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Preface

Genotoxicity refers to the property of chemical agents to induce changes in the genetic information within a cell causing changes which may result in mutations. Genotoxicity is similar to mutagenicity except that genotoxic effects are not necessarily always associated with mutations. Oxidative stress is a complex chemical and physiological phenomenon that accompanies virtually all biotic and abiotic stresses in higher plants and develops as a result of overproduction and accumulation of reactive oxygen species (ROS).

The book "*Induced Genotoxicity and Oxidative Stress in Plants*" is intended to deliver information on genotoxicity induced by a wide spectrum of genotoxic agents and relative oxidative damage in plants with special reference to the metabolism of reactive oxygen species (ROS). Genotoxicity is the ability of different agents to cause damage to genetic material. However, the damage induced in the genetic material includes not only DNA, but also the cellular components related to the functionality and behavior of chromosomes within the cell. For example, proteins involved in the repair, condensation, and decondensation of DNA in the chromosomes, or other structures, such as the mitotic spindle, responsible for distribution of the chromosomes during cell division. Oxidative stress is a complex chemical and physiological phenomenon that accompanies virtually all biotic and abiotic stresses in higher plants and develops as a result of overproduction and accumulation of reactive oxygen species. Stresses induced by various agents may lead to over production of ROS resulting in progressive oxidative damage and ultimately cell death. Despite their destructive activity, they are well-described as second messengers in a variety of cellular processes, due to tolerance to various environmental stresses. Whether ROS would serve as a signaling molecule or could cause oxidative damage to the tissues depends on the delicate equilibrium between ROS production, and their scavenging. Different genotoxic agents, that enter the ecosystem either naturally or through anthropogenic events, tend to impose serious threats on its biotic components specially the sedentary flora. Plants 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 leading to morphological, anatomical, physiological, biochemical, and molecular damage to plants.

In this book, we have chapters that emphasize on the role of different genotoxins and stress induced by them. The chapters are contributed by experienced, highly dignified, and internationally acclaimed scientists, researchers, and academicians around the world. The chapters are designed in such a way that it clarifies the concepts of the specified themes. The book is aimed at several audiences, from breeders to agronomists, from students to researchers, from teachers to academicians working in the fields of agriculture, plant science, environmental biology, and biotechnology. Each group of reader will have different technical backgrounds and expertise. Therefore, the chapters are written on three levels, namely introduction, main text supported by relevant figures and tables, and bibliography.

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About the Editors

Dr Zeba Khan obtained her master's and Ph.D. degree from Aligarh Muslim University. Her professional journey started with joining as a Senior Project Officer in the National Bureau of Plant Genetic Resources, New Delhi, India. She also worked as a Postdoctoral Research Associate under DST-PURSE funded Project at Aligarh Muslim University. During her postdoctoral tenure, she extensively studied the role of nanoparticles on plants and their genotoxical impacts on plant ecology. Her specific research aims in understanding the role of various physical and chemical agents that are responsible for induced genotoxicity and stress in plants as a response to change in the environment and in a way utilization of Genetics and molecular techniques to improve yield and quality of agronomically important crops. During her post-master's tenure, she worked on mutation breeding in Chicory which is an important medicinal herb. She has published a number of research papers, review articles, chapters in reputed journals and books, and presented papers in various conferences, seminars, and symposiums. She has been honored for her outstanding performance in research and had received the young scientist (2018) and environmentalist of the year award (2019) by IFCEE.

Dr. Mohd. Yunus Khalil Ansari worked as a professor in the Department of Botany, A.M.U, Aligarh and retired as a chairperson on 20.08.2018. He has been associated with the teaching in cytogenetics and molecular biology. His research areas are cytogenetics, mutation breeding and biotechnology. He produced several important mutants in more than thirty plants. He has been actively involved in research and published around seventy scientific research articles in reputed national and international journals and was the coauthor of three books. He attended many national and international conferences/symposia and workshop. Professor Ansari has produced 15 Ph.D.'s and 7 M.Phil. students. He was a co-principal investigator in the Department of Biotechnology sponsored project "in vitro propagation of *Pterocarpus* sp. and *Saraca* sp." and worked as a co-investigator in a project sponsored by the Ministry of Environment and Forest on the preserving 30 endangered plants.

Durre Shahwar received her Ph.D. in Botany from Aligarh Muslim University, Aligarh, India. She is the recipient of the Maulana Azad National Fellowship and National Fellowship from the University Grants Commission, New Delhi. Dr Shahwar has been awarded the Junior Scientist of the year award (2018) by International Foundation for Environment and Ecology, Kolkata in 5th International Conference on Environment and Ecology, University Gold Medal for securing first rank in M.Sc. (Botany) in 2014. She has published several research articles and book chapters in peer reviewed national and international journal. She has also participated in various national and international conferences and received life membership of scientific bodies in India. Her research interests are cytogenetic, plant breeding and molecular biology (proteomics and genomic). She is actively engaged in mutation breeding for genetic improvement of legume crop.

