, whether sprayed or absorbed through the stems, were negative. Exposure of cuttings to fumes of malathion resulted in a striking increase in micronuclei frequencies, suggesting that gaseous forms of some pesticides may be particularly effective in the Trad-MCN assay (Ma et al., 1983).

The clastogenicity of Benlate® (benomyl) and thiophanate, two fungicide products used in fruit storage, were evaluated with the Trad-MCN assay at concentrations of 0.05 and 0.07%, respectively (Huang and Chen, 1993a, b). Both agents induced high levels of micronuclei.

Results obtained to date for the genotoxicity of pesticides in *Tradescantia* are summarized in Table 2.4.

Table 2.4- Summary of environmental genotoxicity results using chromosome aberrations in *Tradescantia*, with special reference to the Trad-MCN assay of selected pesticides.

| Agent | Dose range | | Result | | | |
|---|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | Reference |
| **Pesticides** | | | | | | |
| Mevinphos | 3-12 hrs | 200-600 ppm | + | p<.001 | Root tip mitosis. | Ahmed and Grant (1972a) |
| Cyanazine | 3-12 hrs | 200-600 ppm | + | p<.001 | Root tip mitosis. | " |
| Panogen15® (mercurial) | 1-3 hrs | 1 - 5 ppm | + | p < .05 | Root tip mitosis. | Ahmed and Grant (1972b) |
| Malathion | 6 hrs | 5.5-1650 ppm | - | p < .05 | Stem absorption and spray application. | Ma et al. (1983) |
| Malathion | 6 hrs | 0.25-0.65% | + | p < .05 | Heat generated fumes. | " |
| Dichlorvos | 1-6 hrs | | + | | Insecticide. | Ma and Harris (1987a) |
| Benlate® | | 0.05% | + | | | Huang and Chen (1993a,b) |
| Thiophanate | | 0.07% | + | | | " |

Ma et al. (1984) presented the results of 140 Trad-MCN assays performed with a variety of chemical and physical agents. The agents were classified into 9 categories (numbers in brackets indicate number of agents tested in the category): (a) known carcinogens/mutagens [15], (b) common beverages [8],

(c) common chemicals [30], (d) common drugs [32], (e) pesticides [18], (f) common household chemicals [16], (g) ionizing radiation and radioisotopes [3], (h) *in situ* monitoring [13], and (i) complex environmental mixtures [8]. Some positive results within these groupings were: (a) benzo[α]pyrene (50μM), EMS (50 mM), and sodium azide (0.2 mM); (b) ethanol (5%), decaffeinated coffee (25%), and cola (50%); (c) formaldehyde fumes, nitrous oxides, and sulfur dioxide; (d) saccharin, aspirin; (e) Bladex® (cyanazine), dicamba, dichlorvos fumes, maleic hydrazide, *p*-dichlorobenzene, Tordon® (picloran); (f) some air-fresheners and cosmetics; (g) all ionizing radiation; (h) several polluted sites; and (i) several types of combustion exhausts and unconcentrated contaminated waters. Among the negative results were (a) 1,2-benzanthracene, methyl methanesulfonate, and dinitrotoluene; (d) mitomycin C; and (e) atrazine, simazine, and 2,4-D. Of 39 agents tested with the Trad-MCN assay and for which results of Ames tests were available, 26 gave the same results in both tests, representing a congruity of 67%. For pesticides, 11 of 18 agents tested gave positive results. Of eight pesticides tested both in the Trad-MCN and in the Ames test, only one (simazine) gave different results in the two tests (Ma et al., 1984).

The results available to date with *Tradescantia* in the evaluation of the clastogenic properties of selected chemical agents and physiological stresses are presented in Table 2.5.

Table 2.5- Summary of environmental genotoxicity results using chromosome aberrations in *Tradescantia*, with special reference to the effects of physiological stresses and selected chemicals on the Trad-MCN assay.

| Agent | Dose range | | Result | | | Reference |
|---|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | |
| **Selected chemicals** | | | | | | |
| EMS | 24 hrs | 50-100 mM | + | | Aqueous solution absorbed through the stems. | Ma (1979a) |
| 9 chemical categories | | | | | 140 chemicals assayed, 52 were positive, 20 were borderline, and 5 were toxic. | Ma et al. (1984) |
| **Physiological Stresses** | | | | | | |
| Anaerobiosis | 12-48 hrs | Max. 2% Oxygen | + | | Increase chromosome aberrations, including micronuclei in microspores. | Steinitz (1944) |
| Magnesium deficiency | continuous | < 1 ppm | + | p<.001 | Abnormal chromosome replication and micronuclei at meiosis. | Steffensen, (1953) |
| Sulfate deficiency | continuous | 4.0 ppm | + | | Same as above. | Steffensen (1954) |
| Calcium deficiency | continuous | 2.5 ppm | + | p<.001 | Same as above. | Steffensen (1955) |

### *2.3.5. Cosmic Rays and Electromagnetic Fields*

*T. palludosa* was used to study the potential effects of factors associated with space flight, such as acceleration, vibration, weightlessness, and ionizing radiation (Delone et al., 1986). Inflorescences were fixed chemically at several

times from take-off to post-landing, and the mitotic figures of the microspores were analyzed for aberrations. One very special aberration was observed in this material, especially for microspores exposed at early prophase. It consisted of complex nonreciprocal translocations involving spherical fragments. The appearance of such rearrangements was not associated with the duration of flight, or with take-off or landing. It was speculated that the causative agent of these aberrations was a heavy bombardment by cosmic radiation (Delone et al., 1986).

The sensitivity of *Tradescantia* to radiation has been demonstrated for X-rays, external and internal radioisotope sources, and cosmic rays. Likewise, long-wave radio frequencies and short-wave electromagnetic fields occurring in the vicinity of broadcasting antennae have been shown to be harmful to replicating chromosomes. In a series of experiments *in situ* (Haider et al., 1994), *Tradescantia* cuttings were exposed at five distances from the antennae, in Faraday (electromagnetic shielding) and plastic (non-shielding) cages distributed around sites in locations that exceeded the International Radiation Protection Association standards for electric field strength. All treatments resulted in high micronuclei frequencies as compared to laboratory controls ($\alpha$=0.05) and, more importantly, comparison between unshielded and Faraday cage-shielded groups showed highly significant differences ($\alpha$=0.004) (Haider et al., 1994). This result is particularly interesting, for both groups were exposed to exactly the same environmental conditions except the influence of electromagnetic radiation. A dose-response relationship over distance supports the conclusion that the effects observed were due to the electromagnetic fields.

The clastogenic effects of X-rays and other ionizing radiation on *Tradescantia* are summarized in Table 2.6.

Table 2.6- Summary of environmental genotoxicity results using chromosome aberrations in *Tradescantia*, with special reference to the Trad-MCN assay of X-rays and other ionizing radiation.

| Agent | Dose range | | Result | | | |
|---|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | Reference |
| **Radiation** | | | | | | |
| X-rays | 8 min | 75-200 rad | + | | Chromosome breaks, mostly at mitosis. | Sax (1938) |
| X-rays | ~5 min | 77-416 rad | + | $p < .05$ | Chromosome aberrations in pollen tubes and microspores, | Kirby-Smith and Daniels (1953) |
| $^{60}$Co γ-rays | 30 min | 100-400 rad | + | $p < .01$ | and significance measured for coefficients in exponential fit of data. | " |
| $^{32}$P β-rays | 20 min | 100-400 rep | + | $p < 0.1$ | | " |
| $^{60}$Co γ-rays | 16 d | 0.41 rad | + | $p<0.05$ | Micronuclei in microspores. | Sparrow and Singleton (1953) |
| X-rays on 5-FUdR treated cells | 36 hrs 5-FUdR + 2 min X-rays | 100 rad + $10^{-6}$ M 5-FUdR | + | $p < .05$ | Mitotic delay resulting in reduced number of exchanges and chromatid breaks. | Rushton (1969) |
| X-rays | seconds | 20-40 rad | + | | Positive dose response relationship in MCN. | Ma (1979a) |
| X-rays | seconds | 10-58 rad | + | | Correlation coefficient for dose response = .995 | Ma et al. (1980) |
| X-rays + tannic acids | 12 hrs | 35 rad X-rays + 0.1-1.0 mM tannic acids | + | | Synergistic interaction, positive dose - response relationship. | Knasmuller et al. (1992) |
| Cosmic rays | in situ exposure in space satellites | | + | | Unusual chromosome aberrations, i.e., non-reciprocal translocations and spherical fragments. | Delone et al. (1986) |

Table 2.6 (Continued)

| Agent | Dose range | | Result | | | |
|---|---|---|---|---|---|---|
| | Exposure time - max | Concentration | +/- | Statistical Significance | Remarks | Reference |
| Radio-frequency radiation | 30 hrs | Electric-40-170 V/m Magnetic-.01-.11 A/m | + | $p < .05$ | Micronuclei in tetrads. Cuttings in Faraday cage did not differ from laboratory controls. | Haider et al. (1994) |

In a recent study sponsored by the International Programme on Chemical Safety the utility of the Trad-MCN assay (along with three other plant bioassays) was evaluated with four known genotoxic chemicals (Grant and Salamone, 1994) in five different laboratories (Sandhu et al., 1994a; Sandhu et al., 1994b). Even though the results were not identical, there was good agreement among all laboratories, suggesting that the Trad-MCN assay is a reliable short-term bioassay for clastogens (Ma et al., 1994).

The studies reviewed here demonstrate that *Tradescantia* plants, and in particular the Trad-MCN assay, provide a very sensitive, easily manipulated system for the study of genotoxicity, especially under the *in situ* conditions indispensable for environmental studies. The performance attained in such a variety of situations and the extremely low levels of contamination successfully detected in these tests justify the selection of this system as the bioindicator for the studies that follow.

## 2.4. Trad-MCN Complete Assessment of an IPM Program

The induction of mutations is but one of many commonly acknowledged environmental impacts of pesticides (Durham and Williams, 1972; Epstein and Legator, 1971; Yoder et al., 1973). Most pesticides are capable of inducing

mutations in at least one test system (Laborda et al., 1985; Sharma and Panneerselvan, 1990; Waters et al., 1982), though sometimes at doses much higher than the ones observed in field conditions, specially if one considers the dilution of the compounds in soil almost immediately after application. The studies presented in the following sections are an attempt to assess the genotoxicity of pesticides and pesticide residues resulting from recommended agricultural practice. In particular, the possible abatement of such potential genotoxic effects by means of the reduced pesticide usage attained with IPM programs is evaluated.

The first step taken in this assessment was a complete evaluation of the genotoxicity of pesticides as applied in the field (*in situ*), followed by an appraisal of the genotoxic activity remaining in the soil shortly after spraying and an estimate of the clastogenic properties of the commercial formulations used, singly and in combinations. Such an assessment requires an eminent *in situ* approach and necessitates high sensitivity in order to permit detection of very low levels of pesticide contamination, as found for instance in soil extracts. As discussed in the previous sections, the Trad-MCN assay fulfills such requirements and was selected as the indicator system for this segment of the study. Other assays with additional plant species were employed for checking *in situ* the results obtained with the Trad-MCN assay and will be discussed in a later chapter.

### *2.4.1. The IPM Program and IPM Demonstration Field Under Study*

Developing agricultural procedures aimed at reducing pesticide usage has been a primary research objective (Bottrell, 1979; Pimentel, 1993; Pimentel et al., 1993; Smith et al., 1976; Smith and van den Bosch, 1967) and a goal sometimes established as national policy in some countries (Hurst et al., 1992). Perhaps the

most successful pesticide reduction initiative in terms of acceptability by growers and consequent widespread adoption in large scale is the integration of chemical, physical, and biological control practices commonly referred to as Integrated Pest Management (IPM) (Bottrell, 1979; Kuhr, 1994; Pimentel, 1991b). The effectiveness of IPM in reducing pesticide environmental impacts cannot be doubted (Pimentel, 1991a; Pimentel and Andow, 1984). In general, IPM measures can effectively reduce environmental impacts by attenuating soil and water contamination (Pimentel and Levitan, 1986), by diminishing natural enemies and wildlife losses, and reducing domestic animal poisonings (Pimentel et al., 1992), and by minimizing human exposure through contaminated produce and at the working place (Culliney et al., 1992).

Research on IPM normally involves the selection of appropriate techniques of crop management and pest control, taking into consideration local and regional characteristics of climate and soil, and the application of these techniques in an integrated program (Bottrell, 1979; Pimentel, 1985). An additional crucial component commonly appended to IPM research is the maintenance of demonstration fields, as a tool for explicating the program for growers. Such a demonstration field has been established at Cornell University's Musgrave Research Farm in Ithaca, NY, as part of the "Best Integrated Pest Management System for Grain Corn Production" research project. This project has been jointly carried out by the Departments of Soil, Crop & Atmospheric Sciences, Plant Pathology, and Entomology of Cornell University, under the coordination of Dr. W. J. Cox (Bergstrom et al., 1992, 1993, 1994). With the very kind permission of Dr. Cox, this demonstration field has been the site for the development of the present series of experiments.

## 2.5. Material and Methods

### *2.5.1. Demonstration Field Experimental Design*

The four-year (1991-1994) research and demonstration project for grain corn production in which the present experiments were carried out was designed for testing a set of management practices involving crop rotation (comprising continuous corn, continuous soybean, and alternating corn-soybean), several tillage systems, and three chemical input levels (Bergstrom et al., 1992). The total design encompasses an area approximately 150-m long x 30-m wide split in three plots, each directed to the incorporation of one type of management being tested. Since in the present research only the chemical input levels were of interest, the experimental parcel sampled was represented by three 3-m wide sub-plots, representing three independent sampling areas. The *in situ* exposures and soil sampling took place in the years 1993 and 1994, at least 2 yrs into the normal progress of the chemical treatments.

The continuous corn sub-plot was chosen for experimentation in order to assure consistency in relation to pesticide treatment prior to and during the study. The pesticide treatments applied in the continuous corn sub-plot were as follows: a) high input level - commercial seed treatment (captan) + 3-way grower seed treatment (carboxin+diazinon+lindane); soil insecticide (chlorpyrifos); and weed control with broadcast herbicide (cyanazine+metolachlor) application; b) medium input level - commercial seed treatment (captan) + 2-way grower seed treatment (captan+diazinon); and weed control with banded herbicide (cyanazine+metolachlor) application; c) low input level - commercial seed treatment (captan) alone, without any field pesticide application (Table 2.7).

Table 2.7- Pesticide input levels and details of the commercial formulations applied for continuous corn sub-plots assayed for genotoxicity with the Trad-MCN assay.

| Management | Pesticide Input Level | | | Remarks | | |
|---|---|---|---|---|---|---|
| | Low | Med. | High | Trade name / Producer | Active Ingredient (a.i.) | Formulation / a.i. application rate |
| **Seed Treatment** | | | | | | |
| Commercial | + | + | + | | captan | |
| Grower 2-way | | + | | Agrox 2-way Agway™ | captan+diazinon | 37 + 25% appr. 1.5 g/kg |
| Grower 3-way | | | + | Agrox DL-Plus Agway™ | carboxin+ diazinon/lindane | 14 + 15 + 25% appr. 1.4 g/kg |
| **Soil Insecticide** | | | + | Lorsban® 15G DowElanco™ | chlorpyrifos | Granular 1.12 kg/ha |
| **Weed Control** | | | | | | |
| Cultivation | 2x | 1x | | | | |
| Banded Herbicide | | + | | Bladex® 90DF Shell™ plus Dual® Ciba™ | cyanazine+ metolachlor | Water Disp. Granules 2.7 kg/ha Emul.Conc. 2.34 L/ha |
| Broadcast Herbicide | | | + | Bladex® 90DF Shell™ plus Dual® Ciba™ | cyanazine+ metolachlor | Water Disp. Granules 2.7 kg/ha Emul.Conc. 2.34 L/ha |

Planting took place by the end of April under favorable weather conditions. Seed treatment was carried out at the field site, immediately followed by simultaneous seed sowing and pesticide application by a tractor-towed implement.

Soil samples were collected before and after pesticide application and *Tradescantia* plants were exposed shortly after the expiration of the restricted entry interval (REI) of 12 hrs.

### *2.5.2. Soil Sampling, Extraction, and Testing*

Four samples of each pesticide treatment plot were taken from the top 5-cm layer of the soil with a small garden scoop and conserved in paper bags in a -20°C freezer. As soon as feasible after collection, equal portions of each of the four samples were combined for extraction, ground with a pestle and sieved through a combination of 1.168 mm and 420 µm (14- and 35-mesh) sieves. Extraction was performed by mixing 1 part of soil with 2 parts of deionized water (w/v), and agitating the mixture for 24 hrs in a rotary shaker. The mixture was then centrifuged for 20 min at 2400 rpm and the supernatant liquid immediately assayed without dilution.

