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Chapter 2

Detection of Environmental Mutagens Through Plant Bioassays

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http://dx.doi.org/10.5772/intechopen.69274

Abstract

Plants are present in almost all areas of the world and can accumulate many chemical compounds present in the soil, water, and atmosphere. As these chemicals which are potentially mutagenic or carcinogenic are absorbed by the plants sharing the same environment with us, bioassays on plants can be used to detect the presence of environmental hazards. Another reason for selecting plants for assessing adverse effects of these chemicals is the ease of experimentation with plants. Evaluating the effect of a substance on basic plant characteristics such as growth, survival, or reproduction is straightforward and repeatable. Thus, various plant species are commonly utilized as indicators of adverse environmental conditions. This chapter covers the detection of environmental mutagens through plant bioassays, considering the increasing importance of biomonitoring using plants for assessing the mutagenicity of relevant chemicals and industrial waste. From this point of view, a detailed literature search was made on the subject. The genotoxic, cytotoxic, and molecular studies have been investigated and the most useful and important parts and key points of these methods were summarized. This review would be useful for scientists who are planning to conduct research on plant bioassays with different types of methods and chemicals.

Keywords: ecotoxicology, cytotoxicity, genotoxicity, phytotoxicity, mutagens

1. Introduction

Plants are the essential elements of agriculture and forestry and maintain the healthy environment for the rest of the species by producing oxygen and organic carbon compounds. Higher plants are preeminent indicators of genotoxic effects caused by chemical substances existing in the environment and therefore be utilized for detecting environmental mutagens [1]. They are

exposed to many stress factors including chemical compounds and radiation affecting their seed germination, seedling growth, and floral and fruit development. These stress factors can adversely affect the quality and quantity of the product with leading to morphological, anatomical, physiological, biochemical, and molecular damage to plants [2]. There are different kinds of methods for examining phytotoxicity and genotoxicity because usually there is no standard national procedure. Therefore, the parameters of these methods vary depending on the test substances, the test plants, or the individual procedures. Because of its simplicity, low cost, and relatively high sensitivity, application of plant bioassays is usually favored over other available systems in discovering adverse effects caused by chemical substances, or pollution, existing in the environment [3]. Despite these benefits described above, there are also some limitations in using plant bioassays, such as the longer life span of plants than *Escherichia coli* T. Escherich, *Salmonella typhimurium* Lignieres, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, or *Drosophila melanogaster* Meigen; likewise, there are differences between the biochemistry of plants and mammals. Nevertheless, positive correlation results have been observed between plant and mammalian systems in many reports, supporting the preference of plant bioassays in these studies [4]. Hence, plant bioassays are commonly used for screening and monitoring environmental chemicals with mutagenic and carcinogenic potential [5, 6]. The International Program on Chemical Safety (IPCS) makes and supports research programs all around the world and develops methodologies for chemical exposure [4, 7]. Many laboratories from diverse regions of the world have been sponsored by IPCS and participated in evaluating the utility of several plant bioassays for detecting the mutagenicity of environmental chemicals [8]. By means of these studies, many methods were developed to assess toxicity in plants. Some of the recent studies with plant bioassays can be seen in **Table 1**.

Plant bioassays are usually based on the detection of chromosomal abnormalities in mitosis, sister chromatid exchanges (SCEs), and, recently, on the DNA damage analysis. Point mutations

Plant species	Test substance	Method	Reference
Vicia faba L.	Wastewater	Micronucleus method	Liu et al. [9]
Tradescantia pallida (Rose) D.R.Hunt var. *purpurea*	Pesticide	Micronucleus and stamen hair bioassays	Fadic et al. [10]
Triticum aestivum L.	Aniline	Micronucleus, mitotic index, and chromosomal aberration	Tao et al. [11]
Vicia faba L.	Insecticide	Sister chromatid exchange	Quintana et al. [12]
Oryza sativa L. var *nipponbare*	Mercury	Real-time PCR FISH	Zhen et al. [13]
Capsicum baccatum L. var. *pendulum*	Ionizing radiation	TUNEL test	Scaldaferro et al. [14]
Epipremnum aureum (Linden & André) G.S.Bunting	Volatile organic compounds	Comet assay	Naroi-et et al. [15]
Acalypha indica L.	Lead stress	RAPD-PCR	Venkatachalam et al. [16]