Induced Genotoxicity and Oxidative Stress in Plants: An Overview

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Afshana, Mudasir A. Dar, and Zafar A. Reshi

Abstract

Being sedentary, plants always face a vast array of environment-related factors in the form of ultraviolet rays, higher salt concentrations, water scarcity and dehydration, high water potentials, extremely low and high temperature among other air and soil-borne chemicals. Besides this, an increase in the production of industrial wastes, encompassing toxic heavy metals and metalloids constantly put heavy stress loads on plants. Majority of these agents have, very recently, been implicated to harmfully alter the chemical and physical aspects of DNA. This is deemed to happen as a consequence of oxidative stress and reactive oxygen species (ROS) outburst. Consequent to the DNA alterations and genome instability, plants face numerous cytotoxic complicacies which negatively impact their health and hence, yield. Most importantly, the toxic agents induce ROS production, damage other cellular macromolecules, including the vital photosynthetic apparatus. Surging industrialization and widespread use of chemical fertilizers, despite inlaid with some positives, have recently been perceived as serious challenges for plants to cope up with around the globe. To get on well and adapt with the genotoxic agents and the follow-up stress, wide range of efficient counteracting mechanisms spanning over morpho-anatomical, hormonal and biochemical features got evolved in plants. Interestingly, at the molecular level, heavy metal generated genotoxicity and allied disruptions are more than efficiently overcome by changing the activity profile of stress-responsive genes. Another potent way of overcoming genotoxic stress and genomic instability in plants is via epigenetic modifications. Recent advancements in our understanding of environmental stress-induced toxicity and the follow-up compensatory responses (both transcriptional and epigenetic) are anticipated to recognize the

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crucial avenues in the target pathways for elevating the resistance and endurance of crop plants to different environmental stresses.

Keywords

Genotoxicity · Heavy metals · Drought · Salinity · Reactive oxygen species (ROS) · UV radiations

Abbreviations

ABA	Abscisic acid
ADP	Adenosine di-phosphate
AFLP	Amplified fragment length polymorphism
AP-PCR	Arbitrarily primed polymerase chain reaction
APX	Ascorbate peroxidase
ASc	Ascorbate
ATP	Adenosine tri-phosphate
BER	Base excision repair
CAT	Catalase
DNA	Deoxyribonucleic acid
DR	Direct repair
DSBR	Repair of double-strand DNA breaks
FQs	Fluoroquinolones
GR	Glutathione reductase
GSH	Glutathione synthetase
GSSG	Glutathione disulphide
HR	Homologous recombination
LHC	Light harvesting complex
MMR	Mismatch repair
mRNA	Messenger ribonucleic acid
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
PC	Phytochelatins
PCD	Programmed cell death
POX	Peroxidase
PS	Photosystem
RAPD	Random amplified polymorphic DNA
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RuBP	Ribulose 1,5-bisphosphate
SA	Salicylic acid
SOD	Superoxide dismutase
SSR	Simple sequence repeats
UV	Ultraviolet

1.1 Introduction

Being unable to move plants always are bound to cope with a great variety of environmental constraints, limiting their growth and hence, yield (Dutta et al. 2018). Amongst these constraints, harmful UV radiations, salinity, industrial wastes containing toxic heavy metals are most serious with prominent negative impacts on crop plants. A disproportionate fraction of these stress-inducing environmental factors are known to disrupt the physical and chemical parameters of genetic material (DNA). Thus, by altering the genetic material (genotoxic), these are expected to disrupt the morpho-physiology and biochemistry of the subject plants a great deal. Interestingly though all the genotoxic materials change the structure and chemical aspects of DNA, but only some are able to cause mutations. This may better be paraphrased thus, 'All mutagens are genotoxic, but it's not the other way round'. To cope up with the stress causing genotoxic stuff in the environment, plants have evolved enormous counteracting mechanisms which efficiently reduce the level of oxidative stress and greatly help scavenge the harmful reactive oxygen species (ROS). In this chapter, we are interested to understand the influence of various genotoxic agents (physical and chemical) on the performance of crop plants, particularly their yield and how do plants get over with the serious and harmful consequences of genetic material altering agents. Progressive industrialization concomitant with global climate change and other anthropogenic activities has added to the hostilities of atmosphere, hydrosphere and lithosphere, which severely affect the crop plants (Wright and Nyberg 2015). In view of this, environmental stresses and the associated issues like delayed growth and drastic crop yield reduction have emerged as one of the major concerns for the world. Increasing population and the negative impacts of heavy metal-induced stress on plant health impose tremendous roadblocks in meeting the world's ever rising food demands (Wani et al. 2018). Harmful implications of industrial development can be more than compensated by breeding stress-tolerant crop plants in future.

1.2 Different Genotoxic and Oxidative Stress-Causing Agents

1.2.1 Heavy Metals

Heavy metals in the soil compete with essential mineral nutrients for binding sites and are thus absorbed on the root surface (Ramkumar et al. 2020). Straight away after they enter the cells of plants, multifaceted effects of toxic heavy metals in the form of structural and functional disruptions of genetic material and proteins occur. This is materialized directly through attacks on thiol substituents of protein molecules drastically altering their conformational and functional aspects (Bertin and Averbeck 2006). It is well known that heavy metals induce oxidative damages in plasma membranes and other macromolecules including photosynthetic apparatus via increased production of reactive oxygen species (ROS). Decreased membrane

endurance, significant reduction in photosynthetic yield, besides other physiological and biochemical disruptions, is believed to be an immediate outcome of reactive oxygen species formation due to heavy metals. Other important implications linked with ROS production include curtailment in the production of different pigments, imbalanced hormone synthesis, disturbed nutritional status, halted genetic material copying and delayed cell cycle (Sharma et al. 2012). Subject to type and concentration of heavy metal and developmental stage of the plant being exposed, a wide range of stress responses are seen in plant cells. In effect sophisticated heavy metal modulating and ROS scavenging pathways operate in plants to withstand their chemical toxicity (Chan et al. 2016).