### *2.5.3.* Tradescantia *Stock, Maintenance, and General Conditions of Experimentation*

*Tradescantia* clone 4430 was obtained from the stock of Dr. Shahbeg S. Sandhu, from the Genetic Toxicology Division, Health Effect Research Laboratory, U.S. Environmental Protection Agency at Research Triangle Park. Clone 4430 is a diploid hybrid between blue-flowered *T. hirsutiflora* Bush (2461C) and pink-flowered *T. subacaulis* Bush (2441) (Schairer and Sautkulis, 1982) especially suited for laboratory experimentation and described in detail by Emmerling-Thompson and Nawrocky (1980). Because clone 4430 is sterile, there is no risk of losing genetic identity through recombination, and its heterozygosis

for flower color makes it suitable for both the Trad-MCN and Trad-STH assays (Ichikawa, 1992; Ma, 1988).

A population of approximately 500 mature plants containing an average of 4 to 6 stems at different stages of growth was maintained throughout the experimentation period. Such a population was large enough to yield at least 200 cuttings in excellent condition for exposure every other week. The plants were maintained in activated charcoal/Purafil®-filtered air greenhouses at a temperature of 27/21 ± 2°C (day/night), with supplemental lighting provided by GE™ MVT-400 multivapor lamps to maintain a 17-hr day cycle during the entire year. Plants were cultivated in 12.5-cm recyclable cardboard pots containing a soil-peat moss-vermiculite mix (Cornell Mix, Boodley and Sheldrake, 1982) supplemented with Osmocote® (slow release) fertilizer and micronutrient elements. In general, plants were watered every other day and received a dilute fertilizer solution weekly. At every harvest of cuttings (biweekly) all pots were closely inspected and the plants pruned, so as to keep them clean and free of pests. No pesticides were applied to or around the plants during the experimentation period.

For each experiment, 15 to 20 cuttings (per treatment per replicate) containing young inflorescences were harvested 24 hrs prior to treatment and transferred to controlled-environment chambers. These activated-charcoal/Purafil®-filtered air environmental chambers were equipped with GE™ LU-400 Lucalux high pressure sodium and MVR-400 multivapor metal halide lamps, delivering 800 watts $m^{-2}$ at the plant level, and working at an air flux of 6 $m^3$ per minute, resulting in a renewal rate of at least two air changes per minute. The environmental settings were 21/19 ± 0.2°C day/night temperatures, 65/70% day/night relative humidity, and a 17-hr day light cycle.

Before exposure, cuttings were maintained in beakers containing deionized water under continuous aeration with air filtered through activated charcoal/Purafil® for a 24-hr adaptation period. Any wilted cuttings were eliminated at this stage, so that only turgid cuttings with expanding inflorescences were treated. All exposures were performed by diluting the agents to be tested in 1/3-strength Hoagland's solution (Downs and Hellmers, 1975), which corresponded also to the negative control in all experiments. The positive control was represented by a 30-hr treatment (no recovery time) with a 100 ppm solution of ethyl methanesulfonate (EMS - Sigma Co.). Unless otherwise noted a 30-hr exposure period immediately followed by fixation (no recovery time) was carried out. This procedure is recommended when very low levels of active agents are being tested (Ma, 1988).

After treatment the inflorescences were removed from the cuttings and fixed in 1:3 (v/v) acetic acid:ethanol for 24 hrs and then preserved in 70% (v) ethanol. For scoring, inflorescences were dissected and the early anthers squashed in a solution of aceto-carmine stain (prepared by boiling 0.5 g carmine in 100 ml of 45% per volume acetic acid) on a microslide. After placing the coverslip, the microslide was heated repeatedly on a hot plate to about 80°C and gently pressed under absorbent paper in order to remove excess stain (Ma, 1988). Scoring was performed by counting, under 400x magnification, the number of micronuclei present in a random set of 300 early tetrads present in 5 microslides (Ma, 1979b; Ma, 1981) comprising a statistical population of 1500 tetrads per treatment. The number of tetrads containing one or more micronuclei was annotated and the total number of micronuclei was expressed at a 100 tetrads basis. The microslides were coded and scored by the same observer, with the code being revealed only after all treatments had been scored.

In this method, each bud containing the appropriate early tetrads (one valid scored slide) is considered to be a sample population, with buds from the other cuttings representing the replicates of an experimental group or treatment. The mean number of micronuclei/100 tetrads in each treatment was compared by Analysis of Variance (ANOVA) and comparisons between the control and specific treatments were performed with Student's t-tests assuming two-tail distributions (no *a priori* tendencies assumed in the data) and $\alpha$=0.01.

### *2.5.4. Assay of Commercial Formulations*

The three commercial pesticide formulations applied to the fields (Table 2.7) were assayed for genotoxicity under laboratory conditions. These assays involved exposing *Tradescantia* to each of the products in different concentrations and to mixtures of the products in different proportions.

#### *2.5.4.1. Assay of Single Formulations*

Lorsban® 15G (chlorpyrifos) chemical name (IUPAC) *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate, Bladex® 90DF (cyanazine) chemical name (IUPAC) 2-(4-chloro-6-ethylamino-1,3,5-triazin-2-ylamino)-2-methylpropionitrile, and Dual® (metolachlor) chemical name (IUPAC) 2-chloro-6'-ethyl-*N*-(2-methoxy-1-methylethyl)acet-*o*-toluidine were tested for genotoxicity at active ingredient concentrations of 1.0, 10.0 and 50.0 ppm. Bladex® 90DF (cyanazine) and Dual® (metolachlor) were dispersed and emulsified in deionized water at the above-mentioned concentrations. Due to the insoluble nature of the chlorpyrifos- containing Lorsban® granules and the low solubility of this compound (2 mg/L in water), only a nominal concentration of active

ingredient could be assumed for the suspension. *Tradescantia* cuttings were exposed by immersing the stems directly into the aqueous solutions/suspension.

### *2.5.4.2. Assay of Formulated Mixtures*

The first consideration in the design of this assay was about the concentrations or proportions at which the pesticides would be tested. The main objective of this experiment was to determine if any kind of interaction could occur between the products, possibly modifying the effects predicted when each of the formulations was assayed separately. Secondly, the concentrations assayed should reflect the conditions expected when these pesticides are used at the recommended rates of application, thereby mirroring the *in situ* observations.

With these prerequisites in mind, the concentrations of each pesticide to be tested in the mixtures were derived from the rates of application recommended in the label of each product. A dilution factor was calculated assuming a homogeneous distribution of the compounds in the top 5 cm of soil. The recommended dose of each product expressed in weight or volume of active ingredient per hectare was divided by the volume of soil contained in the top 5 cm of 1 ha. These calculations produced a unit-dose for assay, which was mixed in combinations of single or double unit-doses for the three compounds. The computations for definition of these unit-doses are presented in Table 2.8.

Table 2.8- Recommended doses employed and computations performed for determining the concentrations of each pesticide to be combined in the assays of formulated mixtures.

| Compound | Recommended Application Rate | Dilution factor[a] | Unit concentration dose for assay[b] |
|---|---|---|---|
| Chlorpyrifos | 2.3 kg/ha[c] | $5.0 \times 10^5$ | 4.0 |
| Cyanazine | 5.4 kg/ha[d] | $5.0 \times 10^5$ | 10.0 |
| Metolachlor | 3.1 L/ha[e] | $5.0 \times 10^5$ | 6.0 |

All possible three-way combinations of the pesticides, at one- and two-times the unit-doses were assayed, together with the positive and negative controls.

### *2.5.5.* In Situ *Exposure Assay*

*Tradescantia* cuttings (25/replicate/treatment) were brought to the field in beakers containing 1/3-strength Hoagland's solution in an air-tight box on the day following pesticide application. Cuttings were exposed during the night, from early evening to the early hours of the next morning, in a total of 14 hrs. In order to increase pesticide volatilization from the soil, the site where samples were

---

[a] Assuming homogeneous distribution of the compounds in the top 5 cm of soil in one hectare.

[b] Expressed in parts per million.

[c] For sandy clay or silty clay soils with 5% or more organic matter content.

[d] For finely textured soils.

[e] For preplant broadcast application.

placed received approximately 10 L of tap water immediately before placing the cuttings (Prueger and Pfeiffer, 1994).

Treatment consisted of placing the beakers with the *Tradescantia* cuttings on the wet soil along the crop rows. Each beaker was then covered with an inverted 20-cm PVC pot for protection, creating a simple chamber that could accumulate vapors released from the soil. The controls consisted of placing the beakers with the plants on soil covered with a plastic lining. The positive control received an aliquot of EMS to the Hoagland's solution to produce a 100-ppm solution immediately before treatment began.

After treatment, the samples were brought back to the laboratory in the air-tight box, the nutrient solutions were replaced by freshly prepared ones, and the cuttings underwent a 24-hr recovery period in order to allow the exposed meiotic pollen mother cells to reach the early tetrad stage. The inflorescences were then fixed and scored as described earlier.

## 2.6. Results

The range observed in the data for the negative control (spontaneous micronuclei frequency - lowest level under the given conditions) and EMS treatment or positive control (doubled micronuclei frequency - significant response) approximated the spread in the whole data for all treatments, insofar as only low concentrations, low residue levels, and marginally-contaminated fields were evaluated. Since the exposure conditions were kept constant during all laboratory assays, these controls can be used to gauge the sensitivity (the capacity of the test system to detect low levels of an agent) and the reproducibility (the ability to respond consistently to an agent) of the Trad-MCN system as it was employed here.

When considered within each control group (positive or negative), all samples shared the same statistical mean (with the exception of the *in situ* exposure, which showed a slight increase in micronuclei frequency). In only one instance did one sample of the positive control treatment show no statistical difference from a sample of the negative control, coincidentally in the same experiment. This event is represented in Axis E (corresponding to the chlorpyrifos assay) of Figure 2.1.

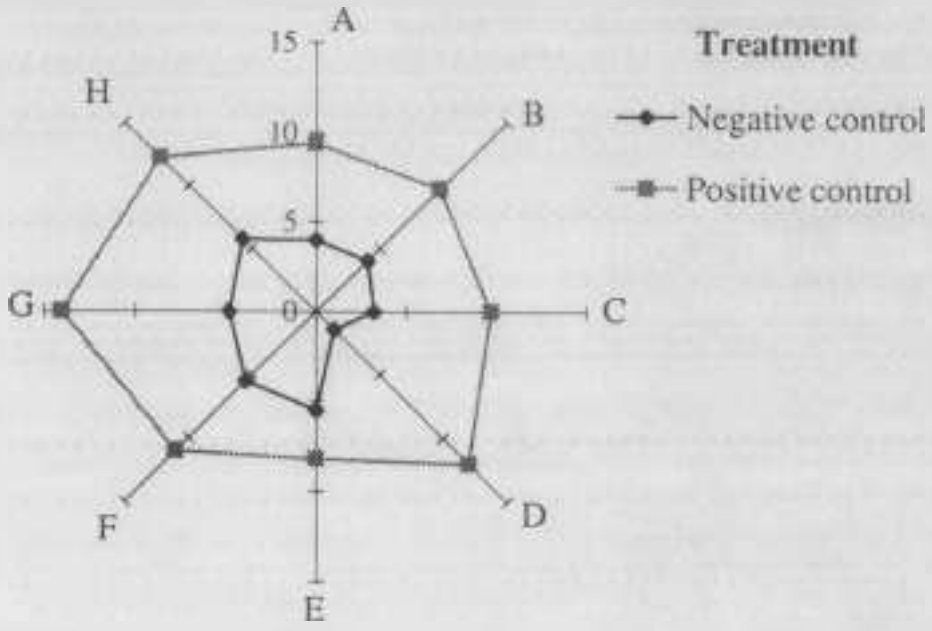


Figure 2.1- Micronuclei frequencies for negative and positive controls in all Trad-MCN assays. The negative controls represent the spontaneous micronuclei frequencies for clone 4430 and consisted of treatment with 1/3 strength Hoagland's solution; the positive controls consisted of 100 ppm aqueous solutions of ethyl methanesulfonate (EMS). *Tradescantia* cuttings were exposed for 30 hrs in a controlled-environment chamber.

The EMS treatment induced a consistent increase in micronuclei frequency, to a level approximately double the spontaneous frequency for clone 4430 (from 4.2±1.4 to 10.7±1.9, for $n$= 40). The spontaneous micronuclei frequency was also

reasonably constant, with the exception of the result shown in Axis D (corresponding to the metolachlor assay) of Figure 2.1, in which a decrease in micronuclei frequency occurred.

It should be noted that the spontaneous micronuclei frequencies observed in the experiments described here are in good agreement with the data available in the literature, e.g., Grant et al. (1992), Ma (1981), and several others.

### *2.6.1. Genotoxicity of Pesticide Residues Extracted from Soil*

Two separate assays were performed with soil extracts, one for samples collected before spraying (corresponding to soil sprayed in the previous season) and one for samples collected shortly after pesticide application. Each assay was accompanied by its own set of controls, and all conditions were kept constant. The results of these two assays are shown in Figure 2.2.

Both the negative and the positive controls for the two assays were very similar, assuring that the conditions of exposure and scoring procedures were comparable. There were no statistically significant differences among the extracts of unsprayed soils; only the EMS treatment showed a statistically significant increase in micronuclei frequency above the control in this experiment. This lack of effect in the unsprayed samples indicated that there were no endogenous agents or residual anthropogenic compounds in the extracts capable of inducing clastogenesis. The extracts of sprayed soils, however, showed significant increases in micronuclei frequency for the medium and high treatments, relative to the negative control and the low pesticide treatment (no pesticide applied to the field).

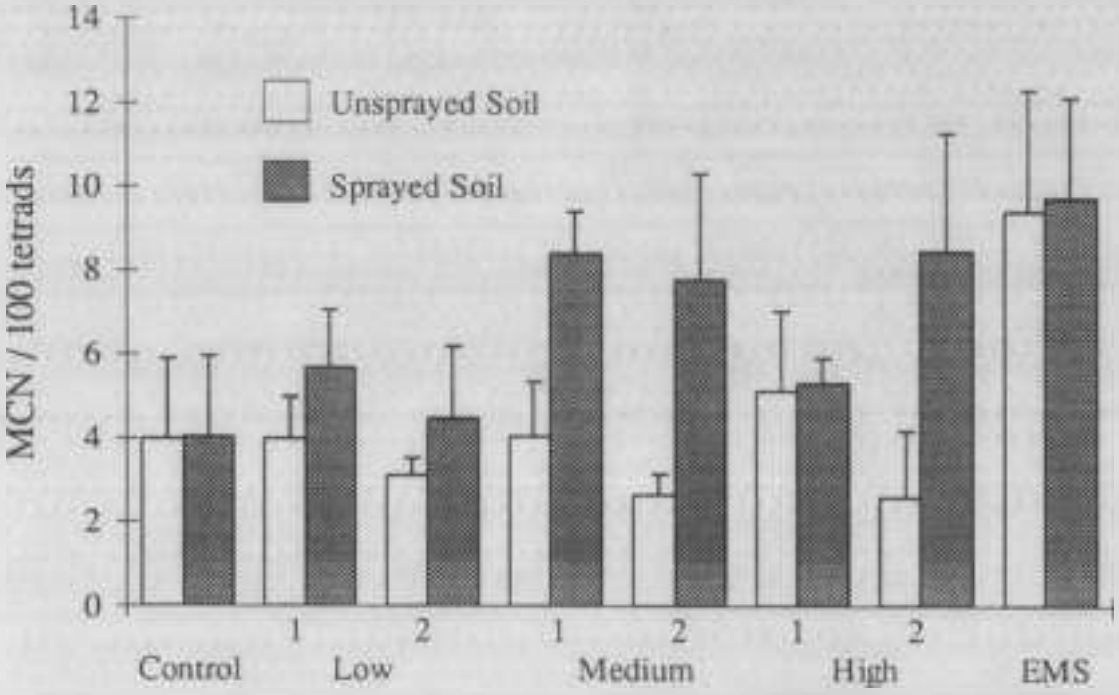


Figure 2.2 - *Tradescantia* (clone 4430) micronuclei production after exposure to aqueous extracts of pesticide-treated soils. The bars represent standard deviations of the mean. *Tradescantia* cuttings were exposed for 30 hrs in a controlled-environment chamber. The results represent 2 separate experiments, each with its own set of negative and positive controls. The Control treatment received only nutrient solution and the positive control (EMS) consisted of a 100 ppm aqueous solution of ethyl methanesulfonate. Unsprayed and sprayed soil samples were collected before and 12 hrs after pesticide application, respectively. The "Low" pesticide treatment received no pesticide, "Medium" received banded cyanazine + metolachlor, and "High" treatment received broadcast cyanazine + metolachlor and chlorpyrifos.