Table 1. Some of the recent studies with plant bioassays.

such as chlorophyll mutations in leaves, waxy mutations, or embryo mutations of *Arabidopsis* are the other detection methods [17]. Seed germination, root elongation, EC50 (the concentration that lowers %50 of the root length) determination, mitotic index, chromosomal abnormalities in different phases of mitosis, seedling growth, and enzyme activity during germination are the preliminary investigations for plant bioassays. In this chapter, some of the most frequently and recently used methods for detection of genotoxicity with plant biosystems are reviewed.

2. Seed germination and root elongation tests

Many plant species have been recommended for ecotoxicity tests using seed germination and root elongation methods. Among them, cabbage, lettuce, and oats are recommended by the US Environmental Protection Agency (EPA) (1983) [18], the Organization for Economic Cooperation and Development (OECD) (1984) [19], and the Food and Drug Administration (FDA) (1987) [20]. Carrot, cucumber, and tomato are also suggested by the EPA and FDA, wheat is accepted by the FDA and OECD, and rice is also mentioned by the OECD. Although not mentioned in any of these documents, millet has been studied at the Illinois State Water Survey for several years [21]. Most frequently used species are *Allium cepa* L., *Lactuca sativa L.*, *Glycine max* (L.) Merr, *Avena sativa* L., *Hordeum vulgare* L., *Pisum sativum* L., *Tradescantia pallida* (Rose) D.R.Hunt , *Vicia faba* L., and *Zea mays* L. The crucifer *Arabidopsis thaliana* (L.) Heynh. is used only for mutation studies as its chromosomes are very small, and the total genome contains only about 70,000 kb in contrast to over a million kilobases in most other plants. The test substance, test duration, test organisms, the species and number of organisms, concentration of the test substance, replicates, randomization, equipment, reliability, environmental conditions (temperature, humidity, watering, lighting, photoperiod, and nutrients), observations, measurements, and analysis of the test results must be done carefully. The seed germination and seedling growth bioassays are more sensitive to separate plant developmental life stages as they integrate the effects of many environmental stress factors on both germination and seedling growth stages, respectively. The early seedling development is a more sensitive endpoint than the seed germination that depends on the energy reserves in cotyledons. Many researchers also found that the different kinds of species used do not respond similarly to toxic chemicals [22, 23]. Seed germination and plant growth bioassays are the most common techniques used to evaluate the toxicity of pesticides [24–27], heavy metals [6], allelochemicals [28], personal care products [29], compost [30], water samples taken from rivers [31], and industrial waste waters [25, 32]. Different plant species have also been used such as cucumber and cress [33], lettuce and soybean [34], red maple, sugar maple, white pine, and pink oak [35] for phytotoxicity tests.

3. Cytogenetic techniques

The frequency and the type of chromosome abnormalities in different phases of mitosis and the micronuclei frequency of interphase cells are analyzed by cytogenetic tests. The DNA damage caused by the genotoxic agents could either be repaired or otherwise could be lead to the DNA alterations. Chromosome abnormalities are the results of DNA double-strand

breaks that were unrepaired or inaccurately repaired. Chromosomes are rearranged since broken chromosome ends become "sticky" and may combine with other broken chromosome ends. After mutagenic treatment, because of the chromosomal rearrangements and acentric fragments, dicentric bridges could be observed in mitotic cells of the first cell cycle. Micronuclei frequency also decreases in the interphase cell in the next cell cycle [36]. The micronucleus (MN) test, *A. cepa* and *V. faba* chromosome aberration test, and the T. MN tests have been recommended as the validated plant bioassays for laboratory testing and in situ monitoring of the genotoxicity of environmental mutagens [7]. Sister chromatid exchange (SCE) test can also be used to detect effects of small doses of pollutants; thus, it is adequate for initial genotoxicity evaluation tests [37]. SCEs result from alterations caused in the gene expression and by the loss of heterozygosity. SCE experiments are traditionally performed and well studied in mammalian cells. For plants, the protocols have been mainly developed in *V. faba* root cells [38].