Heavy metals can affect developmental progression, pace and timing of senescence and production of energy-rich molecules because they are highly active. Due to indiscriminate utilization of heavy metals in industries and agro-technology, their high bioaccumulation and toxic features are among the key abiotic stress agents for life forms (Shah et al. 2010). Many abnormalities in the genetic information have been reported to occur due to either high metal concentration or their unbalanced and inappropriate proportion in different cellular compartments. Toxic metals and other important mineral elements reach cells by common mechanisms of absorption and uptake processes. The amount of heavy metals consumed by plants varies greatly depending upon their concentration and speciation in the soil water. These move from the soil solution to root surfaces, enter the root cells and ultimately reach the shoots through the transpiration stream (Imtiyaz et al. 2016). Excessive metal concentrations induce toxic implications via (1) altered cell membrane permeability; (2) sulphydryl (-SH) cation reactions; (3) reaction affinity with phosphate moieties of ADP or ATP molecules; and (4) critical ion substitution (Kumar et al. 2017).

The effect of their devastating impact on plants mainly includes a powerful and rapid disruption of the developmental progression in both upper and lower plant parts (Alaoui-Sosse et al. 2004). Most importantly they can also cause a drastic reduction in the efficiency of assimilatory apparatus and in some instances evoking premature ageing (Alaoui-Sosse et al. 2004). Heavy metal exposed plants also possess small and thick belowground parts which appear to be or loosely organized (Casella et al. 1988). Amongst all these effects, reduced growth and prior onset of ageing and senescence are taken as the most severe consequences of chronic heavy metal exposure in plants. Meanwhile, the important knowledge of heavy metal induced eco-physiological alterations may have great implications for future research in improving crop yields of plants.

Data from in vivo and, in particular, in vitro research have shown that heavy metals are capable of releasing protein, lipid and thylakoid membrane element components necessary for photosynthetic operation. Previous studies have shown that a surplus of heavy metals get strongly linked with plasma membrane and other cellular structures via oxygen and amino acids (histidine, tryptophan and tyrosine) especially after illumination (Maksymiec 2007). Consequently, the PS II quinone acceptor sites, and/or TyrZ to P680 + electron donation, and electron flow through PSII reaction centre cyt b559 are disturbed. Certain studies have shown the Mg of chlorophyll in many plant species is being substituted with some highly toxic heavy

metals. The decrease in chlorophyll synthesis, following exposure to heavy metals, can ensue because of suppressed synthesis in chlorophyll forming enzymes (Maksymiec 2007). Hg primarily acts on Cu-substituting plastocyanin in its molecule, thus trying to block the electron's passage to PSI (Radmer and Kok 1974). Some in vitro conformational modifications in light harvesting complex II (LHCII) arise due to a complex of cadmium, mercury, lead and some associated proteins (Ahmed and Tajmir-Riahi 1993). Thus, as per Krupa and Baszynski (1995) changes in the various sections of the photosynthetic apparatus could be partly due to the direct intervention of huge amounts of heavy metals.

1.2.1.1 Toxic Implications of some Heavy Metals in Plants

Metal contaminants can be present in soil, air or water and by far soil is the most heavy metal polluted part of the biosphere due to the fact that these metals remain there for longer durations (Lasat 2002). Because of their possible adverse ecological consequences, contamination of croplands by these heavy metal elements and ensuing crop yield reductions has emerged as a grave among the environmentalists. In view of their prevalence in soils and huge toxicity in crop plants, heavy metals are aptly named as soil contaminants.

The warning of heavy metal contamination began with the effects of mercury ingestion which caused Minamata disease. Liu et al. (1994) reported that in many plant species, high concentrations of heavy metals have been found to be chromotoxic and mutagenic. In plants such as *Allium cepa* (Liu et al. 1994) and *Zea mays* L., heavy metal like iron (Pb) usually affects the root growth and cell division (Sagbara et al. 2020).

With the onset of the industrial age, the issue of metal genotoxicity has gained new dimensions. To cope up with the emerging uses and demands for novel materials, huge quantities of new mineral elements, which are not used before, are being mined world over. Such metals are released by air, water and soil into the biosphere and eventually impact the physiological processes of plants, animals and humans. Notwithstanding the fact that radioactive and organic wastes generated toxicity exceeds the heavy metal pollutants mobilized from all combined sources, the potential toxic implications on crop plants and the bioaccumulation of heavy metals along food chains cannot be underestimated (Pacyna et al. 2016).

Several experiments have been done recently in different microbes and animals to test and assess the levels of metal inflicted genotoxicity. Though previously only a few reports highlighted the apparent genotoxic consequences of heavy metal contamination in plant systems, it is now well understood that arsenic, lead and mercury cause a number of breakages (clastogenic) in chromosomes and in some instances alter the genetic material (mutagenic). Besides causing a number of chromosomal and DNA defects, heavy metals are well known for decreasing the rate of division in plant cells (Liu et al. 1995). The degree and extent of genetic material alterations and chromosomal deformities, besides depending on the heavy metal concentration also relies on its oxidation status and exposure time. It has been reasonably concluded that the effect of heavy metals is more apparent and easily recognizable when plants are subjected to high metal concentration treatments for a longer time (Patra et al.

2004). Another twist in the story of heavy metal effects on plants is that the intensity of toxicity is conditioned to diploid chromosome number, lengthwise expansion of chromosomes and the occurrence of metacentric chromosomes (Ma and Uren 1995).