One replicate of the high pesticide treatment (High 1) failed to show an increase in micronuclei frequency. Aside from this exception, the pesticide-treated samples showed statistically significant differences in clastogenicity when compared with the unsprayed samples. These results indicated that a genotoxic activity could be detected for the pesticides applied even after dilution in soil following recommended usage.

### *2.6.2. Assay of Commercial Formulations*

#### *2.6.2.1. Assay of Single Formulations*

Of the three pesticides evaluated for clastogenicity with the Trad-MCN assay, the two herbicides (cyanazine and metolachlor) showed statistically significant ($p<0.01$) increases in micronuclei frequency in relation to the negative control, while the insecticide chlorpyrifos did not. However, an increase in micronuclei frequency was noticed for the 10- and the 50-ppm doses of chlorpyrifos (Figure 2.3).

The negative control treatment in this experiment gave a micronuclei frequency slightly above the levels obtained usually (Figure 2.1, axis 5), which certainly contributed to the lack of statistic significance in this assay. A comparison between the 1-ppm treatment (which showed a micronucleus frequency similar to the spontaneous level for clone 4430 registered throughout this study) and the higher concentrations indicated a statistically significant ($p<0.01$) increase in micronuclei frequency for chlorpyrifos.

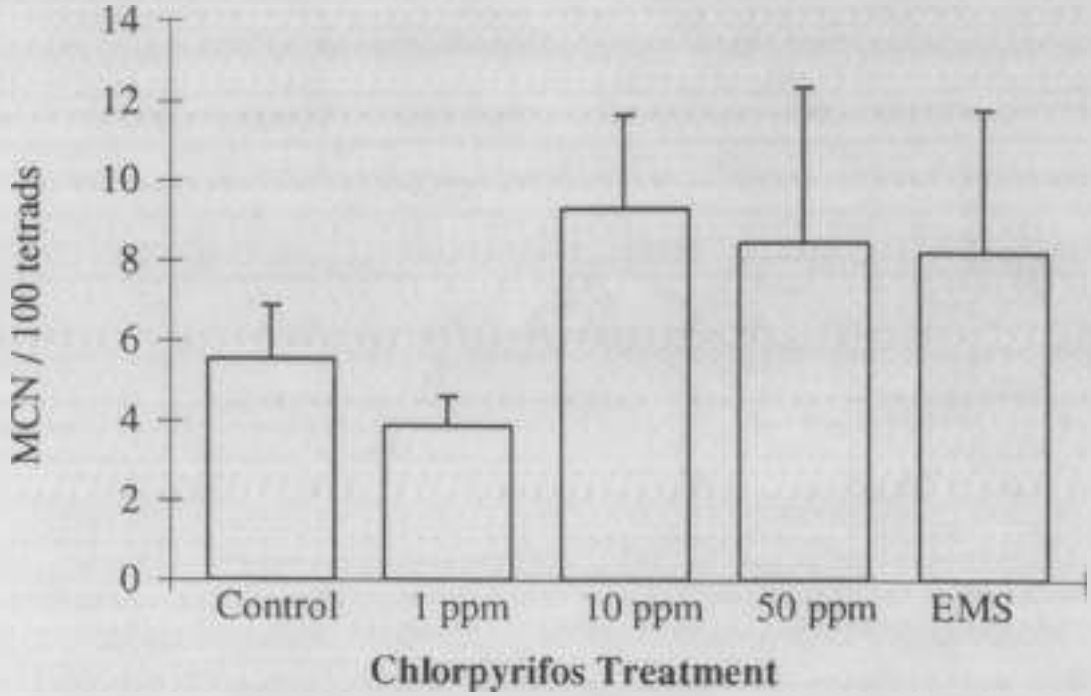


Figure 2.3 - Clastogenic effects of the insecticide chlorpyrifos in the Trad-MCN assay. The bars represent standard deviations of the mean. *Tradescantia* cuttings were exposed to aqueous solutions of chlorpyrifos for 30 hrs in a controlled-environment chamber. The Control treatment received only nutrient solution and the positive control (EMS) consisted of a 100-ppm aqueous solution of ethyl methanesulfonate.

Cyanazine was an effective clastogen at a concentration as low as 10 ppm (Figure 2.4). Even at 1 ppm cyanazine induced an increase in micronuclei frequency ($p<0.05$). At 50 ppm micronuclei induction was decreased in relation to the 10-ppm treatment. Such a tendency was reiterated when the assay was repeated, indicating an acute toxic level of cyanazine to *Tradescantia* pollen mother cells between 10 and 50 ppm.

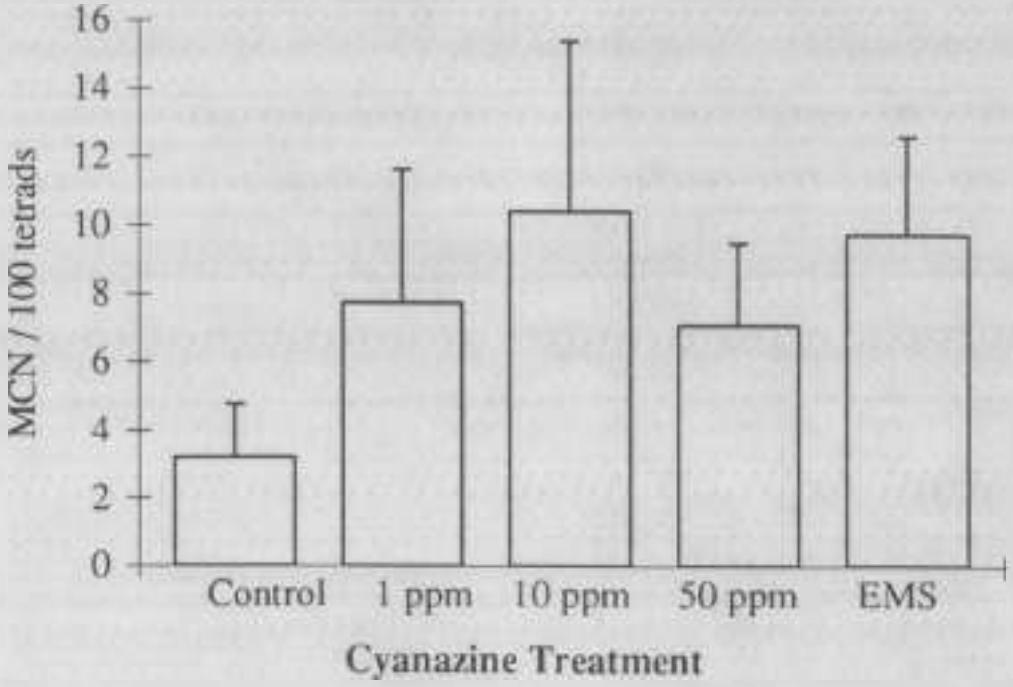


Figure 2.4 - Clastogenic effects of the herbicide cyanazine in the Trad-MCN assay. The bars represent standard deviations of the mean. *Tradescantia* cuttings were exposed to aqueous solutions of cyanazine for 30 hrs in a controlled-environment chamber. The Control treatment received only nutrient solution and the positive control (EMS) consisted of a 100-ppm aqueous solution of ethyl methanesulfonate.

A clear dose-response relationship was observed for the clastogenic effects of metolachlor in the Trad-MCN assay (Figure 2.5). A somewhat wide deviation in the mean micronuclei frequency for the pesticide-treated samples, however, resulted in a failure of the 10-ppm treatment level to show a statistically significant difference when compared with the control ($p=0.054$). A significant increase in micronuclei frequency was observed for metolachlor at a concentration of 50 ppm when compared with the negative control.

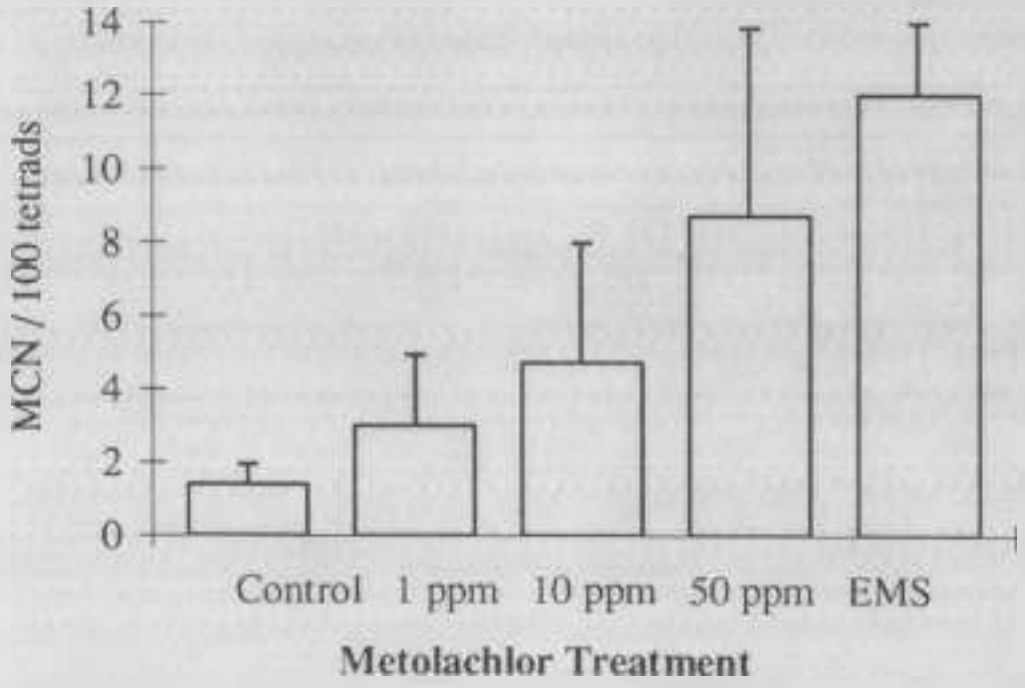


Figure 2.5 - Clastogenic effects of the herbicide metolachlor in the Trad-MCN assay. The bars represent standard deviations of the mean. *Tradescantia* cuttings were exposed to aqueous solutions of metolachlor for 30 hrs in a controlled-environment chamber. The Control treatment received only nutrient solution and the positive control (EMS) consisted of a 100-ppm aqueous solution of ethyl methanesulfonate.

In conclusion, all three pesticides studied were capable of causing some degree of clastogenicity in the Trad-MCN assay at concentrations between 10 and 50 ppm. These concentrations are well below those applied in formulated sprays and approximate those hypothesized to be found in soils shortly after application.

### *2.6.2.2. Assay of Formulated Mixtures*

One lingering question related to the genotoxic properties of the pesticides studied concerns the possible interactions that could be produced when these pesticides are applied sequentially or in mixtures, resulting in modifications of

their individual activities. In order to answer this question, an assay was performed exposing *Tradescantia* to mixtures of the three pesticides at concentrations comparable to those expected to be found in soil shortly after application (Table 2.8). Additionally, these unit-doses were doubled and all possible triplet combinations of 1- and 2-fold the unit-doses were evaluated.

Highly variable responses were obtained in this experiment. Out of eight treatments, two showed a definitely significant positive response, three showed a clear increase in micronuclei frequency ($p<0.05$), and three resulted only in slight increases ($p<0.1$) (Figure 2.6).

There was no clear pattern in these results, albeit the concentrations tested were determined based primarily on hypothesized field occurrence of active ingredients at the recommended dose, instead of the minimum effective doses defined through assays performed for each of the individual compounds. Acute toxicity is one of the factors that could be implicated in this response. Such an interpretation derives from a tendency for the samples receiving two times the unit concentration doses simultaneously for at least two compounds (1:2:2 samples) to show lower micronuclei frequencies than the samples receiving two times the unit concentration dose for only one compound (1:1:2 samples) ($p<0.05$).

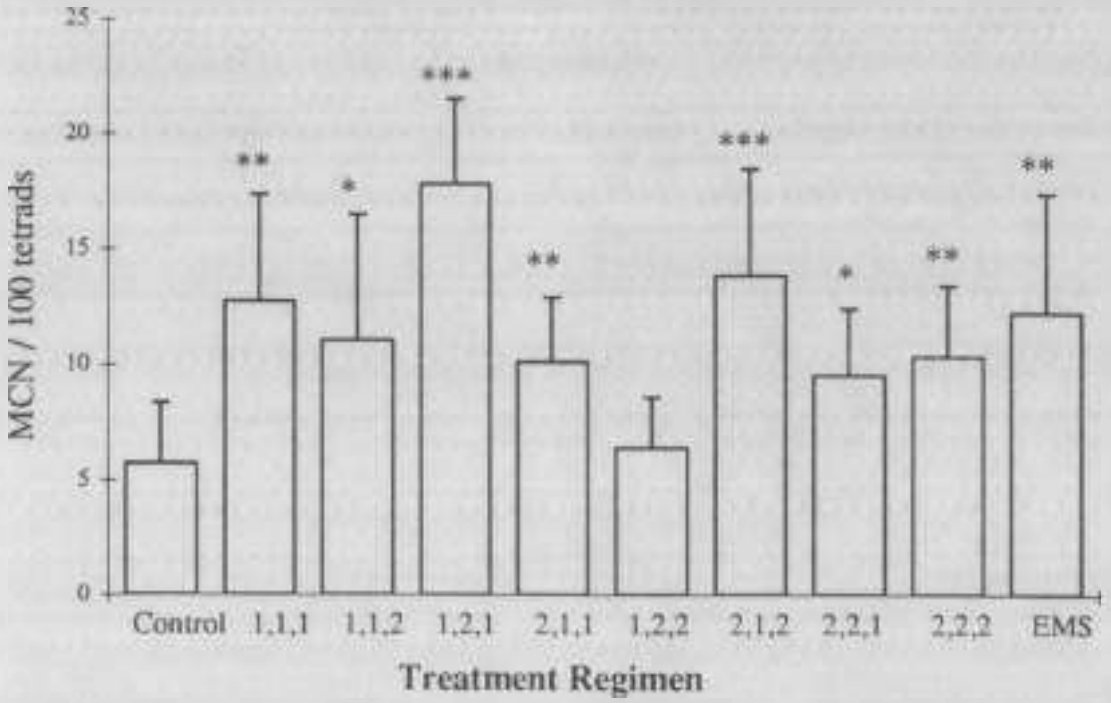


Figure 2.6 - Clastogenic effects of pesticide mixtures in the Trad-MCN assay. The bars represent standard deviations of the mean and the asterisks represent statistic significance levels as $p<0.1$ (*), $p<0.05$ (**), and $p<0.01$ (***). *Tradescantia* cuttings were exposed to aqueous solutions of the pesticides for 30 hrs in a controlled-environment chamber. The Control treatment received only nutrient solution and the positive control (EMS) consisted of a 100 ppm aqueous solution of ethyl methanesulfonate. Numbers on the X Axis indicate the number of unit concentration doses applied for cyanazine, metolachlor, and chlorpyrifos, respectively. Unit concentration doses were calculated with basis on the recommended application rates for each compound and were 10.0 ppm for cyanazine, 6.0 ppm for metolachlor, and 4.0 ppm for chlorpyrifos.

In other words, there was a tendency for mutation to decrease with increasing doses, a possible sign of acute toxicity. No definite conclusions could be drawn from this experiment regarding possible synergistic or antagonistic interactions among the pesticides.

### *2.6.3.* In Situ *Exposure Assay*

Highly significant increases in micronuclei frequency were obtained when *Tradescantia* cuttings were exposed *in situ* to pesticide-treated soils. Both medium and high treatment levels, each represented by two replicates, differed from the low treatment (no pesticide sprayed). Except for one replicate of the high treatment level, all samples also showed a significant increase in relation to the relatively high micronuclei frequency of the negative control. These results indicate that the null hypothesis of no differences in genotoxicity between sprayed and non-sprayed soils is rejected ($p<0.01$), suggesting that an increase in genotoxic activity is effected *in situ* after pesticide treatment of the soil.

There were no statistically significant differences between samples from the medium and high treatment levels (Figure 2.7) which showed higher micronuclei frequencies than the EMS treatment. Although this increase over the positive control was not statistically significant it indicates a micronuclei frequency for the pesticide-treated samples superior to double the spontaneous level for *Tradescantia* under the conditions of experimentation.

The results of this experiment repeated precisely a trend noticed in the soil extracts assay (Figure 2.2), i.e., a slightly higher micronuclei frequency in the Medium treatment level in relation to the High treatment level samples. This result means that the null hypothesis of no difference between Medium and High pesticide treatment fails to be rejected, indicating that there is no abatement in genotoxic activity when the IPM program of pesticide application is implemented, in relation to the pesticide usage normally prescribed for corn.