3.1. *Allium*/*Vicia* chromosome aberration test

Several mutagens can be detected cytologically by cellular inhibition; disruption in metaphase; induction of chromosomal aberrations, numerical and structural, ranging from chromosomal fragmentation to the disorganization of the mitotic spindle; and consequently all subsequent dependent mitotic phases. The microscopic analysis includes mitotic index, micronuclei presence in interphase cells, and chromosomal aberrations in late anaphase and early telophase cells score. Approximately 1000 cells from all the stages of dividing cells in mitosis are counted in order to find the mitotic index value. Chromosomal abnormalities can be determined, and then, they are scored in the first 100 cells in different stages of mitotic division. The mostly used method to determine all of the abnormalities is to scan the slides from right to left, up, and down [39]. The *Allium* material is well known and has been used for the study of basic mechanisms as well as for scoring the effects of chemicals. *A. cepa* (the common onion) has proved to be the most useful and has repeatedly been suggested as a standard test material [40]. The use of *A. cepa* as a test system was introduced by Levan [41], when the effects of colchicine were investigated. Since then, the *Allium* test has been frequently used. Genotoxicity, cytotoxicity, and chromosome abnormalities in plant biosystems are mostly determined in *A. cepa* (2n = 16) and *V. faba* (2n = 12). They are efficient test organisms because of their availability throughout the year, ease of handling, and cultivation. They also do not need to be cultivated in sterile conditions; they have large and small number of chromosomes, which makes the observation of chromosomal damages in the mitotic cycle easier [42]. The *Allium* test has high sensitivity and good correlation when compared with the mammalian test systems. Ma and Grant [43] suggested including *Allium* test as a standard test system to determine chromosome damages induced by chemicals after the evaluation of 148 chemicals by the *Allium* test since 76% presented positive results. It was reported that the sensitivity of the *Allium* test was practically similar as the one observed for human lymphocyte and algae test systems. Rank and Nielsen [44] showed that the *Allium* test was more sensitive than the MicroScreen and the Ames tests. They also reported that there was a correlation of 82% of the carcinogenicity test in rodents in relation to the *Allium* test. The *V. faba* MN test has been shown to be sensitive in evaluating chromosomal aberrations and assessing genotoxicity from both organic and inorganic soil contaminants [45], sediment [46], organic material such as

sewage sludge or composts [47] and water [48, 49]. Many researchers compared sensitivity of the *V. faba* test with other bioassays, i.e., somatic mutation and recombination test (SMART), that utilizes *D. melanogaster* Meigen. and compared with the *V. faba* sister chromatid exchange (SCE) test and MN inductions. Both tests showed 62.5% similarity [38]. Plant genotoxicity assays as the MN test on *V. faba* roots provide quantitative, repeatable, and reliable mutagenic data, and they are sensitive tests to detect new environmental mutagens or combination of different kinds of mutagens [50]. They can be used to develop new techniques for alternative assays in the determination of possible genetic damage caused by environmental pollutants such as pesticides, heavy metals, and more recently personal or health-care products. They can also contribute to an in situ monitoring, which can be carried out on a global scale in media as aqueous biota or soils in relation to human activities [1].

3.2. Tradescantia stamen hair mutation and micronucleus analysis

The genus *Tradescantia*, from the Commelinaceae family, is a higher plant with more than 500 species. Some of these and their clones are used as genetic bioindicators for mutagenic activity, such as *T. pallida* (Rose) D.R.Hunt, for environmental monitoring. It has two assay systems, the *Tradescantia* sp. staminal hair assay and the *Tradescantia* sp. MN assay, developed by Ma [51]. Stamen hair and MN tests have been widely employed for genotoxic effect studies with *Tradescantia* species [43, 52]. Almost all of the parts of the *Tradescantia* species including the root tip and also the pollen tube in development provide the best plant materials for cytogenetic toxicity testing studies. *Tradescantia* species have 12 chromosomes which are easily observable. Sax and Edmonds observed that meiotic chromosomes in pollen development were more easily influenced to breakage than mitotic chromosomes. They especially reported that the dividing chromosomes within the cells at meiosis are approximately ten times more sensitive to breakage than those in the interphase cells [42].