Among the heavy metals Cd, Hg and Pb are known to have immensely harmful and long-lasting genotoxic impacts in plants (Chaoui et al. 1997). For instance, higher oxidation state mercury (Mercuric form), which has a potential capability of getting associated with the genetic material through covalent linkages, causes exchange of sister chromatids in chromosomes (Beauford et al. 2006). Additionally, in a concentration-dependent manner, it causes a significant drop in mitotic index and increases the incidences of aberrations in chromosomes (Patra et al. 2004). Considering the impact of heavy metals on the yield of crop plants, quite recently scores of studies focussed on evaluating the genotoxicity of plants after being exposed to highly toxic heavy metals like Mg, Pb, Cu, Mn and Cd have been carried out. These studies hugely rely on cytological (chromosome abnormalities and formation of micronuclei), molecular (comet assay) and cutting-edge molecular genetic advancements (RAPD, AP-PCR, AFLP, SSR, etc.) (Enan 2006). Heavy metals such as cadmium, lead, chromium and zinc are found to cause drastic negative impacts on seed germination and radical length in *Cicer arietinum* (Gupta et al. 2006). Despite obvious morpho-anatomical anomalies in this species, other cytological defects like bridge formation, laggards, stickiness and fragmentation of chromosomes were also reported (Siddiqui 2015). Likewise, increase in Cd concentration, besides causing membrane lipid peroxidation via ROS, has been implicated in causing genome instability through significant double-stranded DNA breaks in *Vicia faba* (Lin et al. 2007).

1.2.1.2 Response of Plants to Heavy Metal Induced Oxidative Stress

Species survival and persistence of the global biodiversity fundamentally counts on genomic stability due to several protective and repair mechanisms. Due to unprecedented human population explosion and the consequent change in the global environmental and climatic scenarios, enormously huge loads of stress are being directed on plants. Despite lacking the means of locomotion and other avoidance mechanisms plants, however, employ unique defensive and scavenging mechanisms to negate the harshness and hostility of the environment. A rapid outburst of reactive oxygen species intermediates (oxidative outburst) encompassing H_2O_2, $\dot{O}_2$ and $\dot{O}H$ is by and large the most frequent response of plants to environmental stresses like drought, temperature, salinity, radiation, metal, among others (Bolwell et al. 1995). There is a hypothesis named 'general adaptation' syndrome which advocates that different stress types evoke a similar response in plants. This hypothesis holds that the adaptive response in plants depends on the production of reactive oxygen intermediates (ROI) (Leshem and Kuiper 1996). Though disastrous to a number of cellular constituents especially DNA leading to genotoxicity through mutations and apoptosis (Bray and West 2005), ROIs are also known to impart defence (Alvarez et al. 1998), enhance growth and development (Van der Zalm and Schopfer 2004), cause programmed cell death (PCD) (Breusegem and Dat 2006) and initiate responsive signal transduction cascades (Pitzschke and Hirt 2006). One of the principal

counteractive strategies plants opt to respond many adverse environmental stresses is their inherent adaptive response. Most notably the plants which were long thought to be non-responsive have been found to possess diverse adaptive stress response (Panda and Panda 2002). Not surprisingly, therefore, plant cells when subjected to non-cytotoxic low doses of genotoxic substances, they get resistance against heavy doses of either the same or different genotoxin. This behaviour of plants towards genotoxins is specifically termed as genotoxic adaptation. Very recently, however, the above phenomenon has been named as 'conditioning hormesis' in plants (Calabrese et al. 2007). In a range of both prokaryotic and eukaryotic systems, low non-toxic doses of metals, high energy ionizing radiation, oxidative agents, besides other alkylating substances and neutrons trigger comprehensive genotoxic adaptations (Dimova et al. 2008). This has been primarily assessed and tested in the anomalies of spindle association, chromosomal abnormalities, generation of micronuclei, and assays regarding comet and homologous recombination phenomenon (Cortes et al. 1994). Though in vague, breakthroughs in molecular genetic studies hold that the function of genome protection and stability is due to a network of DNA repair pathways, some special proteins, unique polypeptides and epigenetic modifications (Dimova et al. 2008).

Heavy metals are one of the major agents causing lipid peroxidation and bio-membrane damages. The chief decomposition by-product of lipid (polyunsaturated fatty acids) peroxidation, malondialdehyde (MDA) in plants, is considered to be invoked largely due to the heavy metal generated stress (Hassan et al. 2017). For combating heavy metal toxicity, plants, therefore, produce varied types of high affinity low molecular weight thiols which strongly bind damage-causing heavy metals (Ghori et al. 2019). Amongst all these thiols, the most important and common thiols produced in plants include glutathione (GSH) and cysteine. GSH, whose synthesis occurs by the enzymes γ-glutamyl cysteine synthetase (GSH1) and glutathione synthetase (GSH2), both supported by ATP, is a sulphur containing tripeptide represented as γ-glutamate-cysteine-glycine. Besides being a precursor of phytochelatin, GSH significantly also detoxifies cadmium and nickel (Çelik et al. 2020). Phytochelatin polypeptides (γ-Glu-Cys)nGly(n = 2–11), which contain a large proportion of cysteine amino acids, possess strong metal affinities. These phytochelatins, which occur in a wide range of organisms including plants, fungi and many others (Grill et al. 1985; Gekeler et al. 1989) are formed due to the activity of unique enzyme named as phytochelatin synthases. Phytochelatins, in plants, are known to form strong complexes with some deleterious heavy metals in the cell cytoplasm and then subsequently move them into the vacuole (Kumar et al. 2017), offering immense protection.