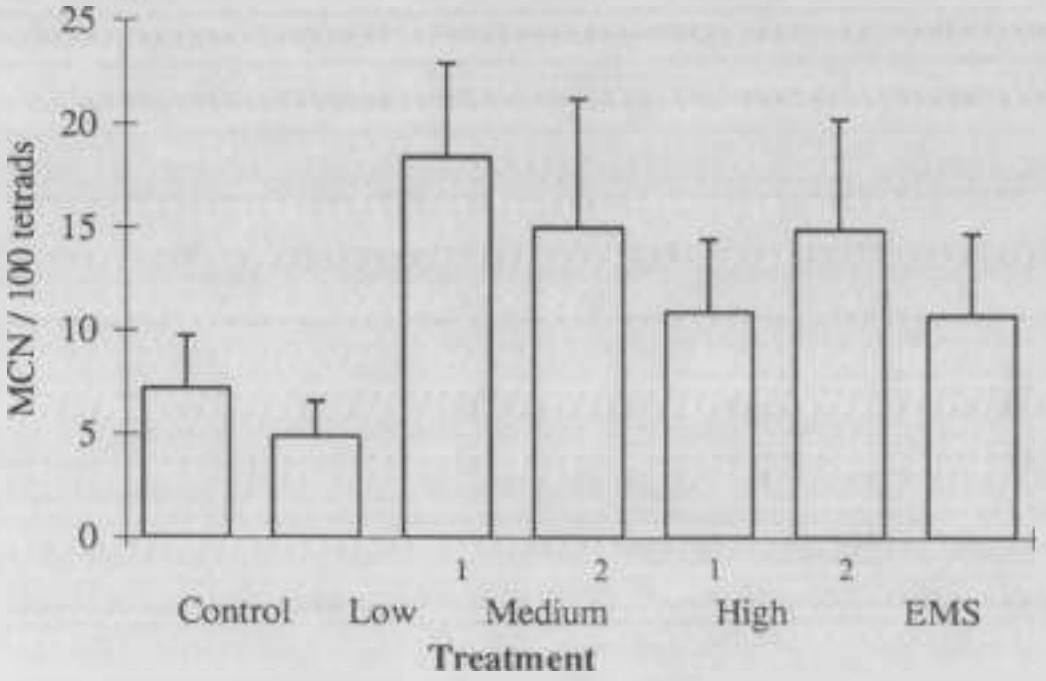


Figure 2.7 - *Tradescantia* (clone 4430) micronuclei production after *in situ* exposure to pesticide-treated soils. The bars represent standard deviations of the mean. *Tradescantia* cuttings were exposed for 14 hrs by placing the beakers containing the cuttings directly on the wetted soil surface. The Control treatment received only nutrient solution and was placed on soil covered with a plastic lining, the positive control (EMS) consisted of a 100-ppm aqueous solution of ethyl methanesulfonate placed also on plastic-covered soil. The Low pesticide treatment received no pesticide, the Medium received banded cyanazine + metolachlor, and the High treatment received broadcast cyanazine + metolachlor and chlorpyrifos. The pesticides had been applied 24 hrs before exposure began.

One could speculate that such a tendency had to do with an effectively higher rate of herbicide application (since the insecticide was not applied to the Medium treatment) for the Medium treatment at the spot where *Tradescantia* cuttings were exposed. This could be the result of the banded mode of application employed in this treatment, in which a much narrower stripe of soil received the pesticide spray, hence locally concentrating the active agents. As the *Tradescantia*

cuttings were placed in the inner part of the sprayed band, a slightly higher dose might have been delivered to these samples in relation to the high treatment level samples, which received broadcast herbicide application.

The Trad-MCN system proved to be highly sensitive and, perhaps more importantly, sufficiently reproducible to detect clastogenic activity for all pesticides studied, as well as for extracts of sprayed soils and *in situ*. The consistency of the results obtained for the controls in the eight different laboratory experiments, and especially for the replicates in the soil extracts analysis and in the *in situ* exposure, emphasizes the usefulness of this system for the evaluation of environmental genotoxicity, specially when low levels of contamination are prevalent.

It should be emphasized, moreover, that the results were consistent in spite of (a) only nominal concentrations could be assumed for the exposures, due to the lack of chemical analysis of the soil samples and pesticide solutions assayed, and (b) commercial formulations were applied instead of pure chemicals, even in the commercial formulations assays, in order to more closely resemble the conditions prevalent in the IPM demonstration field.

## 2.7. Discussion

Whether a chemical is capable of posing a threat to biota depends on partitioning relationships that determine how pervasive the chemical is, its tendency to be immobilized in the media or be absorbed into organisms, and its persistence and toxicity (Gillett, 1983). The *in situ* induction of clastogenicity observed in *Tradescantia* exposed to pesticide-sprayed fields advances a question on how the pesticides reached the target pollen mother cells within the inflorescences.

Even though presenting a relatively low vapor pressure (2.5 mPa) (Tomlin, 1994), close to one-half of the chlorpyrifos applied to agricultural fields may find its way into the atmosphere by volatilization (Whang et al., 1993), especially under the wet soil conditions present at the time of *Tradescantia* exposure. This happens because of the low solubility of chlorpyrifos, which counterbalances the low vapor pressure causing the compound to have a fairly high dimensionless Henry's Law Constant [$H_{c\ calc}$ = -3.75 (Gillett, 1992; Mackay, 1981)]. The Henry's Law Constant is appropriate to assess the fugacity of a compound from solution or from surfaces when water is involved, because water may out-compete chemicals for sorption sites. A $H_{c\ calc}$ above -4.0 indicates that a chemical may volatilize spontaneously from solutions and wet surfaces (Gillett, 1992).

Volatilization was the major route for chlorpyrifos loss from a sandy soil in the first 7 d after application (Chapman and Chapman, 1986). This is not true, however, for cyanazine, which showed no dissipation by volatilization in a laboratory study (Blumhorst and Weber, 1992). With a very low vapor pressure (200 nPa) (Tomlin, 1994) and low $H_{c\ calc}$ (-6.92) cyanazine is virtually non-volatile.

Although metolachlor adsorbs tightly to clays, its adsorption isotherm is typically S-shaped, signifying that it is readily desorbed from soil by water (Chesters et al., 1989). Metolachlor, in spite of its relatively low vapor pressure (1.7 mPa) (Tomlin, 1994) and low $H_{c\ calc}$ (-6.4), volatilized freely (80% in 24 hrs) from a glass surface (Prueger and Pfeiffer, 1994), and losses from soil by volatilization may reach up to 30% (Prueger, 1994). Thus, it is conceivable that, especially for chlorpyrifos and metolachlor, sufficient vapors could have been released from the wetted soils to induce the clastogenic effects detected in the *in situ* assay performed with *Tradescantia*.

The findings of the present experiments corroborate the genotoxic effects detected in a variety of test systems, including plant bioassays, of the three pesticides under investigation. Chlorpyrifos induced an increase in micronuclei frequency in erythroblasts of mouse bone marrow both by intraperitoneal and oral treatments (dermal treatment was negative) with doses below the $LD_{50}$, and with no signs of clinical effects (Amer and Fahmy, 1982). By contrast, chlorpyrifos did not induce increases in either sister chromatid exchange (SCE) frequencies in the chick embryo assay or in Chinese hamster ovary cells. No chromosomal aberrations were found in bovine blastocysts, but the reproductive performance of treated bulls became subnormal after exposure to chlorpyrifos (Muscarella et al., 1984).

The cytogenetic effects on human lymphocytes of a mixture of 15 pesticides commonly found in foods in Italy was studied by Dolara et al. (1994). Chlorpyrifos comprised 2% of the mixture, which was genotoxic only when benomyl was present. Chlorpyrifos was mutagenic in the somatic as well as in the germ cells of *Drosophila*, as determined by induction of mosaic wing spots and sex-linked recessive lethals, at concentrations as low as "5 x $10^{-6}$ %" (Patnaik and Tripathy, 1992).

Shirasu et al. (1976) evaluated the mutagenicity of chlorpyrifos utilizing the microbial test systems *Bacillus subtilis* (rec-assay), *Escherichia coli*, and *Salmonella typhimurium* (reversion assays). None of the assays was positive. On the other hand, chlorpyrifos was reported positive in the primary DNA damage assay with these same organisms and with *Saccharomyces cerevisiae* in another study (Waters et al., 1982). Other assays employed, including reversion assay in *S. typhimurium* and *E. coli*, recessive lethality in *D. melanogaster*, enhanced mitotic recombination in *S. cerevisiae*, and unscheduled DNA synthesis (UDS) in

human lung fibroblasts, all produced negative results (Waters et al., 1982). Chlorpyrifos also failed to induce mutations in *S. cerevisiae* and *S. typhimurium* both before and after activation with plant (1S) and animal (S9) microsomal homogenates, as well as in the *Z. mays* waxy locus test (Gentile et al., 1982).

Significant increases in abnormal mitoses were induced by saturated and 1/8-strength solutions of chlorpyrifos in *V. faba* root-tip meristematic cells both after seed soaking and direct root treatment (Amer and Farah, 1983). Likewise, both mitotic and meiotic genotoxicity were caused by chlorpyrifos in *Hordeum vulgare* L. root tip cells and pollen mother cells after seed soaking and spray treatments (Kaur and Grover, 1985a, b). Positive dose-response relationships were obtained in both tests, with chromosome stickiness being the main effect. A review of the genetic toxicology of pesticides (Sharma and Panneerselvan, 1990) listed several chromosome effects caused by chlorpyrifos, including fragmentation, bridges, vagrancy, disturbed anaphase, and chlorophyll mutant induction in *Gossypium barbadence*, *V. faba*, and *H. vulgare*. The genetic hazard potential of chlorpyrifos was considered uncertain for developmental and reproductive hazards, and positive for hereditary genetic hazard.

The increase in micronuclei frequency observed for a low dose (10 ppm for 30 hrs) of chlorpyrifos, as well as the probable preponderance of this chemical in the positive response of *Tradescantia in situ*, are best supported by the results shown by *Drosophila*. The sex-linked recessive lethal test is the best validated mutagenicity test in *Drosophila*, and a positive response in this test is a strong indication of gene mutation and, more importantly, of chromosome aberration (Patnaik and Tripathy, 1992). Chlorpyrifos was also positive in most plant systems studied to date, although at higher concentrations than the ones assayed in the present research. The stickiness of the chromosomes observed in *H. vulgare*

pollen mother cells even after seed soaking treatment (Kaur and Grover, 1985b) indicates the clastogenic capacity of this chemical in plant cells.

Cyanazine has been shown to induce chromosome damage, represented by increased break frequency, in human lymphocytes *in vitro* at concentrations as low as 1 ppm (Roloff et al., 1992). This result, however, is in disagreement with other data reported for cyanazine genotoxicity as tested in the UDS and SCE assays in human peripheral lymphocytes (concentrations varying from 12 to 100 ppm) and a chromosome aberration analysis in rat bone marrow cells (doses varying from 56 to 224 mg/kg). Cyanazine was ineffective in all cases (Hrelia et al., 1994).

The rate of apparent dominant lethals in *D. melanogaster* was shown to increase when flies were fed a ration containing 0.01% cyanazine. The absence of effects in chromosomal aberration tests, however, led the authors to conclude that the decrease in egg hatchability was more likely due to physiologic toxicity to sperm (Murnik, 1976; Murnik and Nash, 1977).

The possibility of cyanazine being a promutagen was examined in *S. typhimurium* and *S. cerevisiae* assays employing plant (1S) and animal (S9) microsome homogenates, as well as *in situ* using the *Z. mays* waxy locus test (Means et al., 1988). Cyanazine was positive in the *Salmonella* assay only after 1S activation, and in the *Z. mays* assay. Additional evidence of the promutagenic nature of cyanazine was gathered when extracts of cyanazine-treated corn plants were shown to be mutagenic to *Salmonella* (Means et al., 1988).

Cyanazine was reported to induce chromosome fragmentation, bridges, and metaphase arrest in root meristematic cells of *H. vulgare* (Sharma and Panneerselvan, 1990). Cyanazine also induced chromosomal aberrations in

meristematic cells of root tips of *Tradescantia* and *V. faba*. The plants were exposed for up to 12 hrs to cyanazine concentrations varying from 200 to 600 ppm. There was a clear tendency for the effects to decrease as doses increased for the longer exposures in both plant species, implying acute toxicity of the compound (Ahmed and Grant, 1972b).

This low toxicity threshold of cyanazine has been observed also when the commercial formulations of cyanazine were assayed with *Tradescantia* in the present research. The highest micronuclei frequency was repeatedly observed at the intermediate concentration of 10 ppm, while a 50-ppm solution resulted in lower values. Cyanazine has been shown to be a direct acting mutagen as well as a promutagen in all plant assays considered. Moreover, cyanazine was shown to induce mutation *in situ* in the *Z. mays* assay at doses recommended in normal agricultural practice (Means et al., 1988).

Metolachlor induced chromosome damage in human lymphocytes *in vitro* at concentrations as low as 0.1 ppm (Roloff et al., 1992). The ability of animal microsomal homogenates to activate metolachlor into mutagenic metabolites was shown in *S. typhimurium* and in *S. cerevisiae* (Plewa et al., 1984). Metolachlor was also mutagenic in *S. typhimurium* directly (without activation). Moreover, a mixture of metolachlor and cyanazine induced mutations *in situ* in the *Z. mays* waxy assay (Plewa et al., 1984).

The mutagenic activity of metolachlor at such low levels and with these varied test systems is in agreement with the results obtained with the Trad-MCN assay. A positive dose-response relationship was observed in *Tradescantia* treated with metolachlor solutions with concentrations varying from 1 to 50 ppm. Although metolachlor has been shown to be activated by microsomal homogenates, similar to cyanazine, no external activation system is normally

needed in the Trad-MCN assay because the plant cuttings carry their own enzymatic complement (Ma, 1981).

The biological impact of these pesticides in the environment is a function of the concentration at which they are found, their availability, and their intrinsic biological activity. The three pesticides under investigation must be considered potentially genotoxic, since all of them have shown positive results in test systems ranging from prokaryotes to *in vitro* mammal assays, and two of them (cyanazine and metolachlor) were positive in *in vitro* human assays (Roloff et al., 1992).

In addition to the potential genotoxic impacts these compounds may have at the level of the agricultural field assessed here, their distribution in the environment may pose additional threats. Both cyanazine and metolachlor were among the most frequently detected pesticides in river water and in paired drinking water samples in a 6-yr study conducted in Canada (Frank et al., 1990). These compounds were present in almost 30% of the samples analyzed in both river and drinking water at concentrations of up to 10 µg/L for cyanazine and up to 28 µg/L for metolachlor. The maximum acceptable (interim) levels of these herbicides in Canada are 10 and 50 µg/L for cyanazine and metolachlor, respectively, or equal to the maximum level detected for cyanazine, and only less than twice the levels found for metolachlor (Frank et al., 1990). These two herbicides are also among the most common contaminants in rainwater (Richards et al., 1987). Cyanazine appeared in 25% of 325 rainwater samples collected in Iowa, at concentrations as high as 28 µg/L. Metolachlor occurred in 20.7% of the samples, with a maximum concentration of 2.70 µg/L (Nations and Hallberg, 1992). Cyanazine was also reported as a common contaminant in groundwater (Isensee et al., 1988). Although lower than the concentrations normally referred to

in evaluations of mutagenicity in laboratory conditions, the widespread presence of these compounds in the environment may have adverse consequences.

In the present assessment of the potential genotoxic effects posed by chlorpyrifos, cyanazine, and metolachlor to *Tradescantia*, each compound exerted some clastogenicity, even at the low concentrations found after recommended agricultural usage. It has also become apparent that, as detected through the application of a very sensitive bioassay system, the reduction in pesticide usage attainable through IPM programs may be insufficient to eliminate genotoxicity in the sprayed fields, as no reduction could be detected from the high to the medium pesticide treatment levels applied in the IPM program evaluated. This result is reinforced by the low treatment (no pesticides applied) not differing from the negative control either for the extracts of treated soils or for the *in situ* exposures.

Due to its extraordinary sensitivity, the Trad-MCN assay may be classified as a risk-averse system for environmental genotoxicity assessment (Ennever et al., 1988; Grant and Salamone, 1994). However, given the value of pragmatically achieving safer pesticide handling practices and considering the value of sound biological assessments in the judgment of any pesticide reduction program, additional evaluations are needed. Of special interest would be an evaluation of the possible mutagenic activity pesticides could have on the crop plants being treated, and the effectiveness of an IPM program in reducing the potential genotoxicity imposed by these pesticides to crop plants and weeds present in agricultural fields. In the experiments described in the following chapter maize, soybean, and *Tradescantia* were used to check *in situ* the results just described for the Trad-MCN assay.

