Genotoxicity of the Herbicide 2,4-Dichlorophenoxyacetic acid (2,4-D): Higher Plants as Monitoring Systems

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ABSTRACT : Higher plants provide valuable genetic assay systems for screening and monitoring environmental pollutants. They are now recognized as excellent indicators of mutagenic effects of environmental chemicals and are applicable for the detection of environmental mutagens both indoor and outdoor. 2,4-dichlorophenoxyacetic acid (2,4-D) is a herbicide commonly used in agriculture. The residues of 2,4-D are present in air, water, soil and edible plants. It constitutes a real hazard to the public health because it's wide spread use in agriculture. Genotoxic effects of 2,4-D on plant cells and potential of higher plants as a biomonitoring system for detecting chemical mutagens are evaluated. It is recommended that higher plant systems have been accepted by regulatory authorities as an alternative biomonitoring system for the detection of possible genetic damage resulting from pollution and the use of environmental chemicals.

Keywords : 2,4-Dichlorophenoxyacetic acid (2,4-D), Genotoxicity; Plant Biomonitors.

INTRODUCTION

Many pesticides are not mutagens by themselves but become active by metabolic transformations. Thus, a promutagen may be converted into a mutagen through metabolic activation. Plant activation is the process by which a promutagen is activated to a mutagen by plant enzymatic systems. Many food plants are exposed to pesticides and other chemicals used in agriculture (Plewa, 1978; Plewa and Gentile, 1982). Genetic toxicology is a multidisciplinary field of research involved in detecting DNA damaging and protective compounds, understanding the biological consequences of DNA damage and their molecular modes of action that lead to alterations and repair of the genetic material. The detection of genotoxic effects requires the use of concentration procedures or of highly sensitive detection systems that can be used for *in situ* monitoring. Since all currently available concentration procedures lead to the loss of potentially active compounds, the second alternative is preferable. A number of plant

bioassays have been developed, that can be used for *in situ* environmental monitoring (Uhl et al., 2003). Most of the indicator species have been employed in genetic research for many decades and important features include the availability of information on their genomic structure, a small number of chromosomes which is ideal for aberration analyses. The chlorinated aromatic hydrocarbon acid pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) has become substantial environmental pollutants since they are widely used as hormonic herbicides. While at low concentrations 2,4-D acts as an auxin analogue promoting plant growth, at high concentrations it is lethal and used as herbicide against broad-leafed and woody plants (Sinton et al., 1986; Devine et al., 1993; Tripathy et al., 1993). This review presents literature data that refer to genotoxic potential of 2,4-D and its molecular mode of action on plant cells.Types of plants used as biomonitoring systems are discussed. Examples of mutagenic studies with 2,4-D indicating the usefulness of higher plants as a monitoring system are reviewed.

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Background of 2,4-D

2,4-D, a member of the chlorophenoxy family of herbicides (CAS: 94-75-7, chemical formula: $C_8H_6Cl_2O_3$; molecular weight: 221) was the first successful selective herbicide developed. It was introduced in 1946 and rapidly became the most widely used herbicide in the world. Registered forms of 2,4-D include 2,4-D acid, 2,4-D dimethylamine salt (DMAS), 2,4-D isopropyl acid (IPA), 2,4-D triisopropyl acid (TIPA), 2,4-D ethylhexyl ester (EHE), 2,4-D butoxy ethyl ester (BEE), 2,4-D diethyl amine(DEA), 2,4-D isopropyl ester (IPE), and 2,4-D sodium salt. 2,4-dichlorophenoxyacetic acid (2,4-D) is a moderately persistent chemical with a half-life between 20-200 days. Unfortunately, the herbicide does not target only weeds. It can cause low growth rates, reproductive problems, changes in behavior, or death in non-tagged species.The production and degradation of 2,4-D leads to the creation of many compounds including chlorophenols (Michalowicz, 2005; Bukowska, 2006).