Ma and Grant [43] have prepared a historical perspective, detailing the importance of this plant in mutation studies. Firstly, the heterozygosity for flower color in *Tradescantia* sp. clones was used for these studies, and then, the stamen hairs have been determined to be good indicators of mutations. Clone 4430 is a hybrid of *Tradescantia hirsutiflora* Bush. and *Tradescantia subacaulis* Bush. reproduced only asexually, through cloning. This test uses the stamen hairs of *Tradescantia* sp. inflorescences to evaluate the frequency of somatic mutation, induced for mutagens, through changes in the color of stamen hair cells from blue to pink, due to the expression of a recessive gene of these cells. The frequency of micronuclei in tetrad cells of male meiotic cells in *Tradescantia* induced by the tested mutagen was determined [42]. The *Tradescantia* sp. MN test may be used for in situ exposure conditions to evaluate air or water pollution or under laboratory conditions for testing radioactive or chemical agents [53, 54]. The *Tradescantia* sp. stamen hair mutation (Trad-SH) assay (clone 4430) was evaluated for its efficiency and reliability as a screen for mutagens in an IPCS collaborative study on plant systems. The results of the study confirm that the Trad-SH assay is an unsuspicious system for screening potential environmental mutagens. A survey of the current literature indicates that the Trad-SH assay could be used for in situ monitor of liquid, gaseous, and also radioactive pollutants as well although the study was carried out under laboratory conditions [55].

3.3. Sister chromatid exchange

The sister chromatid exchange (SCE) test is developed from the semiconservative DNA replication model which we could see the separation of DNA. The cytogenetic monitoring of exposure to potential mutagens in the environment could be done by SCE which is a highly sensitive cytogenetic tool for detecting DNA damage. It involves firstly the breakage of both DNA strand and then an exchange of whole DNA duplexes. The symmetrical exchange during S phase at one locus between sister chromatids that does not alter chromosome length and genetic information is defined. Taylor was the first scientist who made the SCE test visualized for plant cells, but he used tritium and autoradiography, which provided poor spatial resolution [56]. After Taylor, it was discovered that sister chromatids could be differentiated and revealed SCEs in combination with Hoechst dye 33258 incorporation of the DNA base analog 5′-bromodeoxyuridine (BrdUrd) staining [57]. BrdUrd is a synthetic nucleoside that is an analog of thymidine and is actively incorporated into the newly synthesized DNA during replication process. It is commonly used in the detection of dividing cells in living organisms during the S phase of the cell cycle substituting for thymidine. The standard fluorescence plus Giemsa (FPG) staining method also will enable visualization of SCEs in metaphase spreads of growing cells in medium containing BrdUrd with a light microscope [56]. The frequency of SCEs per chromosome set increases after treatment with genotoxic agents. SCE method was first applied in mammalian cells, and later, it has been shown that it can be applied in plant cells.

Especially plant species that have relatively large and a low number of chromosomes such as *A. cepa* and *V. faba* are used for SCE analysis [57, 58]. *Crepis capillaris* (L.) Wallr. is also a good material for analyzing the frequency of SCE with 2n = 6 chromosome number. It allows studying SCE frequency in each chromosome type, since it has three pairs of morphologically differentiated chromosomes [59, 60].