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# 3. *In situ* Assessment of Pesticide Mutagenicity in an Integrated Pest Management Program with the *Tradescantia* Stamen Hair Mutation, the Maize Waxy Mutation, and the Soybean Leaf Mosaicism Assays

## 3.1. Introduction

Studies on the genotoxicity of the pesticides applied to an IPM program for corn and soybean utilizing the *Tradescantia* micronucleus (Trad-MCN) assay were described in Chapter 2. It was shown that it is possible to detect genotoxicity by exposing the plants *in situ* in recently-sprayed fields, to extracts of soil samples collected shortly after spraying, and to solutions of the commercial pesticides.

However, due to the extreme sensitivity of the Trad-MCN assay, a question remains regarding whether less sensitive subjects, or more specifically the crop plants actually being exposed to those pesticides in a regular basis, could respond in a comparable fashion under the same conditions. This information would be valuable for examining the possibility that an increased mutation rate could occur in pesticide-treated fields, and whether the reduction in pesticide application rate attained with an IPM program could effectively lessen such genotoxic response.

In order to address these questions, three bioindicator plants were chosen for an *in situ* assessment of mutagenicity in an IPM demonstration field for corn and soybeans. *Tradescantia* clone 4430 was once again utilized, with mutation being evaluated in the stamen hairs (Van't Hof and Schairer, 1982). This assay was chosen as a check for the results obtained with the Trad-MCN assay (Chapter 2), which employed the same clone 4430 under very similar conditions, and because of the large data base available for this assay in environmental mutagenesis studies.

The second assay employed was the soybean (*Glycine max* [L.] Merr.) leaf mosaicism test (Vig, 1982b), and the third was the maize (*Zea mays* L.) waxy forward mutation assay in the microgametophytes (Plewa, 1982). The latter two assays were chosen as representatives of the crops being tested in the IPM program. Details of the experimental conditions in the field, the pesticides applied and the treatment levels, and the design of the IPM program were given in Section 2.5.1.

In the present Chapter, the three plant bioassay systems are briefly reviewed, with emphasis on environmental mutagenesis studies, and the experimental conditions and results of the exposure of these plants *in situ* in the IPM demonstration field will be presented and discussed.

### *3.1.1.* Tradescantia *Stamen Hair Assay*

The stamen-hair mutation assay (Trad-STH) is a point mutation (mitotic) assay in which expression of the heterozygous dominant blue character of the stamen hair cells is prevented, resulting in the appearance of the recessive pink color (Nayar and Sparrow, 1967). Early studies with this system centered on the assessment of the genotoxic and cytotoxic effects of ionizing radiation and employed the meristematic cells of the stamen hairs of *Tradescantia* clone 02 as a higher organism surrogate for microbial cultures. In this assay, full growth of the hair was considered as equivalent to colony formation and stunted hairs as equivalent to nonsurvivors in cell cultures due to severe, highly deleterious or lethal events. In addition to mutation (color change) being used as an endpoint, genotoxic changes such as the expression of giant, twin or triplet cells, branching of the hair and other growth anomalies were recorded along with loss of reproductive integrity as indicators of genotoxicity (Nayar and Sparrow, 1967).

The genetic basis for the expression of pink cells in the stamen hairs of *Tradescantia* clone 4430 was established by means of reciprocal test-crosses with the parental pink- and white-colored *T. subacaulis* Bush (Emmerling-Thompson and Nawrocky, 1980). Pink pigmentation was determined to depend on a pair of alleles at a single locus, with blue (B) being dominant to pink (b), and clone 4430 was shown to be homozygous dominant for the white locus.

Sparrow and Underbrink (1972) studied the effects of neutrons and X-rays in the Trad-STH assay (clone 02), defining a linear dose-effect relation for both agents and a doubling dose as low as 1 rad for X-rays. The spontaneous mutation frequencies of several species and hybrids of *Tradescantia* were determined based on many years of experimentation at Brookhaven National Laboratory (Sparrow and Sparrow, 1976). Hybrids (i.e., clone 4430) and putative hybrids (i.e., clone 02) showed lower frequencies and a narrower variability in spontaneous mutation as compared with clones of pure species and were considered more suitable subjects for experimentation.

The effects of background radioactivity were studied by cultivating *Tradescantia* on monazite sand (Nayar et al., 1970). Mutation was found to increase in all exposed samples, and radionuclides absorbed into the plants were much more effective than external radiation alone. These results were later confirmed by exposing plants to soil samples drawn from the nuclear bomb experimental site at Bikini Island (Ichikawa and Ishii, 1991a). Soil samples which caused significant increases in mutation frequency were shown to contain $^{137}Cs$ and $^{60}Co$, among other radionuclides. The Trad-STH assay was used *in situ* as a monitor for ionizing radiation in the vicinity of nuclear power plants in a large study carried out in Japan (Ichikawa, 1981). Significantly increased mutation frequencies were correlated with wind direction and operation periods of the

nuclear facilities. Cebulska-Wasilewska (1992) observed an increase in the spontaneous mutation frequencies of *Tradescantia* correlated with the contamination caused in Cracow by the blowout of the nuclear reactor in Chernobyl (a 700-km distance).

The applicability of the Trad-STH assay to chemical mutagenesis studies was proposed by Underbrink et al. (1973) and tested in a comparison of the effects of ionizing radiation and gaseous EMS and DBE (Nauman et al., 1976). The responses to chemical agents showed characteristics similar to X-rays (exponential rise followed by saturation in mutant cell frequencies), and clone 4430 was more sensitive than clone 02. These results were later confirmed with a variety of chemicals and radionuclides (Tano, 1987, 1990; Tano and Yamaguchi, 1985). In these studies, mutagens were applied topically, directly onto the inflorescences. Doses as low as 5 to 20 pg for N-nitroso-N-methylurea and N-nitroso-N-ethylurea and 100 pg for EMS were effective and detectable in the Trad-STH test. The detection limit for external radiation was below 1 rad.

This high sensitivity of the Trad-STH assay to chemical mutagens was first shown after the accidental exposure of plants (clone 02) to fumes entering the air supply of a building at Brookhaven National Laboratory. A sudden increase in spontaneous mutation frequency raised the suspicion that led to the discovery of the contamination (Sparrow and Schairer, 1971). Additional studies with the Trad-STH assay evaluating the mutagenesis of chemical agents involve maleic hydrazide, EMS, N-nitroso compounds, and several organic solvents, among other agents (Badaev et al., 1989; Gichner et al., 1994; Gichner et al., 1982; Gichner et al., 1988; Kuglik et al., 1994; Veleminsky et al., 1987; Villalobos-Pietrini et al., 1986).

The Trad-STH has been shown to be capable of activating promutagens into direct-acting mutagens. Benzo-α-pyrene, atrazine, and several N-nitroso compounds were mutagenic when tested without prior treatment with microsomal fractions (Veleminsky and Gichner, 1988). A review of the mutagenicity of ionizing radiation and chemical agents in the Trad-STH assay was presented by Ichikawa (1992).

Perhaps the most important contribution of the Trad-STH assay was in the series of studies on atmospheric pollution carried out with a mobile laboratory (Schairer, 1979; Schairer and Sautkulis, 1982; Schairer et al., 1982). Air drawn from polluted sites around the U.S. induced higher mutation frequencies than filtered air samples from the same locations or air samples from the control site in the Grand Canyon. The mutagenicity of polluted atmospheres has been detected also in the vicinity of an oil refinery and petrochemical complex (Lower et al., 1983a), a lead smelter (Lower et al., 1978; Lower et al., 1983b), a pharmaceutical factory (Cebulska-Wasilewska and Guminska, 1987), and a municipal waste incinerator (Ma et al., 1993).

The mutagenic effects of chemical smokes used by the U.S. Army were evaluated with the Trad-STH assay. Positive responses were found for fogoil and tank diesel, as well as for their combination (Schaeffer et al., 1987). Ozone at concentrations occasionally found in polluted areas (300-800 ppb) was not mutagenic in the Trad-STH assay (Gichner et al., 1992), even though it had been reported as positive at higher concentrations (Schairer, 1979). A review of the Trad-STH as an assay for gaseous mutagens was presented under the U.S. EPA Gene-Tox Program (Van't Hof and Schairer, 1982).

In addition to studies of gaseous mutagens, the Trad-STH assay has been used to assess the mutagenicity of aquatic environments. Episodes of mutagenicity

in the water of a reservoir in Missouri were shown to be correlated with events facilitating the transfer of mutagens from the contaminated sediment to the water column (Lower et al., 1985). Grant et al. (1992) evaluated *in situ* the genotoxicity of the water in an area of Lake Superior in the vicinity of a pulp and paper mill using the Trad-STH, the Trad-MCN (micronuclei) and the *V. faba* chromosome aberration assays. Even though the Trad-STH was sensitive enough to detect mutagenicity, it was inferior to the other assays in terms of amenability for field manipulation. Remotely located field sites cause difficulties in cultivating the plants for the long (14-d) recovery period required in this test.

There is a scarcity of information on the mutagenic effects of pesticides in the Trad-STH assay. Tomkins and Grant (1972) studied the mutagenic effects of menazon (a *s*-triazine aphicide), metobromuron (substituted urea herbicide), and Daconil 2787® (chlorothalonil, chlorinated aromatic hydrocarbon fungicide) by wrapping the inflorescences of *Tradescantia* with cotton soaked in pesticide solutions (1500 ppm). No positive responses were found. The benzimidazole-derived fungicide Benlate® (benomyl) was tested for mutagenicity with the Trad-STH assay (clone KU 20) at doses commonly used in agriculture (0.5-4.0 g/L) (Sakamoto and Takahashi, 1981). Again, no positive responses were recorded. Contrasting with these negative results, seven out of nine insecticides were positive when tested with the Trad-STH assay using clone 4430 (Huang and Chen, 1993). Dichlorvos (0.1%), omethoate (0.04%), methamidophos (9.05%), Meobal® (3,4-xylyl methylcarbamate) (0.05%), mevinphos (0.006%), Amobem® (chloramben, actually a herbicide) (0.045%) and thiophanate-methyl (0.07%) gave positive results, while trichlorfon (0.1%) and Bassa® (2-*sec*-butylphenyl methylcarbamate) (0.02% - toxic) did not. Atrazine was reported to be mutagenic after chronic exposure (Schairer and Sautkulis, 1982), and cyanazine also has

been reported to be mutagenic in the Trad-STH system (Veleminsky and Gichner, 1988).

These studies show that the Trad-STH assay provides a sensitive, well standardized system for the examination of mutagenicity. This was recently shown in a study sponsored by the International Programme on Chemical Safety (Sandhu et al., 1994a; Sandhu et al., 1994b), when the Trad-STH test was evaluated in five different laboratories with four known chemical mutagens (Grant and Salamone, 1994). The agreement among the laboratories, although not perfect, was good enough to substantiate the reliability of the Trad-STH assay for the evaluation of chemical mutagens (Ma et al., 1994). The limited availability of data concerning pesticides in this system is counterbalanced by the large database on *in situ* exposure to environmental agents.

### *3.1.2. Soybean (*Glycine max *[L.] Merr.) Leaf Mosaicism Assay*

At least 11 loci affect the development of chlorophyll in soybean. One of these loci, designated $Y_{11}$, expresses incomplete dominance and is adequate for the evaluation of genotoxic events. Heterozygous plants ($Y_{11}y_{11}$) display a light green color easily distinguishable from the dark green ($Y_{11}Y_{11}$) and the greenish-yellow ($y_{11}$ $y_{11}$) homozygotes (Vig and Paddock, 1968). Chromosomal mechanisms such as deletion, duplication, translocation, and non-disjunction may alter the genotype of the primordial cells of the leaf, resulting in the appearance of dark green, yellow, or double spots on the light green background of a heterozygous plant. Seeds or primordial leaves of soybean (clone T219) seedlings treated with genotoxic agents may show increases in the frequency of spots (mosaicism), on the two single leaves and the first trifoliate compound leaf, permitting the evaluation of chromosomal damage. The size of a spot on a leaf

depends on how early in the development of the leaf the causative chromosomal event took place, reflecting the number of cell divisions from the moment of chromosomal damage to the completion of leaf expansion.

Another important mechanism for the development of mosaicism is somatic crossing over, in which cells attain complementary homozygosis, resulting in dark green/yellow mirror images called twin (or double) spots. Not all crossing over events may end up as twin spots, due to possible curtailment of mitotic activity in one kind of daughter cells. In general, however, it is possible to speculate on the main genetic mechanisms induced by a given agent by the types and proportions of spots present in the leaves (Vig, 1982b).

The relationship between mitotic anomalies and leaf mosaicism in soybean was established in a comparison of chromosome aberrations in the root tips and the frequency of leaf spotting (Vig, 1969). The responsiveness of leaf mosaicism in soybean to genotoxic agents was demonstrated when mitomycin C, an agent known to induce crossing over, caused a multifold increase in frequency of spots, especially twin spots (Vig and Paddock, 1968). The alkylating agent EMS caused predominantly single spots, as also did the chromosome-breaking agent daunomycin, indicating that mutations, deletions, and other anomalies are less effective in inducing twin spots (Vig and Paddock, 1970). Methyl methanesulfonate (MMS) was the most effective sulfonate tested in soybean. By way of contrast to EMS, MMS induced predominantly twin spots, probably due to single-strand breaks and chromatid exchanges (Vig et al., 1976).

The promutagen dimethyl nitrosoamine (DMNA) was shown to be activated to a direct-acting mutagen in soybean plants. A characteristic saturation curve resulted after treatment with increasing doses of DMNA, indicating saturation of the enzymatic apparatus, while the related agent methylnitrosourea,

which does not require activation, did not show saturation (Arenaz and Vig, 1978). Veleminsky and Gichner (1988) reviewed the evidence for promutagen activation in the soybean system. Even though the known promutagen benzo-α-pyrene did not cause increases in spot frequency in soybean, several nitrosoamines, DBE, furylfuramide, and azide did. Other agents inducing high frequencies of leaf spots were colchicine, EMS and other sulfonates, several nitroso compounds, γ-rays, X-rays, UV light, and $^3H_2O$, among others (Vig, 1978).

In order to better understand the genetic mechanisms involved in the appearance of leaf mosaicism in soybean, the effects of selected chemicals were reviewed and correlated with their most likely modes of action (Vig, 1981, 1982a, 1985). The capability of providing information on the genetic mechanisms involved in genotoxicity of a chemical is the main advantage of the soybean system. An analysis of all genetic mechanisms possibly involved in mosaicism and the most prominent spotting responses expected to arrive from each genetic anomaly was rendered in a review of the soybean leaf mosaicism system presented for the U.S. EPA Gene-Tox Program (Vig, 1982b).

The ability of the soybean system to predict carcinogenesis was evaluated in a test of a well-known liver carcinogen, the methionine analogue L-ethionine. A linear dose-response relationship was observed with concentrations ranging from 0.5 to 2.5 mg/ml (Fujii, 1981). The cytosine analogue 5-azacytidine, a negligibly mutagenic chemical capable of inducing demethylation of DNA, caused a linear dose-related increase in soybean leaf mosaicism with doses varying from 10 to 100 μg/ml. All types of spots were affected. Treatment with the base analogs 5-bromouracil and 5-fluorouracil also induced increases in leaf mosaicism, but not in twin spots (Katoh et al., 1993).

The DNA repair inhibitor cordycepin-3'-deoxyadenosine induced mutations in the soybean system (Inoue et al., 1986), but the strong tumor initiator dimethylbenzanthracene did not, even when combined with tumor promoters and other mutagens, such as ionizing radiation (Fujii and Inoue, 1987). The mutagenicity of heterocyclic amines formed in broiled meats was studied with the soybean system. A positive dose-effect relationship was obtained with concentrations as low as 0.01 to 0.1 µg/ml, and the incorporation of rat liver S9 microsomal fraction improved the response. The frequency of twin spots did not rise, indicating that mutation (forward and reverse), but not crossing over , was the main genetic mechanism involved in these effects (Katoh et al., 1992).

An attempt to use the soybean leaf mosaicism test in an *in situ* environmental mutagenesis study indicated a major deficiency of this bioassay. Even though simply soaking the seeds in the solution of an agent may be sufficient to cause an observable response in the seedling, *in situ* assessments commonly necessitate much longer exposures, sometimes 40 d or more. Under such conditions, the plants become subject to pests and unfavorable weather and can grow so poorly as to prevent evaluation, as reported in a study of the mutagenicity of the atmosphere in the vicinity of a lead smelter (Lower et al., 1978).

The soybean leaf mosaicism assay has been used in the assessment of the mutagenicity of pesticides. Of six pesticides tested with concentrations commonly used in agricultural sprays, Ekatin® (thiometon) was positive, while Anthio® (formothion), Baycid® (fenthion), diazinon, EPN (dichlofenthion), and Karphos® (isoxathion) were negative. None of the compounds was positive in the microbial *Salmonella* reversion assay, even after treatment with plant and animal S9 microsomal fractions, indicating that the mutagenic activity of thiometon may be specific for plants (Fujii and Inoue, 1983).