Application of 2,4-D

According to US Environmental protection Agency (EPA, 2005), 2,4-D kills plants by increasing three characteristics of the plant: i) the plasticity of the cell walls, ii) the amount of proteins being made in the plant, iii) the amount of ethylene being produced by the plant. The effect of these changes is to cause cells to divide and the plant to grow uncontrollably. The end result is that the tissues of the plant are damaged and death occurs. Upon 2,4-D treatment, its molecules penetrate into plants through the above-ground tissue. Transport of 2,4-D within a plant terminates in zones of active growth, where the pesticide suppresses processes of oxidative phosphorylation and synthesis of nucleic acids in intensively dividing cells, producing a reduction in the amount of auxins. This leads to the formation of mis-shaped leafs, injured reproductive organs and die-back of plants (Khalatkar and Bhargava, 1982). After 64 years of use, 2,4-D is still the third most widely used herbicide in the United States and the most widely used worldwide. Its major uses in agriculture are on wheat and small grains, sorghum, corn, rice, sugar cane, low-till soybeans, rangeland, and pasture. It is also used on rights-of-way, roadsides, non-crop areas, forestry, lawn and turf care, and on aquatic weeds. Currently over 600 end-use products are registered for use on over 300 sites.

Molecular mode of action

Unlike other herbicides that have specific targets in the cell (by interacting with some metabolic pathways) the intimate mode of action of herbicide 2,4-D is less known. Some authors described a mutagenic and genotoxic potential for 2,4-D which increases the mutation frequency in a concentration-dependent manner (Kumari and Vaidyanath, 1989; Pavlica et al., 1991). In plants, 2,4-D stimulates some enzymatic activities such as phospholipase A2 (Scherer and Andre, 1989), cytochrome P-450 and several hydroxylases (Mougin et al., 1991; Topal et al., 1993), and an NADH oxidase localized on the external cell surface (Hicks and Morre, 1998). Grossmann et al. (2001) reported that the application of auxinic herbicides, together with an abscisic acid (ABA) and ethylene-mediated effect, led to an overproduction of H_2O_2 which was involved in the induction of tissue damage and cell death. However, in plants there is very little biochemical and molecular information on the effect of 2,4-D on different reactive oxygen species (ROS)-mediated enzymatic systems, proteolytic activity, and lipid peroxidation, as well as on the oxidative modification of proteins and its further degradation by herbicide-induced proteases (Mohandas and Grant, 1972; Ateeq et al., 2002). The action of 2,4-D in plants concerns mostly disrruption of the hormonal equilibrium of the auxin-cytokinin system (Grabinska-Sota et al., 2003). At present it is known that 2,4-D can disturb some chemical reactions, it binds enzymes and change their activity, interacts with phospholipids. The toxicity of many compounds concerns the formation of (ROS), including superoxide anion, hydrogen peroxide, superoxide radical and hydroxyl radical (Mates, 2000; Bukowska, 2006). 2,4-D can bind to certain phospholipids and disturb physical

interactions in membrane, which probably increases the availability of lipids to peroxidation (Pogosyan et al., 1984). The induction of lipid peroxidation in pea microsomes may be a result of the production of free radicals formed during metabolism of carbon. Investigations led by Watahiki et al. (1995) show that 2,4-D inhibits glutathione s-transferase (GsT) activity. The authors suggested that it may occur when 2,4-D effects as a non-substrate ligand that modifies GsT activity or binds itself as a substrate with GsT and then GsH. The mechanisms of genotoxicity and mutagenicity of 2,4-D are poorly understood, and the available genotoxicity data is controversial. Generally, many reports confirmed genotoxic properties of 2,4-D and those could be considered as the ultimate 2,4-D may be connected with amino acids asparaginic acid and glutaminic acid and alanine, and also isoleucyne, phenylalanine and tryptophane. Among animals and plants 2,4-D connects proteins to form complexes (Sugaya and Sakai, 1996). The incoherent genotoxicity results may be attributable to i) Methodologies and treatment protocols, ii) The selection of compositionally different 2,4-D salts and acids could lead to different absorption and metabolism, iii) Type of solvents in each experiment could lead to different absorption and metabolism

rates vi) Dose concentration, type of plant cells and type of tissue.