4. Molecular techniques

4.1. Fluorescent in situ hybridization

The classical cytogenetic techniques were usually used for detecting the changes in chromosomal number and morphology. However, chromosome staining with the traditional methods such as Feulgen or orcein staining can fail in the analysis of small changes in chromosome structure. The fluorescent in situ hybridization (FISH) allows the detection and a more detailed localization of chromosomal rearrangements, both in interphase and mitotic nuclei, which gives new possibilities to study chromosomal aberrations [61]. Additionally, it helps to reveal the mechanisms of the formation of chromosomal abnormalities in plant mutagenesis. Although there are a few number of DNA probes for particular plant chromosomes, *A. thaliana* is a good example when FISH employing chromosome region-specific DNA probes (e.g., centromere, telomere, rDNA) is helpful in chromosome aberration analysis. The translocations in chromosomes of tetraploid plants of *A. thaliana* have been detected by FISH [62]. The effects of maleic acid hydrazide on hairy root tip meristem cells of *C. capillaris* were studied with FISH

using rDNA and telomeric sequences as a probe and spontaneous chromosomal rearrangements were determined [63]. It is also important to analyze the chromosomal rearrangements in interphase cells treated with mutagenic chemicals that may cause a decrease in the frequency of cell divisions. The basic steps of this procedure are the same as the other organisms, but several cytogenetic laboratories modified various techniques for plant cells.

4.2. TUNEL test

Another test used to identify apoptosis that has found application in plant genotoxicity studies is the terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) test [64]. TUNEL assay detects DNA fragmentation by the help of fluorescence microscope. TUNEL test is used to detect DNA damage associated with nonapoptotic events such as necrotic cell death induced by exposure to genotoxic chemicals. It is not limited to the detection of apoptotic cells [65] and has also ability to stain cells going through active DNA repair [66]. The regulated cell death plays an important role during development of plants, and it is also essential for plant-specific responses to biotic and abiotic stress factors. The terminal deoxynucleotidyl transferase catalyzes the polymerization of labeled nucleotides to DNA strand breaks in situ. For TUNEL test, successive hand-cut sections of each axis of embedded plant material are stained with propidium iodide (PI) in order to stain the nuclei of dead cells to red and DAPI (4',6-diamidino-2-phenylindol) which can pass through the normal cell membrane and stains the nuclei to blue. DAPI can be used to stain both live and fixed cells. The detection of DNA breaks at a single nucleus can be achieved with TUNEL test within a short time, and the screening of labeled nuclei is easier than other methods. It is recommended for the preliminary genotoxicity investigation of the new identified chemicals [67].

4.3. Single-cell gel electrophoresis (comet assay)

DNA damage in higher plant cells was evaluated by the frequency of chromosomal aberrations in metaphase chromosomes, abnormal anaphase and telophases, and micronuclei; however, these tests measure unrepaired genome damage in cells which have reached mitosis. DNA damage may be originated from DNA metabolism spontaneously or from the effects of environmental factors. There are different kinds and levels of DNA repair mechanisms in cell nucleus to prevent these damages. When the repair mechanisms are ineffective or there was a heavy DNA damage, it may lead to the inhibition of replication, transcription, or protein synthesis; however, in the long term, chromosomal abnormalities or mutations could be formed. It is a sensitive and fast fluorescent technique, which is used to determine the amount of DNA damage on single cell level. After its introduction as "alkaline comet assay," it has been developed with many modifications for investigating the process of apoptosis and became a workable technique for detecting a variety of DNA damages in plant cells. It allows the determination of double- and single-stranded DNA breaks in a single cell and also helps to measure the level of the migration of DNA by using horizontal gel electrophoresis system [68]. The length of the tail and the amount of the DNA in the head and in the tail are measured to assess the toxicity in a computerized image analysis system. The tail moment (TM) can be calculated to show DNA damage [69]. The comet assay allows fast detection of DNA damage, shortly after the injury, before DNA is repaired, and

without any need to wait for progression into mitosis [70]. The presence of a cell wall and the absence of free cells in plant tissues cause technical difficulties for performing the comet assay. Over the past few years, many scientists have improved the methodology for the comet assay on plant cells. Navarrete et al. [70]. developed a simple and efficient mechanical extraction to isolate cell nuclei to overcome these problems. This technique was then improved by Gichner. The different internal parameters such as nucleus isolation methods, filtration and lysis steps, agarose concentration, and the external parameters such as room temperature and light intensity were evaluated during these studies [71].