The paucity of data on *in situ* evaluations of genotoxicity with the soybean leaf mosaicism assay attest to difficulties in using this system in such studies. The susceptibility of soybean (clone T219) to pests and unfavorable weather, and the obvious interference that would arise with protective pesticide sprays are the main reasons for such difficulties. There might be a special concern in relation to the complex of mosaic virus-related diseases (Sinclair and Backman, 1989) which might cause confusion in scoring due to the symptoms induced by the virus, which include mottling, commonly with a yellow pattern. The characteristic crinkling of the leaves caused by the virus and a tendency to produce colored bands along the major veins, however, make it easy to differentiate the disease symptoms from the spots caused by gene conversion in $Y_{11}y_{11}$ leaves, which do not cause wrinkling and normally do not show any definite patterns.

The use of the soybean leaf mosaicism assay in the present study of *in situ* effects of pesticides was justified because soybean was one of the particular crops present in the IPM program under investigation. Results from this system would parallel the susceptibility shown by the crop plants, complementing the information provided with the more sensitive *Tradescantia*.

### *3.1.3. Maize (*Zea mays *[L.]) Waxy Pollen Grain Assay*

Because it is a classical subject for the study of genetics, more is known about the genetic makeup of maize (*Zea mays* [L.], $2n$=20) than of any other plant species (McClintock, 1984). Based on this extensive knowledge, at least 13 specific loci have been frequently used in mutation assessment in maize, involving alterations in color and/or morphology in leaves, endosperms, and pollen grains (Plewa, 1982). Among these varied endpoints, alterations in the pollen grains come forth as the most advantageous kind of trait. Firstly the haploid nature of

pollen greatly facilitates the expression and detection of mutations and other genetic events occurring in the mother diploid sporophyte and subsequently passed through to the microgametophyte (Nilan et al., 1981). Secondly, as genetic damage is analyzed directly in the pollen grains, the statistical resolution is greatly enhanced, for literally millions of individual subjects can be looked upon in the pollen population derived from a single exposed plant (Nilan et al., 1981).

Present in at least 15 angiosperm genera (Nilan and Rosichan, 1982), the most common trait lending itself to the analysis of mutagenesis is the Waxy (*wx*) locus (located in maize at position 59 of chromosome 9) which controls the production of the amylose component of the starch (Plewa, 1985a). The waxy trait of maize was introduced from China by the beginning of this century and was promptly recognized as different from the common American varieties of floury, sweet, flint, or pop. The waxy maize (*wxwx*) is characterized by the sole presence of amylopectin in the starch, giving an appearance of hard wax to the endosperm. In contrast, starchy (*Wx-x*) kernels present a mixture of amylopectin and amylose. Due to the presence of amylose, the endosperm of kernels carrying the *Wx* allele stain black when reacted with iodine, as opposed to the reddish-tan color produced by waxy kernels. Genetic studies showed that the waxy allele is recessive to starchy (*Wx*) and segregates as a Mendelian monohybrid. It was soon discovered that the waxy phenotype could be detected in pollen grains by the iodine test. As functional haploids whose starch type is determined by their own genetic constitution and not by the parental sporophyte, pollen grains offer a unique opportunity for detecting mutations altering the starch composition (Plewa, 1985a).

Two directional changes can be evaluated in the pollen grains of maize. A genetic reversion of *wx* to *Wx* can be detected by scoring black iodine-stained

pollen grains from homozygous plants producing reddish-tan (*wx*) pollen grains. Conversely, a forward mutation of *Wx* to *wx* can be detected by scoring reddish-tan pollen in the population of black-stained pollen grains from homozygous starchy (*WxWx*) plants. The forward-mutation assay (*Wx*→*wx*) is less specific than the reversion test, because not all genetic alterations in *wx* result in reversion to the capacity of producing amylose, whereas a mutation in *Wx* causing a loss in amylose production will be manifested as waxy (Plewa, 1982).

The amenability of maize plants to field conditions, coupled with the availability of inbred Early-Early Synthetic varieties, able to set tassels in ca. 40 d (Plewa, 1985b) and the ability to enzymatically activate promutagens into direct-acting mutagens (Plewa and Gentile, 1976b) render the waxy pollen assay in maize especially suited for the *in situ* study of mutagenic pesticides under agricultural conditions (Grant, 1982). This was confirmed when pre-emergence field-applied atrazine (0-10 lbs/acre) induced a dose-related increase in reversion frequency in maize exposed *in situ*. Atrazine alone normally does not induce mutation, but was shown to be activated into a direct acting mutagen by maize (Plewa and Gentile, 1976b).

The sensitivity of the maize waxy locus was shown in a multitechnique study comparing this assay with the Ames and the *Tradescantia* assays in an *in situ* screening of the mutagenicity of sewage sludge (Hopke et al., 1982). Sludge was added to the soil at a highest proportion of 1:3, and potted plants were watered with a maximum of 1/3 dilution of sludge. Mutation frequencies (forward and revertant) increased by up to two orders of magnitude even for the lowest concentration, while increasing concentrations of sludge caused a decrease in mutation frequency and an increase in pollen abortion, indicating toxicity of the sludge. These results were corroborated by the other species assayed.

In another multitechnique study, concentrated water samples (XAD-2 resin, with a concentration factor of 3000) from a lake surrounded by corn and soybean crops and treated municipal waters derived from the lake were assayed with the Ames test, and unconcentrated samples were assayed with the maize waxy reversion test. All the concentrated samples from the lake and some of the samples of tap water were highly mutagenic in the Ames test after activation with S9 microsome homogenates, but none of the unconcentrated samples induced mutation in maize. Chromatographic and mass spectrometric analysis of the water showed the presence of a variety of man-made pollutants, especially some known mutagenic pesticide residues (Heartlein et al., 1981).

In a series of *in situ* assessments of the environmental mutagenesis associated with industrial complexes, the maize waxy-revertant assay was compared with the *Tradescantia* stamen hair and the *Glycine* leaf mosaicism assays. Maize was more sensitive than *Tradescantia*, while soybean failed to produce scorable plants in an assessment of the mutagenicity associated with a lead smelter (Lower et al., 1978; Lower et al., 1983b). Revertant mutation dropped with distance from the smelter, and a monotonic variation was found for mutation and concentration of metals (Cd, Cu, Pb, and Zn) in the assayed soils, suggesting that soil metal contamination was the cause for the mutagenicity observed.

The same trend, with maize being more sensitive (26 times the control values for the exposed plants) than *Tradescantia* stamen hair mutation (5 times the control values), was obtained in an assessment of the mutagenicity of the environment in the vicinity of an oil refinery and a petrochemical complex (Lower et al., 1983a). It is likely that these outcomes were produced by airborne pollutants, attesting to the amenability of maize for responding to atmospheric

exposure. Constantin (1982) reviewed these studies evaluating atmospheric mutagens with the maize waxy assay, as well as the data available for γ-radiation and some pesticides. A linear increase in forward mutation was caused by increasing doses of γ-radiation (0 to 400 rad) in 2-hr exposures. Among the pesticides, *s*-triazines, heptachlor, chlordane and their combinations gave positive results, whereas metolachlor was negative.

Special attention has been devoted to the capacity of maize to activate promutagens, especially pesticides, into direct acting mutagens (Plewa, 1978). Most commonly, maize plants are exposed to pesticide-treated soils, or the plants are sprayed directly, and extracts of the plants are evaluated for mutagenesis with microbial bioassays in a modification of the host-mediated assay in mammals (Epstein and Legator, 1971). Alternatively, maize (or other plant) extracts are homogenized for microsomal preparation, and the chemicals to be tested are activated before being assayed (Rasquinha et al., 1988) in a fashion comparable with the use of S9 mouse liver microsomal homogenates commonly applied in the Ames test.

In one example of this type of assay, atrazine was shown not to induce mitotic gene conversion in *S. cerevisiae* when applied directly. However, water extracts of maize plants grown in atrazine-treated soils induced a dose-related increase in gene reversion in the yeast. Moreover, atrazine caused increased revertant mutation in the pollen grains of the exposed maize, indicating that the activated agent(s) would remain in the plant at least until microsporogenesis (Plewa and Gentile, 1976a).

A large number of pesticides have been evaluated for mutagenicity using this plant (and animal) activation/microbial assay technique (Gentile et al., 1982; Plewa et al., 1984; Plewa et al., 1979). In general, the results for homogenate-

activated chemicals in plant and animal tissues concur for both microbial and maize waxy assays. One very interesting exception is the *s*-triazine class of herbicides, which usually causes increased mutation after plant homogenate activation but frequently fails to do so after animal homogenate activation (Plewa et al., 1984).

This conclusion was contested by Sumner et al. (1984), who exposed maize to atrazine-treated fields *in situ* and evaluated the plant extracts for mutagenicity with the Ames test. No mutation frequency increases were noticed. By contrast with these findings and corroborating previous results, the water-soluble extracts of maize exposed to the three most important *s*-triazine herbicides (atrazine, simazine, and cyanazine) were mutagenic in the Ames test (Means et al., 1988). Employing $^{14}C$-labeled atrazine, it was shown that 89% of the mutagenic metabolites could be found in one fraction of the extract and that the mutagen(s) generated by all three compounds were similar.

Numerous studies are available involving maize homogenates and/or intact plants in the activation of promutagens, with subsequent assessment of mutagenicity with microbial bioassays. These studies include classic promutagens such as 2-aminofluorene, benzo-α-pyrene, pentachlorophenol, and *m*-phenylenediamine (Plewa et al., 1993; Plewa et al., 1988; Sandermann, 1988; Ysern et al., 1994), nitrosoamines (Callen, 1982), acetanilide herbicides (Gentile et al., 1977), and a variety of pesticides (Rasquinha et al., 1988). A review of this kind of study was provided by Veleminsky and Gichner (1988).

The importance of these studies lies partly in demonstrating that plants, including maize, can activate and subsequently channel hazardous chemicals into man's food chain (Sandermann, 1988, 1992). This capability has been substantiated in comparisons of animal and plant metabolism (Sandermann, 1982).

Although the major enzymatic reactions are quite similar, plants incorporate the significant process of compartmentation, accumulating metabolites and their conjugates that would normally be excreted in animals (Menn, 1978).

## 3.2. Multitechnique Assessment of Pesticide Mutagenesis in an IPM Demonstration Field *in Situ*

The three plant bioassays introduced above were employed in the *in situ* assessment of mutagenesis induced by the pesticides applied in an IPM program for corn and soybean. For details of the IPM program, the pesticides applied, and the demonstration field experimental design, see sections 2.4.1 and 2.5.1, and Table 2.7.

## 3.3. Material and Methods

### *3.3.1. Plant Stocks, Multiplication and Maintenance*

#### *3.3.1.1.* Tradescantia

*Tradescantia* clone 4430 was utilized in the *in situ* stamen hair mutation assay. Clone 4430 is a diploid hybrid between blue-flowered *T. hirsutiflora* Bush (2461C) and pink-flowered *T. subacaulis* Bush (2441). For more information on the origin, characteristics and maintenance of this clone, see section 2.5.3.

#### *3.3.1.2. Soybean*

*Glycine max* (L.) Merr. genetic line T219H was obtained from the USDA-ARS Field Crops Research Unit, Iowa State University, Ames, IA, from the stock

of Dr. Reid G. Palmer. Line T219H was provided as seeds harvested from a self-pollinated population of heterozygous (light green, $Y_{11}y_{11}$) plants. The seeds were propagated in the greenhouse (see section 2.5.3 for greenhouse conditions) by selecting and cultivating the heterozygous plants. These heterozygotes segregate as 1 dark green:2 light green:1 yellow lethal. As only the light green seedlings show mosaicism, the number of seeds sown for experimentation must be twice the number of subjects to be exposed and scored. Harvested seeds were air-dried and stored in paper bags in a well-ventilated area.

#### *3.3.1.3. Maize*

Maize (*Zea mays* L.) seeds of the Early-Early Synthetic variety were obtained from the Maize Genetics Stock Center, University of Illinois at Urbana-Champaign, from Mr. E. B. Patterson. The lineage employed in the experiments was homozygous *Wx*, consequently amenable for the forward mutation test. The seeds were propagated in a greenhouse (see section 2.5.3 for greenhouse conditions), and the harvested seeds were air-dried and kept in paper bags in a well-ventilated area.

### *3.3.2. Exposure, Sampling, and Analysis*

#### 3.3.2.1. Tradescantia *Stamen Hair Assay*

For each experiment, 25 to 30 cuttings (per treatment) containing young inflorescences were harvested 24 hrs prior to treatment and transferred to a controlled-environment chamber (for details see section 2.5.3). The cuttings were maintained in deionized water and aerated for 24 hrs for adaptation. The cuttings then were transferred to aluminum foil-wrapped beakers containing 1/3 dilution

Hoagland's solution and brought to the IPM demonstration field in an air-tight box on the day following pesticide application. Exposure consisted of placing the beakers containing the inflorescences on the humidified soil and covering them with inverted 20-cm PVC pots for 14 hrs (see section 2.5.5 for details). After exposure, the samples were returned to the laboratory in an air-tight box, the nutrient solutions were replaced by freshly prepared ones, and the cuttings were aerated during the recovery period.

The Trad-STH assay requires a recovery period of at least 14 d, the length of time needed for the flower buds containing the primordial stamen hair cells at the time of treatment to blossom, exposing the developed stamen hairs to be scored. During this recovery period, the cuttings were aerated in the controlled-environment chamber and the nutrient solutions were replaced daily. Wilted leaves were removed. From Day 8 on, the newly opened flowers were collected and the stamens were severed, placed on a microslide in a drop of 1:1 (v:v) glycerine:ethanol solution, and scored for pink events under 40x magnification with a stereo-microscope. The number of hairs per stamen for each treatment-day was estimated by counting the hairs in two anti-sepal and two anti-petal stamens of two randomly selected flowers (Ichikawa and Ishii, 1991b). A pink event (mutated cell) consisted of one or a string of adjacent pink cells between the dominant blue cells, at any position in the hair (Underbrink et al., 1973). At least five flowers were scored per treatment per day, totaling around 1800 hairs/treatment/day.

The number of pink events counted per flower was transformed to pink events/1000 hairs. The mean mutation frequency of each treatment was compared for all days pooled and for the day presenting the highest mutation frequency. Analysis of Variance (ANOVA) was used for comparing the means of all

treatments; Student's t-test, assuming two-tail distributions (no *a priori* tendencies assumed in the data) and $\alpha$=0.05, was employed when comparing control and treatment means.

### *3.3.2.2. Soybean Leaf Mosaicism Assay*

Seeds of soybean (T219H) were brought to the field on the second day after pesticide application and were planted manually amid the commercial corn seeds sown for the IPM program experiments. A total of 600 seeds were sown, representing 100 seeds for each of the three pesticide regimes, split into two blocks. Such a design should yield around 50 scorable seedlings, or 250 leaves per treatment per replicate, considering the genetic segregation expected for $Y_{11}y_{11}$.

Besides the presence of the "low" pesticide treatment level in the field, which represented a control for *in situ* exposure (no pesticide applied), a set of laboratory controls was concurrently established in the greenhouse. Soybean seeds were soaked in deionized water for 24 hrs for the negative control, and in a 100-ppm aqueous solution of EMS for the positive control. These seeds (100 per treatment) were washed with distilled water and planted individually in 8 cm recyclable cardboard pots containing a soil-peat moss-vermiculite mix (Cornell mix) supplemented with Osmocote® (slow release) fertilizer and micronutrient elements. Upon germination, the light green ($Y_{11}y_{11}$) phenotypes were selected while the homozygous seedlings were discarded. The seedlings were cultivated until the first compound trifoliate leaves expanded completely and were scored for spots with hand-held magnifying lenses. Scoring consisted of annotating, for each plant and leaf, the number and type (dark green, yellow, or double) of spots larger than 1 mm. Only spots clearly shown as a sector of differently colored cells as a

result of chlorophyll deficiency (yellow) or excess (dark green) were scored, excluding any apparently damaged areas of the leaf.

Field-sown seedlings were checked weekly for development and every other day when the compound leaves began to expand. Upon completion of leaf expansion, the seedlings were scored and eliminated from the population. Only leaves with at least 80% of their surface undamaged were considered for scoring.

The mean number of spots in each treatment, for total spots as well as for each spot type (dark green, yellow or double spots), were compared statistically by ANOVA ($\alpha$=0.05). In addition, to exclude any bias possibly introduced by the presence of multi-spotted leaves, the proportion of leaves presenting at least one spot (binomial response, mutated or non-mutated) was compared with the Chi-square ($\chi^2$) test.