Metabolism of 2,4–D in plant cells

Plants hydrolyse 2,4-D esters to 2,4-D, which is the active herbicide. Further metabolism in plants occurs through three mechanisms, i) side-chain degradation, ii) hydroxylation of the aromatic ring, iii) conjugation with plant constituents (Loos, 1969). Degradation of the side-chain of 2,4-D has been observed in many plants. Luckwill and Lloyd-Jones (1960) suggested two degradation pathways leading to the formation of 2,4-dichlorophenol. Thomas et al. (1964) and Feung et al. (1973) identified 2,5-dichloro -4-hydroxyphenoxyacetic acid as major phenolic acid metabolites. Evidence was found by Fleeker and Steen (1971) indicating hydroxylation resulting in the elimination of the 4-chloro constituent from the aromatic ring, in addition to migration of the chlorine at the 4-position to an adjacent carbon on the ring. Studies indicate that resistant crops such as grasses form water-soluble conjugates with sugars; whereas sensitive broad-leaved crops such as beans

Table 1. Types of plants used as biomonitoring systems

Latin name	Common name	No. (χ)*	Family
Zea mays	Maize	2n=20	Poaceae
Tradescantia	Spiderwort	2n=24	Commelinaceae
Pisum Sativum	Pea	2n=14	Fabaceae
Secale cereale	Rye	2n=14	Poaceae
Impatiens balsamina	Balsam	2n=16	Balsaminaceae
Allium cepa	Onion	2n=16	Alliaceae
Allium sativum	Garlic	2n=16	Alliaceae
Arabidiopsis thaliana	Mouse ear cress	2n=10	Brassicaceae
Hordeum vulgare	Barley	2n=14	Poaceae
Lycopersicum esculentum	Tomato	2n=24	Solanaceae
Crepis capillaris	Hawksbeard	2n=6	Asteraceae
Lilium longiflorum	Easter lily	2n=24	Liliaceae
Triticum aestivum	Wheat	2n=42	Poaceae
Oryza sativa	Rice	2n=24	Poaceae
Vicia faba	Broad bean	2n=12	Fabaceae
Phaseolus vulgaris	Common bean	2n=22	Fabaceae
Glycine max	Soybean	2n=40	Fabaceae
Nicotiana tabacum	Tobacco	2n=48	Solanaceae
Sorghum vulgaris	Sorghum	2n=20	Poaceae

*: Number of chromosomes

form water- insoluble amino acid conjugates (Montgomery et al., 1971; Feung et al., 1973 and 1975).

Types of plant biomonitors

Several higher plants provide unique and valuable systems for detecting and analyzing the effects of chemical mutagens (Table 1).

Such plants include maize (*Zea mays*), barley (*Hordeum vulgare*), tomato (*Lycopersicon*), mouse-ear cress (*Arabidopsis thaliana*), soybean (*Glycine max*), broad bean (*Vicia faba*), spiderwort (*Tradescantia*), onion (*Allium cepa*), Hawk's beard (*Crepis capillaris*), lily (*Lilium*), pea (*Pisum sativum*), and tobacco *(Nicotiana tabacum)*. Several numbers of assays have been validated and standardized to stimulate routine use in the detection of environmental mutagens (Grant, 1994). The most commonly assays used for studying mutagenicity of various pollutants in plants are based on the detection of chromosomal aberrations in *Allium cepa* (Fiskesjo, 1995; Ma et al., 2005), *Tradescantia* (Ichikawa, 1992), *Vicia faba* plants (Kanaya et al., 1994) or *Zea mays* (Grant and Owens, 2006). An *Allium cepa* chromosome aberration test that can serve as a rapid screen for toxic effects of chemicals is among them (Grant, 1994; Bolle et al., 2004). Due to its sensitivity, the *Allium cepa* test was the first of nine plant assay systems evaluated by the Gene-Tox Program of the US Environmental Protection Agency (Grant, 1994). *Tradescantia* is another important plant for mutagenesis studies. This plant makes it possible to perform chromosome aberration, stamen-hair mutation and micronuclei formation assays (Rodrigues et al., 1997). Various chemicals have scored positive in the *Vicia faba*-based sister chromatide exchange assay (Rank et al., 1994; Ma et al., 2005). In some systems, e.g. in tests with maize, morphological changes of the pollen are used, or in the case of *Arabidopsis thaliana*, changes in the color of the embryos. Soybean (*Glycine max*) and tobacco (*Nicotiana tabacum*), formation of mosaicism which leads to leaf spots varying in their color and morphology; detection of somatic crossing over, chromosome deletions, nondisjunction and point mutations