The detoxification mechanisms evolved in plants in response to heavy metals involves binding (chelation) and in some cases sub-cellular localization. Multiple heavy metal detoxification mechanisms, acting in coordination and intricately networked, help plant to survive in heavy metal contaminated environments via repair of damages to their genome (Moura et al. 2012). Surprisingly, both short- and long-term processes underlying these repair mechanisms are operative in plants at various levels. Amongst the immediate or short-term processes include the rapid

changes in the transcriptional status of stress-regulated genes, ultimately affecting plants metabolism and physiology (Wada et al. 2004). In contrast, the long-term heavy metal initiated plant cell responses comprise various types of genetic modifications among which epigenetic modulations are significantly implicative (Schroeder et al. 2013). Need-based expression changes in stress-induced genes, which is long debated to be an intimate consort of stress response in plants, involves both universal and gene-specific regulatory mechanisms. Quite rationally it's therefore impressed upon that coordinated and profusely networked domains of stress perception and signalling pathways, involving cross talks at various steps, are actually behind the scenes of counteractive plant responses to different heavy metals (Wada et al. 2004).

1.2.1.3 Glutathione-Induced Stress Tolerance in Plants against Heavy Metals

In almost every part of the cell including cytoplasm, chloroplast, endoplasmic reticulum, vacuole and mitochondria, glutathione (GSH) has been reported to occur (Vogelsang and Dietz 2020). It is the most common non-proteinaceous thiol group present in plant cells and its wide range of biochemical functions have largely been assigned due to the thiol group. The nucleophilic nature of thiol group grants GSH the ability to form links, named as mercaptide linkages, with both metals and some select electron loving molecules (electrophiles). Unique chemical behaviour, relatively high stability and considerably large solubility in water allows the plants to use this compound in overcoming the negative impacts of oxidative stress of heavy metals, alongside some organic chemicals of endogenous or exogenous nature (Sarwar et al. 2017). Many studies suggest that overexposure to harmful metals directly or indirectly through their influence on metabolism leads to the formation of ROS. In plant systems, GSH acts by controlling the levels of one potentially severe oxygen species H_2O_2 (Gechev et al. 2006). By doing this a significant fraction of reduced form (GSH) gets converted to its oxidized state (GSSG), which is mandatory for the operation of some redox signalling pathways in plant systems (Millar et al. 2003). This change in the relative amounts and hence the ratios of reduced to oxidized forms (GSH/GSSG) of glutathione, indicating the cellular redox balance is thought to be associated with ROS perception in plants. Reduced glutathione (GSH) with strong antioxidant properties directly reduces most of the ROS generated during stress episodes (Millar et al. 2003).

In addition to scavenging most of the ROS, GSH also functions as an immediate precursor for the formation of phytochelatin. Phytochelatins (PCs) which are small peptides possessing unique metal linking properties were at the outset found in the higher plant cell suspensions, exposed to Cd (Su et al. 2020). Following this many other eukaryotes including higher plants were shown to contain PCs (Gekeler et al. 1989). In addition to Cd, heavy metals like Hg, Cu, Zn, Pb and Ni were also reported to induce PC formation. Formation of PCs from GSH in plant cells when treated with heavy metals involves phytochelatin synthase (PCS) enzyme. Straight away multitudes of physiological studies have implicated the physiological importance

of PCs in metal detoxification pathways alongside the maintenance of ionic balance (Hirata et al. 2005).

1.2.2 Ultraviolet Radiations

Genome stability, an important predictor of plant developmental progression and health, is closely linked with crop productivity. However, a wide range of well-known genotoxic agents (both chemicals and radiations) cause chemical and physical alterations in DNA structure and hence decrease its stability (Prasad et al. 2008). The genotoxic agents change genome integrity via oxidations in the individual bases, severely affecting the vital DNA copying processes and information transfer to mRNA(transcription) which causes the cell to die (Cadet and Davies 2017). Amongst the radiations, UV-B from sunlight with strong penetration power affects the plants and animals. These radiations are known to inhibit growth and development in plants due to reduced genome stability via oxidation and formation of cross-links between DNA bases (Bornman et al. 2019). Consequent upon these integrity and stability issues of genome, a spectrum of other physiological changes like recession in normal protein formation patterns, destruction of plasma membrane constituents and photo-assimilatory complexes occur that negatively influence the developmental pace of the whole organism. On the whole, the radiation-induced DNA damages can have a wide range of genotoxic and cytotoxic implications on the overall performance of plant cells. Left unrepaired, DNA structure and stability anomalies are expected to induce a series of functional and metabolic disruptions in plant cells (Burdak-Rothkamm and Rothkamm 2018).

1.2.2.1 Repair of DNA Damage Caused by Oxidative Stress and Induced Genotoxicity

To get along and adapt to the harmful effects of radiation caused DNA damages, the plant cells possess an in-built array of DNA repair systems, credibly increasing the chances of unaltered genetic transmission across generations (Vishwanatha et al. 2016). On recognizing the DNA damage, the eukaryotic cells delay their division and instead enter a checkpoint to repair the damages through the activation of a signal transduction cascade. The checkpoint proteins, including a conglomerate of sensor kinases, adaptors and many down-regulated effector protein kinases, help the cells to respond to DNA damages before entering the division phase (Petsalaki and Zachos 2020).

Several DNA repair pathways, working at different levels, are operative in an organism. They can be categorized as: (A) Direct repair (DR) which is essentially an enzyme (photolyase)-mediated, light-dependent photo-reactivation process (Jiang et al. 1997); (B) Mismatch repair (MMR), comprising base excision repair (BER) and nucleotide excision repair (NER) systems; in this repair system, damaged DNA bases and nucleotides are removed and replaced with correct ones (Shuck et al. 2008) and (C) Repair of double-strand DNA breaks (DSBR), which depends on the process of non-homologous end joining (NHEJ) and homologous recombination

(HR) (Puchta and Hohn 1996). All these pathways, though specific and uniquely efficient, are crucial to ensure the continued existence and stability of genomes. However, some kind of links in the execution of different DNA repair pathways has been reported in a number of studies. Molinier et al. (2008), using a genetic approach found a crosstalk of (DR), a prospected nexus between NER and HR mechanisms, with RAD1–RAD10 endonuclease intervention has also been stressed upon (Dubest et al. 2002). In spite of some initiatives taken, detailed understanding of plant-specific DNA repair mechanisms had to go a long way.