#### *3.3.2.3. Maize Waxy Pollen Grain Forward Mutation Assay*

Early-Early Synthetic maize seeds were sown amid the commercial corn seeds planted for the IPM program experiments on the second day after pesticide application. Each seed was marked with a small flag, and upon germination each seedling was tagged in order to permit differentiation from the commercial corn plants. There were 50 seeds in each of the three pesticide treatment regimes in two blocks, or a total of 300 seeds for three replicated treatments.

Laboratory controls were established in addition to the *in situ* control for the low pesticide level. Maize seeds were soaked in deionized water (negative control) or in a 100-ppm EMS aqueous solution (positive control) for 24 hrs, thoroughly washed in distilled water, and sown individually in 12.5-cm recyclable cardboard pots containing a soil-peat moss-vermiculite mix (Cornell mix)

supplemented with Osmocote® (slow release) fertilizer and micronutrient elements.

Upon tasselling, portions of the inflorescences with unopened florets (pre-anthesis) were collected and fixed in 70% by volume ethanol. Scoring consisted of randomly selecting five florets, washing them in 70% ethanol to remove any extraneous pollen grains, and dissecting them to extract the anthers. The anthers were then minced in a petri-dish containing gelatin-iodine stain (500 mg potassium iodide + 95 mg iodine in 25 ml water, and 1.5 g of bacteriological gelatin in 25 ml water, mixed immediately before use) under a stereo-microscope. Any fragments were removed and the stained pollen grains were mounted on a microslide (Plewa, 1982).

The total number of pollen grains on the slide was estimated by counting (under 40x magnification) all full (viable) pollen grains in 20 randomly chosen 1-$mm^2$ grids of the microslide. The microslide was then carefully scanned for mutant (tan-colored pollen grains among the black-colored non-mutated population) and aborted pollen grains, which are easily recognizable by their withered appearance. Approximately 20,000 pollen grains were examined per tassel (per plant), and ten plants per treatment, totaling an average of 200,000 pollen grains per treatment. The frequency of mutated pollen grains was calculated for each plant, and the mean mutation frequency for the ten plants compared between treatments.

Due to the presence of plants showing no mutated pollen grains (an occasional zero among the normally large numbers resulting from the numerous pollen grains on a slide), a transformation was performed in the data before statistical analysis in order to normalize the variance. A transformation indicated

for counts of rare events was chosen from Snedecor and Cochran (1967) as follows:

$$Y = sqrt(X) + sqrt(X+1)$$

where $X$ is the observed and $Y$ is the transformed mutation frequency. This transformation resulted in a normalization of the variance caused by the presence of samples containing no mutated pollen grains. The transformed mutation frequencies and the rate of pollen abortion computed per plant was compared statistically between treatments by ANOVA ($\alpha$=0.05).

## 3.4. Results

### *3.4.1.* Tradescantia *Stamen Hair Assay*

A first step in the analysis of the results of the Trad-STH assay is the determination of the day in which the majority of the stamen hairs being formed at the time of exposure express themselves in the scorable flowers. Day 10 after exposure yielded the highest mutation rate for all treatments combined, indicating that this day was the most appropriate for analysis (Figure 3.1).

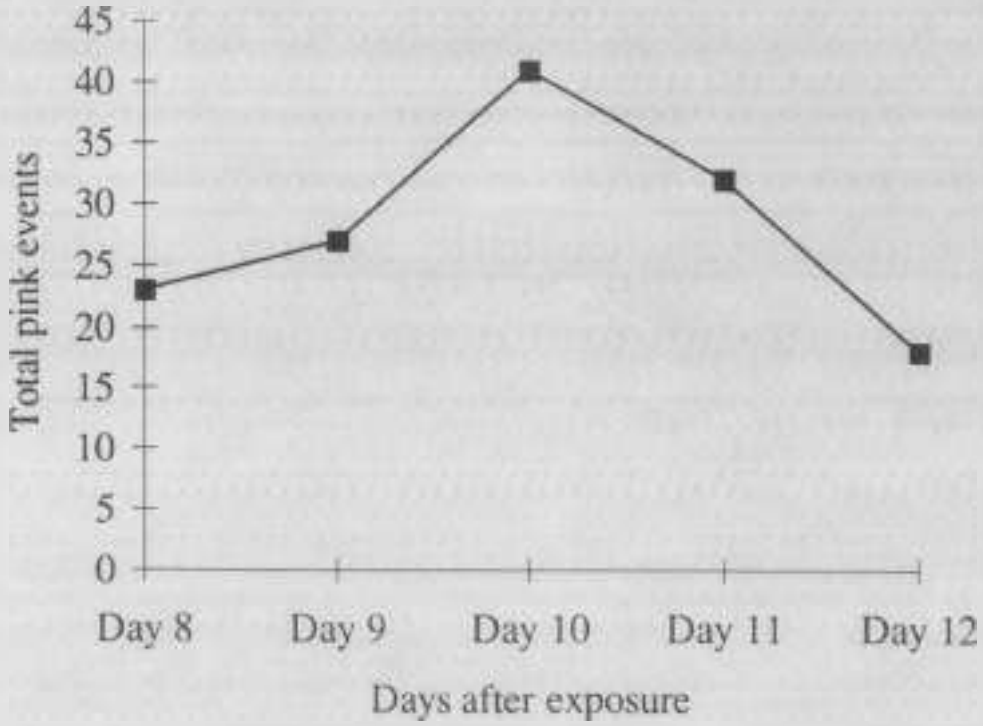



Figure 3.1 - Combined mutation rate in the Trad-MCN assay after *in situ* exposure to pesticide-sprayed soil. *Tradescantia* cuttings were exposed for 14 hrs by placing the beakers containing the cuttings directly on the wetted soil surface. The peak in total pink events at Day 10 indicates completion of the recovery period or the most sensitive day for scoring.

A statistically significant ($\alpha$=0.05) increase in mutation frequency was observed from the low to the high pesticide treatment levels in the Trad-STH assay 10 d following exposure (Figure 3.2). Contrary to the results obtained with the Trad-MCN assay (section 2.6.3), no increases occurred in mutation frequency for the Medium pesticide treatment level with the Trad-STH assay.

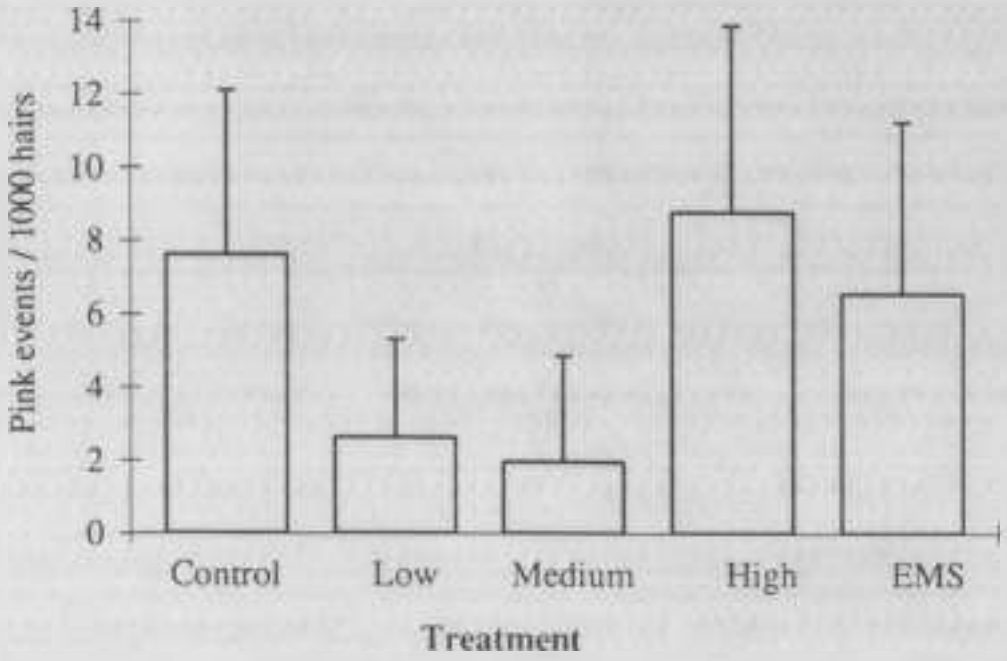



Figure 3.2 - *Tradescantia* stamen hair mutation at Day 10 after *in situ* exposure to pesticide-sprayed soil. The bars represent standard deviations of the mean. Day 10 represented the end of the recovery time, when the combined mutation rate for all samples was highest (see Figure 3.1). *Tradescantia* cuttings were exposed for 14 hrs by placing the beakers containing the cuttings directly on the wetted soil surface. The Control treatment was placed on soil covered with a plastic lining and the positive control (EMS) consisted of a 100-ppm aqueous solution of ethyl methanesulfonate placed also on plastic covered soil. The Low pesticide treatment received no pesticide, the Medium received banded cyanazine + metolachlor, and the High treatment received broadcast cyanazine + metolachlor and chlorpyrifos. The pesticides were sprayed 24 hrs before exposure began.

The Low pesticide treatment (no pesticides applied to the soil) induced a level of mutation consistent with the spontaneous mutation frequency of clone 4430 (Sparrow and Sparrow, 1976), suggesting that the exposure conditions were adequate. The EMS (positive control) treatment was effective in causing a significant increase in mutation over the spontaneous level (represented by the Low treatment), confirming Day 10 as the time at which the stamen hairs being formed at exposure time reached full development.

There was an inexplicably high mutation level associated with the negative control in this experiment, confounding the results. This high mutation level observed for the negative control was also detected when the results for all scoring days were pooled for analysis as can be seen in Figure 3.3.

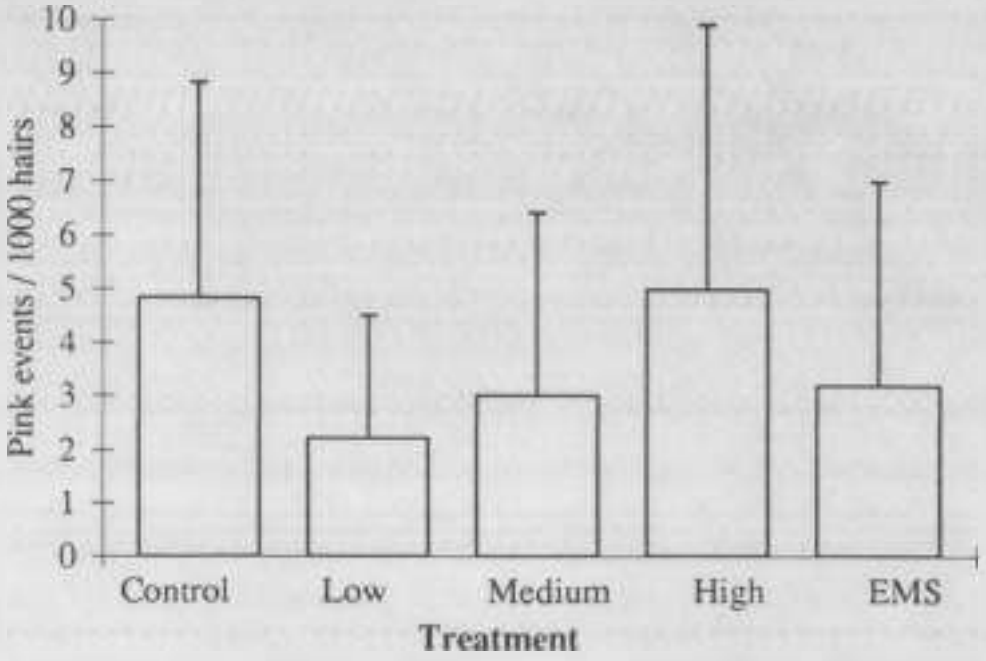



Figure 3.3 - Combined *Tradescantia* stamen hair mutation for Days 8 through 12 after *in situ* exposure to pesticide-sprayed soil. *Tradescantia* cuttings were exposed for 14 hrs by placing the beakers containing the cuttings directly on the wetted soil surface. The Control treatment was placed on soil covered with a plastic lining and the positive control (EMS) consisted of a 100-ppm aqueous solution of ethyl methanesulfonate placed also on plastic covered soil. The Low pesticide treatment received no pesticide, the Medium received banded cyanazine + metolachlor, and the High treatment received broadcast cyanazine + metolachlor and chlorpyrifos. The pesticides were sprayed 24 hrs before exposure began.

Again, the same trend was observed, with mutation frequency increasing from low to high pesticide treatment levels and with the negative control showing a mutation rate comparable to the positive control. Even though weakened by the

mutation level of the control, the results obtained with the Trad-STH assay show a trend that support the results of the Trad-MCN assay under the same conditions.

### *3.4.2. Soybean Leaf Mosaicism Assay*

Due to unfavorable weather, only a small portion of the soybean seedlings reached the growth stage required for scoring under field conditions (complete expansion of the first trifoliate leaf). Also, a large proportion of the leaves of the surviving seedlings were severely damaged by insects, reducing considerably the statistical population available for analysis. Unfortunately, the plants in the High pesticide treatment plot suffered the most severe attack, and no seedlings remained for scoring in this treatment.

In order to assemble the minimum leaf population for analysis, leaves from the two experimental blocks (replicate plots) established for each treatment were combined. Hence, only one statistical group remained available each for the Low and Medium pesticide treatments, accompanied by the laboratory negative and positive controls. In general, plants exposed *in situ* showed relatively lower spot frequencies than the laboratory-grown plants, probably due to a poorer leaf expansion and seedling development in the field. Due to this difference between field and greenhouse grown plants, the statistical comparisons were performed only within groups.

The spot frequency observed for the laboratory negative control was usually similar to the Medium pesticide treatment in the field, which in turn normally showed twice as many spots as the Low pesticide treatment. The laboratory positive control was usually significantly greater than the negative control (Figure 3.4).

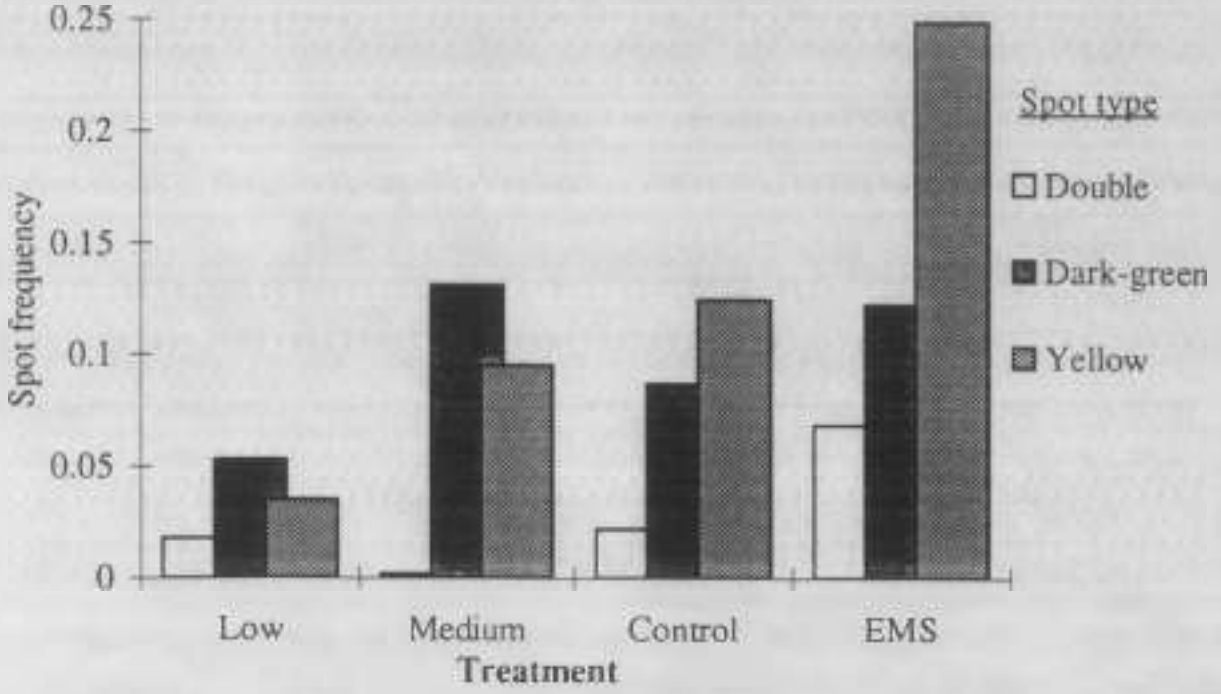



Figure 3.4 - Frequency of different types of spots on leaves of $Y_{11}y_{11}$ soybean seedlings grown in pesticide-sprayed soil *in situ*. The Control treatment consisted of seedlings grown in soil-peat moss-vermiculite mix in a controlled-environment chamber. The positive control (EMS) consisted of seeds soaked for 24 hrs in a 100-ppm aqueous solution of ethyl methanesulfonate and then grown as the Control. The Low pesticide treatment received no pesticide, and the Medium received banded cyanazine + metolachlor. The pesticides were sprayed 24 hrs before the seeds were planted in the field.