are used (Vig, 1982). Rice (*Oryza sativa*), induction of chlorophyll mutations and occurrence of mutations in the waxy locus of pollen are used (kumari and Vaidyanath, 1989). In recent years, significant progress in generation and development of transgenic plants as biomonitors has been made (Lebel et al., 1993; Kovalchuk et al., 1998 and 1999; Ries et al., 2000, Kovalchuk et al., 2001, Besplug et al., 2004; Li et al., 2006, Boyko et al., 2007; Van der Auwera et al., 2008). One of the important advantages of transgenic biosensors is the ability to customize the assay in accordance with monitoring needs. Transgenic plant biomonitors used for the evaluation of genotoxicity are *Arabidopsis thaliana* and *Nicotiana tabacum* plants (Kovalchuk and Kovalchuk, 2008). The *Lycopersicon esculentum* assay is a very good plant bioassay for assessing chromosome damage both in mitosis and meiosis and for somatic mutations induced by chemicals. *Pisum sativum* has been used for studying all the cytological endpoints that follow treatment of chromosomes by chemical and physical agents. (Grant and Owens, 2002). Detailed descriptions of these assays can be found in Plewa (1982); Sandhu et al. (1989); Grant (1994); Kanaya et al. (1994); Ma et al. (1994 and 1995).

Advantage and disadvantages of plants as biomonitors

The major advantages of plants that should be considered in mutagen screening and monitoring are summarized in Table (2). The advantages in utilizing plant systems have been reviewed by many authors (Mann and Story, 1966; Nilan, 1978; Conte et al., 1998). The most serious disadvantage of a plant system for the detection of genetic risks to man is the lack of similarity between vegetative and mammalian metabolism. Plant assays lacks general acceptance because of the problem that faces all higher plant test systems: namely, plant cells are distantly separated physiologically and phylogenetically from human cells (Nilan, 1978). However, it has been shown that plants have a higher degree of relevance than might be expected from the pharmacokinetic properties between plants and animals and appear to be most suitable for a risk-averse program,

Table 2. Genotoxic potential of 2,4-D in plant cells

Plant	Type of changes	References
Allium cepa	Chromosomal aberrations Chromosome fragmentation Chromosome stickiness and clumping	Mohandas and Grant, 974 Kumari and Vaidyanath,1989 Grant, 1978
Allium cepa	Chromosomal aberrations Clastogenicity Sister-chromatid exchange Mitotic index	Ateeq et al. 2002 Dolezel et al. 1987
Arabidipsis thaliana	Homologous recombination (A---> G mutation)	Filkowski et al. 2003
Oryza sativa	Chromosomal aberrations	Kumari and Vaidyanath,1989
Pisum sativum	Chromosomal aberration in meiosis C-mitosis DNA damage	Grant and Owens, 2001 Romero-Puetas et al.2004
Secale cerale	Endoreduplication	Grant, 1978
Triticum aestivum	Sister-chromatid exchange	Murata, 1989
Sorghum	Meiotic chromosome aberration	Pelwa 1978
Hordeum vulgare	Mitotic chromosome aberration	Geras'kin et al. 2006

as negative results are unlikely, but quite predictive when they occur (Ennever and Rosenkranz, 1986; Ennever et al., 1988). The relevance of higher plant genotoxic bioassays has been discussed in detail (Fiskesjo, 1995; Grant, 1994; Grant and Owens, 2002).

Types of changes induced by 2,4-D

Chromosomal aberration: In plant cells 2,4-D induces mitotic and meiotic irregularities both in vivo and in vitro (Khalatkar and Bhargava, 1982). Furthermore, the most frequent types of aberrations induced are fragments, erosion, bridges, laggards, micronuclei as well as Chromosome aberrations have been used as a measure of reproductive success in plants for many years and have been correlated with morphological and taxonomical changes, fertility-sterility relationships, mutations, and other characteristics polyploidy and aneuploidy (Fiskesjo et al., 1981; Pavlica et al., 1991). Contamination of the soil by 2,4-D herbicide at the application rates recommended for agricultural use resulted in a significant increase in aberrant cell frequency. In *Allium* root tips 2,4-D caused changes in mitotic activity, as well as changes in chromosome and chromatin structure, and also changes during the cell cycle (Geras'Kin et al.,

2006). The mutagenic activity of 2,4-D was assessed utilising cytogenetic, chlorophyll mutation, specific-locus and pollen viability endpoints in *Allium cepa* and *Oryza sativa* (Pavlica et al., 1991). In the Allium root-tip assay the frequency of aberrations was estimated. With an increase in concentration there was rise in frequency of aberrant cells. In the case of mutation assays, with increasing concentration, there was a concomitant increase in mutation frequency. Highly significant chlorophyll-deficient and waxy mutants besides sterile pollen were observed (Kumari and Vaidyanath, 1989).