1.2.3 Temperature

1.2.3.1 High Temperature Stress

Higher temperature stress and its adverse impacts on physiology (photosynthesis, respiration), metabolism of proteins and other important membrane constituents severely limit the growth and distribution of plants in natural environments (Georgieva 1999). During high temperature, oxidative stress occurs due to overproduction of reactive oxygen species (ROS) which modifies the synthesis of macromolecules and nucleic acids (Khan and Shahwar 2020). Raised temperatures cause injury to plant cells by enough formation of active oxygen species like superoxides, peroxides and hydroxyl radicals, impairing the structure as well as function of vital cellular constituents (Van Breusegem et al. 2001; Liu and Huang 2005). Upon exposure to extremes of temperature, an outburst of highly active oxygen species production occurs in plants cells which subsequently result in cell damage and undesirable physiological alterations. Long-term exposures to temperature extremes and the consequent increase in ROS formation can drastically cause enzyme inactivation, lipid peroxidation, protein and DNA damages. For compensating the negativity of higher temperatures in plant species, a number of detoxification mechanisms (enzyme or non-enzyme dependent) have evolved which convert a considerable fraction of harmful oxygen entities to relatively benign molecules (Sairam and Tyagi 2004). Enzymatic antioxidants like superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase actively detoxify the highly reactive superoxide and H_2O_2 (Mittler 2002). Treatment of plants with salicylic acid (SA), abscisic acid (ABA) and calcium chloride additions shows some promise of enhancing the thermal resistance in a number of crop plants (Larkindale and Knight 2002; Chakraborty and Tongden 2005). Increase in thermal tolerance is particularly vital and indispensable for plants as they can't move to favourable environments in response to the daily temperature fluctuations.

Photochemical reactions and associated carbon metabolism reactions are more likely to get affected if temperatures go beyond 30 °C (Wang et al. 2009). Additionally, the water status of leaf cells and intracellular carbon dioxide are markedly affected due to high temperature generated heat stress-induced stomatal closure (Greer and Weedon 2012). All these effects in consortia lead to an apparent reduction in photosynthetic rate and hence delays developmental progression by

stalling growth. While the underpinning procedure involved in photosynthetic inhibition due to heat stress in plants is largely unclear, reduction in the rate of carbon fixation during photosynthesis due to inhibition of RUBP is believed to be mostly the most plausible reason (Kurek et al. 2007). One more likely explanation suggests that the heat stress significantly halts the process of electron transfer in light reaction of photosynthesis and decreases the operation of rubisco enzyme (Makino and Sage 2007). Amongst all the photosynthetic components PSII (crucial for photosynthetic electron transport in photosynthesis) is the worst affected by elevated temperature stress (Havaux 1996). In chloroplasts, the most severely affected enzymes due to heat stress are PSII, Rubisco and ATP synthase (Asthir 2015).

1.2.3.2 Low Temperature Stress

Cell damage, decreased production and limited distribution of plants in natural environments are also thought to be an immediate outcome of low temperature (0–15 °C) stress (Theocharis et al. 2012). Cold stress initiated damages in the cellular structures of non-adapted plants are observed very early (few hours after subjecting to cold). Moreover, it is a well-known fact that cold temperature treatment for a small duration induces only some transitory alterations while long-term exposures cause necrosis or death. Cold acclimation in plants has been recently related to the attainment of resistance to low temperatures (Theocharis et al. 2012). Reorganization of molecular and physiological features is believed to be the key behind cold tolerance and cold counteractive measures in some plants.

In addition to direct damages to cellular constituents, cold also severely impacts PSII restoration and damage repair. A number of reports confirmed that low-temperature stress inhibits the repair of PSII rather than causing photo damage to it. Protein labelling studies in Synechocystis cells showed a considerable suppression in de novo synthesis of D1 protein at lower temperatures (Allakhverdiev and Murata 2004). Another well-known fact is that extreme low temperature blocks the formation of D1 protein of PSII that is intensely associated with the assembly of photo system II constituents and repair (Kanervo et al. 1997).

1.2.3.3 Temperature Stress-Related Antioxidant Responses in Plants

By and large, the major outcome of oxidation related stresses in plants includes surged ROS production which consequently disturbs the structural and metabolic balances (Munné-Bosch and Alegre 2002). However, to a considerable extent these negative effects of temperature in a large number of plants are compensated (Janská et al. 2010). Plants are known to alter their metabolism for protecting vital proteins and other indispensable cellular structures, maintaining their turgor and osmotic balances (osmotic adjustments) and in some cases cause the modification of antioxidant system to properly stabilize the redox balance and maintenance of cellular equilibrium (Janská et al. 2010; Hasanuzzaman et al. 2013). Quite surprisingly temperature initiated stress effects in a large number of plant species have been observed to be alleviated by changes in the activity profile of a set of temperature stress-responsive genes (Semenov and Halford 2009).

Plants are believed to increase their thermostability and antioxidant potential just to reduce the incidence of temperature-related structural and physiological perturbations (Xu et al. 2013). A wide range of essential antioxidant enzymes in plant cells are drastically affected within the temperature range of 0–50 °C. The activity of CAT, SOD and APX increases upto a temperature of 50 °C and thereafter shows a considerable decline. On the contrary, the activity of POX and GR diminishes with rising temperature and have been shown to perform better in the temperature range of 20–50 °C (Chakraborty and Pradhan 2011).