Even when combined replicates were considered, the populations of plants exposed *in situ* were not large enough to give statistically significant differences in most comparisons. With the exception of double spots, the mutation frequency observed for the Medium pesticide treatment level was more than double the frequency for the Low pesticide treatment. Nevertheless, only in the case of total spots did the means of the Medium and Low treatments differ significantly ($\alpha$=0.1). That this result stems from the small population size is inferred from the comparisons between the positive and negative laboratory controls. Although the actual differences between these treatments were not proportionally as great, the

positive and negative controls differed significantly ($\alpha$=0.05) in all but the comparison of dark green spots (Table 3.1). The statistical comparisons carried out with the $\chi^2$ test rendered the same results, pointing out a significant difference between the positive and negative laboratory controls and no difference for the means of the Low and Medium pesticide treatments in the field.

Table 3.1- Summary of spot frequencies and relative proportion of the different types of spots on the leaves of $Y_{11}y_{11}$ soybean plants (var. T219H) exposed to pesticide-treated soils *in situ*.

| Treatment | Number of leaves analyzed | Frequency of spots/leaf (%)[a] | | | | Relative proportion of spots | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Db | DG | Y | Tl | DG/Db | Y/Db | DG/Y | Tl/Db |
| Low | 57 | 1.7 | 5.2 | 3.5 | 10.5 | 3.1 | 2.1 | 1.5 | 5.9 |
| Medium | 84 | <1 | 13.0 | 9.5 | 23.8[b] | >10 | >10 | 1.4 | >10 |
| Control | 185 | 2.1 | 8.6 | 12.4 | 23.2 | 4.1 | 5.9 | 0.7 | 11 |
| EMS | 205 | 6.8[c] | 12.2 | 24.8[c] | 43.9[c] | 1.8 | 3.6 | 0.5 | 6.45 |

a - Db refers to double spots, DG refers to dark green spots, Y refers to yellow spots, and Tl refers to total spots.

b - Differs significantly from Low ($\alpha$=0.1).

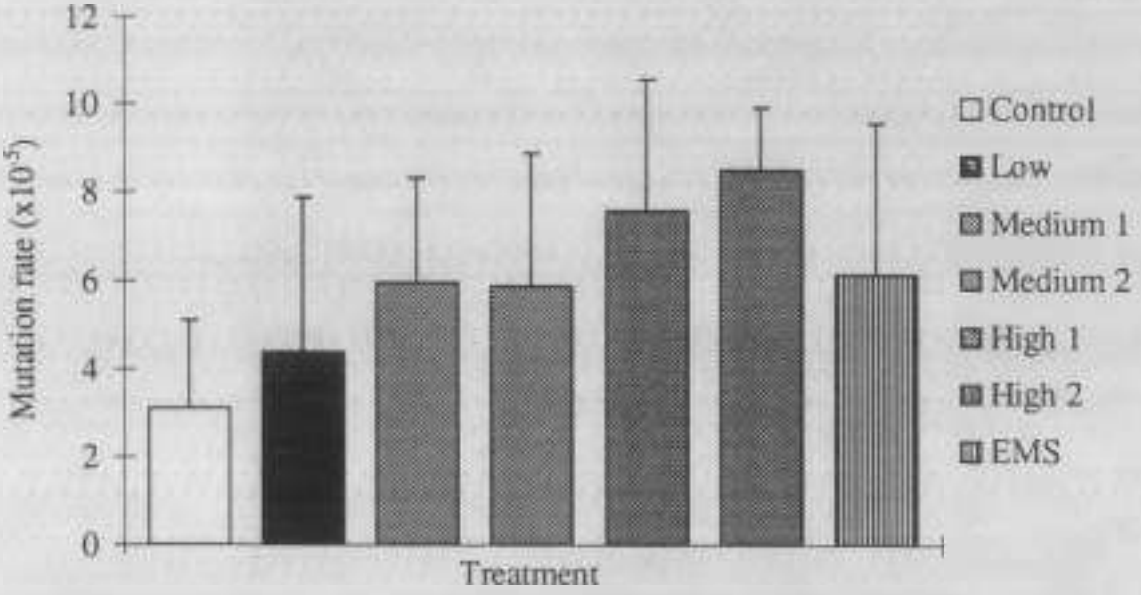
c - Differs significantly from Control ($\alpha$=0.05).

Analysis of the relative proportions of different types of spots (Table 3.1) shows the simple conversion from light green to either dark green or yellow as the most common outcome induced for the pesticide treatments as well as for EMS. As can be seen in the DG/Y column of Table 3.1 only a maximum of 50%

displacement was observed between dark green and yellow spots for any treatment, an indication that mutations and other genetic mechanisms capable of precluding the expression of one allele were the most common effects induced by all the treatments. The relative scarcity of double spots, which comprised only a small fraction of the total spots (column Tl/Db, Table 3.1), suggests that somatic crossing-over was not an important genetic mechanism induced either by the pesticides or by EMS (Vig et al., 1976). In summary, the soybean leaf mosaicism assay did not perform well *in situ*, due to the susceptibility of the seedlings to weather and predators. However, the system was sensitive enough to display broad differences between treatments even *in situ*, although failing to provide statistical significance due to the small population able to reach a growth stage suitable for scoring.

### *3.4.3. Maize Waxy Pollen Grain Forward Mutation Assay*

A statistically significant ($\alpha$=0.05) increase in forward mutation frequency occurred when maize plants were exposed *in situ* to the pesticide-sprayed field. If one considers the sequential pesticide treatments (from low to high) as increasing doses (Table 2.7), a dose response-like rise in mutation frequency occurred. The mutation frequency observed for the negative control ($2.66 \times 10^{-5}$), as well as for the Low pesticide treatment level ($6.45 \times 10^{-5}$), were well within the range of spontaneous mutation frequency commonly described in the literature (Hopke et al., 1982; Plewa and Gentile, 1976b). The positive control (EMS) showed a significantly higher mutation frequency than the negative control. The Medium pesticide treatment was intermediate between the Low and the High treatments, while these last two treatments were themselves statistically different (Figure 3.5).




*Transformed data: y=sqrt(x)+sqrt(x+1). Actual range=2.6 - 18.9*

Figure 3.5 - Forward waxy mutation in pollen grains of corn plants grown in pesticide-sprayed soil *in situ*. The Control treatment consisted of plants grown in soil-peat moss-vermiculite mix in a controlled-environment chamber. The positive control (EMS) consisted of seeds soaked for 24 hrs in a 100-ppm aqueous solution of ethyl methanesulfonate and then grown as the Control. The Low pesticide treatment received no pesticide, the Medium received banded cyanazine + metolachlor, and the High received broadcast cyanazine + metolachlor and chlorpyrifos. The pesticides were sprayed 24 hrs before the seeds were planted in the field.

There were no physiological toxic effects induced by the pesticides to the development of the microgametophytes, at least as indicated in terms of changes in pollen grain abortion rates. The average rate of pollen abortion (Figure 3.6) did not differ significantly between treatments and remained below 2%, inferior to the abortion rates normally cited (Heartlein et al., 1981; Hopke et al., 1982).

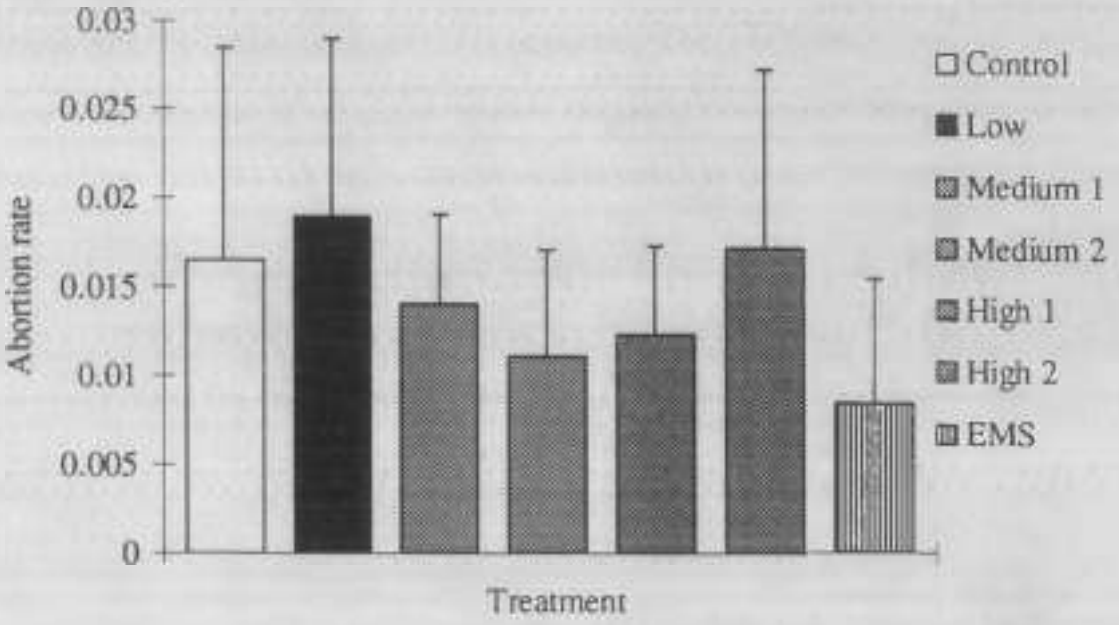



Figure 3.6 - Pollen abortion rate of corn plants grown in pesticide-treated soil *in situ*. The Control treatment consisted of plants grown in soil-peat moss-vermiculite mix in a controlled-environment chamber. The positive control (EMS) consisted of seeds soaked for 24 hrs in a 100-ppm aqueous solution of ethyl methanesulfonate and then grown as the Control. The Low pesticide treatment received no pesticide, the Medium received banded cyanazine + metolachlor, and the High received broadcast cyanazine + metolachlor and chlorpyrifos. The pesticides were sprayed 24 hrs before the seeds were planted in the field.

These results show that even after one single application at recommended doses at the beginning of the season, pesticides remain active and may induce mutations in exposed plants, in this case in the pollen grains formed last. Additionally, the response pattern shown by the maize assay, with no significant reduction in mutation frequencies from High to Medium pesticide treatment levels, corroborates the results obtained with the highly sensitive Trad-MCN assay. The similarity between the results obtained with these two essentially different assays strongly suggests that the reduction attained with the IPM

program being tested (from High to Medium application rates, Table 2.7) may be insufficient to abate the environmental mutagenesis of the pesticides used.

### 3.5. Discussion

The *in situ* exposure of *Tradescantia*, soybean and maize plants to pesticide-treated soils results in mutations detectable in all three assay systems. Two of the assays, however, yielded questionable or marginal data, due to an inexplicable high frequency of mutation events for the negative control in the Trad-STH assay, and to the small population of soybean seedlings that reached the scorable stage. The results from the maize assay indicated an increase in the mutagenicity caused by pesticide treatment of the soil.

There is no previous literature that reports the utilization of the Trad-STH assay in the *in situ* evaluation of pesticide mutagenicity. Nevertheless, this assay has been shown to be adequate for the appraisal of pesticide mutagenicity (Gichner et al., 1982; Huang and Chen, 1993; Sakamoto and Takahashi, 1981; Tomkins and Grant, 1972). Of the three pesticides to which *Tradescantia* was exposed in the present experiments, only cyanazine had been tested previously with the Trad-STH assay (Veleminsky and Gichner, 1988), and showed a positive response.

The Trad-STH assay proved adequate for *in situ* exposure under agricultural conditions, tolerating well the long recovery period required after field exposure and successfully setting sufficient flowers for scoring, besides being responsive to the low levels of pesticides present at the time of exposure. The final results of this assay, however, were unreliable due to the high mutation frequency of the negative control.

The soybean leaf mosaicism assay has been sparsely used as an indicator of pesticide mutagenicity, and no data are available on the utilization of this system for the *in situ* evaluation of pesticides. A major constraint of this test system is the difficulty of growing healthy seedlings in the field without employing protective chemicals that would potentially interfere with the results. The susceptibility of the heterozygous plants to insects and other pests seemed to be more severe than for the more vigorous homozygous plants.

Despite the diminished statistical power caused by the small number of seedlings that attained the developmental stage appropriate for scoring, the results obtained with the soybean leaf mosaicism assay suggest that the pesticide application rate prescribed in the IPM program under investigation may induce an increased level of mutation in relation to a no-pesticide regime. The frequency of spots occurring on leaves of seedlings growing on sprayed soil was more than twice that of the non-sprayed soil. It became clear, however, that some sort of protection must be afforded to the seedlings for *in situ* exposure, because the poor development under unfavorable field conditions seemed to interfere negatively with the production of spots, as suggested by the higher spot frequency obtained with the greenhouse grown seedlings in relation to the field-grown seedlings.

The proportions of the different types of spots observed in the soybean assay are an indication of the type of genetic mechanism likely to be in effect for that particular chemical or exposure condition. As a general rule, single (dark green and yellow) spots are caused by mutational events, such as deletion, duplication, translocation, and non-disjunction. These mutations interfere with the expression of one allele of the pair $Y_{11}y_{11}$, while allowing the other to predominate and determine the chlorophyll content of the spot and hence its coloration. Double spots, on the other hand, result from crossing-over, even

though not all crossing-over events may manifest as double spots, due to a possible failure of one of the primordial cells carrying the altered genotype to develop. In the present experiments, single spots greatly predominated over double spots, both for the *in situ* exposure to pesticides and for the EMS treatment.

As a known alkylating agent, EMS has been shown to induce mainly single spots on soybean leaves (Vig, 1981; Vig and Paddock, 1970), due to the induction of chromosome fragments and their subsequent loss. Such a mode of action is in agreement with the capacity of EMS, and apparently also of the pesticides under investigation, to induce micronuclei in the Trad-MCN assay, as reported in the previous chapter. Even though not especially adaptable to field exposure the soybean leaf mosaicism assay provides the advantage of allowing for speculation on the genetic mechanisms involved in the mutational outcomes observed. The simple use of physical barriers, such as cloth nets for protection against pests, could greatly enhance the applicability of this bioassay for *in situ* studies.

The maize waxy pollen grain assay has been extensively used for the *in situ* assessment of pesticide mutagenicity, alone or incorporating microbial assays. All three pesticides applied in the IPM program under investigation had been previously evaluated for mutagenicity with the maize waxy pollen assay. Cyanazine was shown to be mutagenic in the reversion test, as well as in two microbial assays (*Salmonella* and *S. cerevisiae*) after being activated with maize microsomal homogenates or with extracts of maize plants exposed to the compound (Plewa, 1978). In the same series of experiments, metolachlor gave negative results. The mixture of cyanazine+metolachlor was positive, but whether such an effect was caused by the *s*-triazine alone or by any additive effect of metolachlor could not be resolved.

In a later study (Plewa et al., 1984), metolachlor mixed with the non-mutagenic herbicide dicamba produced negative results. This time, however, extracts of maize exposed to metolachlor, as well as microsomal homogenate-activated metolachlor, were mutagenic in the *Salmonella* and *S. cerevisiae* assays. Chlorpyrifos was shown not to induce mutation in the maize waxy pollen reversion test, nor in the *Salmonella* or in the *S. cerevisiae* mutation assays (Gentile et al., 1982).

The results of the present experiments corroborate the adequacy of this assay for the *in situ* assessment of pesticide mutagenicity. The short generation time (ca. 40 d from seed to tasselling) of the Early-Early synthetic variety allows for relatively expeditious experimentation and differentiation between the experimental subjects and commercial varieties when they are mixed in the field, as in the present study.

A clear and significant increment in mutation frequency was observed for the High pesticide treatment level in relation to the non-sprayed (Low pesticide treatment level) soil. The pesticide application rate recommended in the IPM program under investigation (Medium treatment level) showed a mutation frequency intermediate between High and Low pesticide treatment levels. The decrease in relation to High treatment, however, was not statistically significant, which agrees with the results previously described for the Trad-MCN assay. In other words, the results yielded *in situ* by the maize waxy pollen assay suggest that the reduction in pesticide dosages attained with the implementation of the IPM program proposed may not be sufficient to prevent mutagenicity of the pesticides.

The conclusion that a partial reduction in pesticide application rate may be ineffective in abating mutagenic impacts indicates that a different strategy is

warranted if concern about environmental mutagenesis should result in changes in agricultural management. Such a strategy should rely on extensive testing and selection for field use of alternative, non-mutagenic compounds. Furthermore, the agreement observed among the several test systems employed in the present investigation attests to the appropriateness of plant bioassays for the assessment, especially *in situ*, of environmental mutagenicity in agricultural fields.

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