Homologous recombination: Filkowski et al. (2003) employed the transgenic *Arabidopsis thaliana* point mutation and recombination tests to monitor the genetic effects of the A--->G mutation associated with herbicide 2,4-D. They found that 2,4-D had a significant effect on the frequency of homologous recombination.

Endoreduplication: in which chromosome duplication occurs without nuclear division has been reported after 2,4-D treatment (Dvorak, 1968).

Chromosome Stickiness and Clumping: Grant (1978) suggested that chromosome stickiness a rises from improper folding of the chromosome fiber into single chromatids and chromosomes. As a result there is an intermingling of

the fibers and the chromosomes become attached to each other by means of subchromatid bridges. Chromosome stickiness and clumping have been reported following treatment with 2,4-D.

Chromosome fragmentation: Zeljezic and Garaj-Vrhovac (2004) reported that 2,4-D caused an increase in chromatid and chromosome breaks, number of micronuclei and number of nuclear buds. Results from multiple breaks of the chromosome in which there is loss of chromosome integrity. Fragmentations can range from partial to total disintegration of the chromosome. Chromosome fragmentations in plant cells have been observed in root tip cells of *Allium cepa* after 2,4-D treatment (Grant, 1978).

DNA strand breaks: The only routine genomic DNA damage test currently carried out with plants is the single cell gel electrophoresis (SCGE or comet) assay. The alkaline SCGE assay detects single and double stranded DNA breaks and conformation changes of genomic DNA by measuring the level of DNA migration through an agarose gel in an electric field (Tice et al., 2000). Chemical mutagen induction of comets in tobacco leaves and roots was highly correlated with the induction of leaf mutations (Gichner and Plewa, 1998; Gichner et al., 1999). 2,4-Dichlorophenoxyacetic acid (2,4-D) interaction with DNA was studied by Ahmadi and Bakhshandeh (2009). Analyses of fluorescence spectra, viscosity measurements, and alternative current voltammetry interactions indicated that 2,4-D is a groove binder of DNA.

Sister-chromatid exchange (SCE): Somatic recombination and sister chromatid exchanges (SCE) can result in chromatid alteration that can affect the expression of genes by the loss of heterozygocity. The effect of 2,4-dichlorophenoxyacetic acid (2,4-D) at low concentrations on cell cycle duration and sister-chromatid exchange (SCE) frequency was studied using meristem root-tip cells of Garlic (*Allium sativum* L.) 2,4-D induced a marked prolongation of the cell cycle. At the same time, small but statistically significant increases in SCE frequencies were observed at 5 µM and 15 µM 2,4-D concentrations (Dolezel et al., 1987). Results for genotoxixcity of 2,4-D are shown in Table 3.

Conclusion

Several plants that have already been used as biomonitors are described. These plants can be successfully used for both sensing environmental pollution and monitoring the effectiveness of polluted areas decontamination. The results from higher plant bioassays could make a significant contribution in protecting the public from agents that can cause mutation. The advantages haunted by higher plant genetic assays make them ideal for use by scientists in developing countries. Environmental pollutants can have destructive effects on higher plants; at high concentrations they can cause genotoxic effects. Plants, as biological

Table 3. Advantages of plants as bimonitoring systems

· Regeneration from single haploid and diploid cells.	· The major plant test systems have numerous genetic end points for determining the effects of mutagens.
· The cost of culturing, the cost and time of training technicians to handle a variety of end points following mutagen treatmentand space requirements are relatively small.	· When seeds are used, the mutagenic effects can be studied under wide range of environmental conditions, such as large differences in pH, water content, temperature, and metabolism.
· They can be used for outdoor monitoring	· They do not contaminate easily.
· Several plant test systems provide unique advantages for in situ monitoring	· Some plants have short generation times
· Plants are able to process complex pollutant molecules	· The chromosome organization of plants is similar to that of human and mammalian cells.
· Responses to given mutagens similar to those of other non-mammalian and mammalian systems.	· They are eukaryotes and They are easy to culture
· Several provide chimeral situations and cells from chimeras can be regenerated	· Several yield relatively good genetic information.

indicators, can measure genotoxic potential effects of pollutants, when they are used to measure effects of herbicide

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