Besides depending on the exposure time, magnitude of temperature also influences the response of antioxidant formation in many plant species. For instance, the Pepper plants, treated with 8 °C for 3 consecutive days show the oxidation and peroxidation associated symptoms during the first day (Airaki et al. 2012). During the first 24 h, formation of CAT and APX gets invoked, raising the concentration of Asc and GSH. The oxidative stress-related effects in pepper plants got receded in the second and third day of low temperature treatment owing largely to early adjustment of their antioxidant metabolism during the early hours due to adjustment of their antioxidant metabolism (Airaki et al. 2012).

1.2.4 Pesticides

In the face of development and expansion of our economy, we have unknowingly put our life supporting natural resources like water and soil at risk. Among the plethora of industries polluting the precious water and soil resources, pesticide formulation plants are highly perilous. Worldwide as well as in India pesticides like organo-chlorines and phosphates are well-represented contaminants of aquatic and terrestrial ecosystems (Jayaraj et al. 2016). Pesticides present in soils and water in the form of suspended or dissolved particles get accumulated in the edible parts of crop plants, causing a serious threat to the well-being of humans. Recent spike in agriculture production through mechanisation and indiscriminate use of hazardous pesticides and chemical fertilizers have tremendously contributed to water pollution in developing countries. Many pesticide residues which are known to have harmful DNA alteration potencies cause serious mutations (Rahman and Debnath 2015).

Pesticides include a broad range of chemicals used to protect crop plants from fungi, insects, herbs, etc. Amongst these fungicides, herbicides and insecticides constitute the mostly widely used chemicals effective against disease caused by fungi, herbs and insects, respectively (Dhanamanjuri et al. 2013). Unfortunately, the excess use of these chemical pesticides has led to their accumulation in the soil (Ahemad 2011), thereby reducing the fertility of soil. Furthermore, the indiscriminate use of these chemicals is known to have induced significant resistance in the insect pests and other fungi, reducing their effectiveness which is reflected in their tremendous usage. Also it has been ascertained that most of these agrochemicals, besides removing harmful agents, also decline the population of some beneficial insects (Kim et al. 2017). Out of the total 4.6 million tonnes of pesticides used annually worldwide, almost 85% are alone used in agricultural fields (Zhang et al.

2011). Moreover, amongst all kinds of pesticides, herbicides and fungicides are disproportionately used globally (De et al. 2014). Large-scale use of these agrochemicals is supposed to have some serious consequences in plants with apparent disruptions of important physiological and biochemical processes. This occurs due to disruption of membrane structure, reduced photosynthetic yield, and compromised pigment production, disruption of hormone and nutrient status, and halting of DNA synthesis, gene expression and cell proliferation (Shakir et al. 2016). Exposure to herbicide 2,4-D in chicory has been found to induce chromosomal variations in chicory (Khan et al. 2009) A serious concern related to herbicide use is that many of these act non-specifically (Xia et al. 2006), causing considerable economic losses in multiple crop farming. Agrochemicals have been reported to affect plant health by casing genotoxic damage of fundamentally important bio-molecules including DNA by spiking up the pace of reactive oxygen species production (ROS) (Sies 2015). ROS-induced cellular damages especially of membrane proteins and nucleic acids eventually cause a wide spectrum of oxidative and genotoxic responses in plant cells. In response to pesticide-mediated oxidative stress and cellular damages, plant cells exhibit some antioxidant defences (Banerjee et al. 2001). These defences which are both enzymatic (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) and non-enzymatic (phenylpropanoids, carotenoids, glutathione and proline) effectively inactivate and detoxify the harmful free radicals which are later on scavenged (Yusuf et al. 2011). Besides, agrochemicals have also been implicated to have some cytotoxic effects in a number of plant species (Pandey 2008). Excessive exposure to pesticides in *Allium cepa* and *Vicia faba* has been known to cause serious chromosome structural aberrations (Mesi and Kopliku 2013). These structural alterations in chromosomes are reflected in the form of mutations (Fatma et al. 2018). Owing to the above fact, agrochemicals are widely assessed for their mutagenic potencies in crop plants (Larramendy et al. 2015). Therefore, in addition to reducing crop pests, many of the agrochemicals are strongly associated with some chronic crop damages and are hence absolutely concerning. These severe drawbacks of chemical pesticides call for the creation of alternatives which are target specific, environment friendly, cost effective and above all without any genotoxic side effects (Rahman and Debnath 2015). Despite a handful of studies, precise comprehension of the underlying pesticide-induced crop damage mechanisms is yet to be understood. In an attempt to investigate the various kinds of cytotoxic and genotoxic effects of pesticides on the genome of crop plants, *Trigonella foenum graecum L.* (fenugreek), native to tropical regions, was being exposed to fungicides like tricyclazole and thiabendazole and insecticides including plethora and slash-360. It was found that the exposure fungicides and insecticides in this plant species causes a number of abnormalities among which chromosomal breakdown, membrane disruption and generation of ROS are highly consequential (Mahapatra et al. 2019).