4.4. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) technique

RAPD-PCR is a PCR-based and quite reproducible technique that yields information on a large number of markers without having to obtain DNA sequence information for primer design [72]. Many scientists used RAPD-PCR technique commonly for a variety of purposes such as cultivar identification, genetic diversity assessment, and the construction of phylogenetic relationships [73], and it has been successfully utilized in genotoxicity identification of toxic chemicals. A number of selective and sensitive assays for DNA analysis in ecotoxicology have been developed with the improvement of recent molecular biology techniques. DNA-based techniques such as RFLP, QTL, RAPD, AFLP, SSR, and VNTR are being used to investigate the variations at the DNA sequence level. RAPD-PCR can be used to detect genotoxicity, and differences in RAPD profiles can clearly be shown when comparing DNA fingerprints from untreated and treated individuals to genotoxic agents [74]. Many studies support the view that the RAPD analysis is a highly sensitive method for the detection of DNA damage induced by environmental pollutants like toxic chemicals. RAPD markers are at this moment low valuable markers due to the lack of repeatability. A few work is usually published at this moment using this kind of markers. This kind of study using other DNA markers will be of much more interest.

4.5. Real-time polymerase chain reaction (RT-PCR) technique

Plants have risk of DNA damage due to continuous exposure to environmental mutagens, and thus a variety of repair mechanisms should operate to maintain genome integrity. *A. thaliana* is a mostly studied plant for the repair mechanisms after exposure to several mutagens such as UV-B radiation [75], heavy metal contamination [76], and wound stress [77]. In the first step of the DNA damage response, DNA lesions or replication inhibition must be detected. The DNA damage response is controlled by the activation of several regulatory kinases and also checkpoint proteins that lead to specific cell cycle arrests as well as changes in the chromatin structure at the site of DNA damage. The transcriptional regulation of the genes could be determined by RT-PCR in order to evaluate the mechanism of plant response to genotoxic agents. To investigate effects of mutagens on the transcript levels of some gene-encoding antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), they were studied in Ref. [78]. The mutants of *A. thaliana* that are hypersensitive to UV radiation (designated uvh and uvr) have been isolated to investigate the respond of plants and its pathways to UV radiation. UVR2 and UVR3 products were previously identified as photolyases that remove UV-induced pyrimidine dimers in the presence of visible light [76]. Hu et al. (2007)

investigated the role of calmodulin (CaM) and the relationship between CaM and hydrogen peroxide (H_2O_2) in abscisic acid (ABA)–induced antioxidant defense in leaves of *Z. mays* [78].

5. Flow cytometry

Flow cytometry (FCM) is a rapid and multiparametric technique that theoretically has the potential to detect minute variations in nuclear DNA (nDNA) content, as well as chromosomal damage, in exposed organisms. It can also provide information on polyploidization and evaluate cell cycle dynamics in plants. Pfosser et al. [79]. evaluate the sensitivity of FCM by detecting the variations in DNA content as small as 1% in aneuploid wheat-rye lines. Relatively to DNA damage, Rayburn and Wetzel correlated the coefficient of variation of the G0/G1 peak with chromosomal aberration in aluminum-exposed plants, as this parameter is able to detect broken and rearranged chromosomes in daughter cells [80]. Monteiro et al. also detected an increase in the full peak coefficient of variation (FPCV) of the G0/G1 peak of lettuce plants exposed to Cd [81].

6. Conclusion

Hundreds of new industrial chemicals have been continuously produced to facilitate our lives, and we are not able to be aware of their damage before we investigate their effects on organisms. Plant bioassays serve as a tool to demonstrate the cytotoxic and genotoxic effects of environmental pollutants by means of clear-cut evidence of chromosome damage and gene mutation. These studies could also be useful to establish a database for environmental conditions in the various regions of the world. Some of these simple and clear-cut indicators revealed by plant bioassays could also be used to demonstrate the genotoxic effects of environmental pollution to the general public. The kind of education that is required is not only about teaching people how to detect and eliminate pollutants but also to educate the general public on the root cause of pollution problems. Pollution is related to every facet of human life, and it is life itself that generates pollution. Regulations and guidelines are essential to cure the symptoms of pollution. Plant bioassay studies deserve to be included by the enforcement agencies, particularly of the developing countries, for their regular monitoring of pollution sites.

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