1.2.5 Salinity

Salt stress is regarded as one of the major global issues having detrimental effects on crop plants. According to an estimate almost 50% of the global agricultural land will be harmed due to rising salt quantities (Wang et al. 2003; Bartels and Sunkar 2005). Escalated salt concentration in soils is strongly associated with a number of crop injuries among which oxidative stress, formation of reactive oxygen species and membrane protein disruptions are concerning (Munns 2006; Muchate et al. 2016). Building up of excess salts in the root systems of plants, through stoppage of water and mineral uptake, disturbs the osmotic equilibrium (Paranychianakis and Chartzoulakis 2005). It has further been reported that excess salts leads to enormous harmful effects on the integrity and functioning of DNA, RNA, represses synthesis of proteins, impedes the continuity of cell cycle, retards germination of seeds and decreases the productivity (Rodríguez-Eugenio et al. 2018; Anuradha and Rao 2001). To ensure their survival, plants constantly adapt by activating a series of genes including protein kinases. These protein kinase genes have recently been shown to function in various signal transduction cascades which govern cell proliferation and initiation of stress response (Zhu 2016). Currently newly identified variants of nutrients and fertilizers are being given exogenously to plants by researchers to improve their salt tolerance and hence productivity (Zhu 2016). There is concrete evidence in favour of l-carnitine exogenous treatment scaling up the pace of cell cycle by increasing mitosis under saline circumstances (Surai 2015). During episodes of salt stress in mammalian cell lines, it has been observed that l-carnitine activates a number of antioxidant enzymes which are actively associated in the manufacture of numerous protective molecules (Surai 2015). By controlling cell cycle through some unknown transitions, antioxidant compounds enhance the salt tolerance in plants and thus reduce the incidence of salinity associated oxidative damages (Benjamin et al. 2019). Similar studies by Charrier et al. (2012) suggest that in *Arabidopsis thaliana*, carnitine treatment of seedlings greatly supports development, besides giving protection against excess salts and the associated oxidative damages. In view of the stimulatory effect of carnitine on seed germination and cell proliferation in *Arabidopsis thaliana*, its 1 mM concentration is appropriately suggested to be the best stress reducing remedy in other plant cells.

It has been observed that when cells located at the root tips of barley were treated with high salt concentrations, they undergo chromosome breakdown. A handful of studies revealed that abnormally high salt levels are mutagenic due to induction of structural aberrations or even changing the number of chromosomes (Tabur and Demir 2010). Quite interestingly, it has been well reported that increased concentration of salts raises the percentage of chromosome abnormality (Marakli et al. 2014). Amongst all sorts of abnormalities, disorderly prophase was the most prominent type of chromosomal alteration in salt stressed seeds of barley. Furthermore, salt stress has been acknowledged to generate a significant number of ring-shaped chromosomes in this species. Surprisingly the prior treatment of salt stressed root meristem tips of barley with l-carnitine significantly reduced the frequency of

oxidative stress initiated chromosomal anomalies and other genotoxic effects (genotoxic index).

1.2.6 Antibiotics

There is a growing concern among the scientific community regarding an increase in the traces of pharmaceutical products in the environment (Pico and Andreu 2007). So far a number of drugs have been reported to occur in soil sediments, wastewaters of domestic and industrial origin, natural water bodies and interestingly in the living organisms of aquatic ecosystems (White and Rasmussen 1998). Many antibiotics are known to occur in huge amounts in organic fertilizers (Hamscher et al. 2002), domestic sewage and sludge treated soils (Golet et al. 2003). It is well known that a significant fraction of drugs including antibiotics find their way into the wastewaters through the excreta. Drugs like fluoroquinolones (FQs) have been detected in appreciable amounts in the raw sludge and water samples of natural reservoirs in Switzerland (Golet et al. 2002, 2003). Furthermore, addition of this drug laden sewage sludge to the agricultural soils pollutes the soil and underground water resources (Hamscher et al. 2005).

The ever-increasing ecological concern related to the presence of pharmaceutical traces in the wastewaters of hospitals is that several antibiotics and cytostatic drugs exhibit DNA damaging properties in both prokaryotic and eukaryotic cells (Giuliani et al. 1996). It has been found that the wastewaters of health care institutes contain considerable quantities of ciprofloxacin which was later found to be the principal genotoxic agent in these effluents (Hartmann et al. 1999). Drugs like fluoroquinolones were shown to cause untimely replication of genetic material, induce DNA cuts, inflict chromosome damages and form micronuclei (Bredberg et al. 1991). Considering the huge genotoxic potential of quinolones and fluoroquinolones, evaluation of their impacts on plant roots through direct exposure was impressed upon. Subsequently a test based on micronuclei formation in *Vicia faba* was devised by Marcato-Romain et al. (2009) to assess the genotoxic implications of drugs like quinolones and fluoroquinolones. This test is enough sensitive for the assessment of both clastogenic and aneugenic effects of drugs on plant genomes (El Hajjouji et al. 2007). Micronuclei basically arise because of chromosomal cuts and abnormal mitosis.

An important group of antibiotics having structural resemblances to nalidixic acid (NA) effectively interact with the DNA gyrase enzyme and inhibit its activity (Curry et al. 1996). Another group of highly active compounds affecting a broad range of bacterial species include the fluorinated quinolones and naphthyridines where the seventh carbon position is linked to a cyclic amino group as its enrofloxacin (ENR) (Radl 1990) and its principal metabolite ciprofloxacin (CIP) (Gorla et al. 1999). The mammalian topoisomerase II which is similar to other gyrase enzymes and many other enzymes assisting replication are known to strongly cross-react with quinolones (Bredberg et al. 1991). It is supposed that this compound invariably leads to stabilization of Gyrase-DNA complexes which subsequently causes

topoisomerase II induced DNA cleavage (Robinson et al. 1991). Fluoroquinolone compounds were also shown to have a considerably strong reactivity towards enzymes involved in the DNA replication (Bredberg et al. 1991). In view of their topoisomerase II inhibition properties, these chemical compounds induce a series of genotoxicity-related phenomenon like breakage of DNA strands during its replication, non-disjunction and compression of chromosomes during the process of meiosis (Ferguson and Baguley 1994; Heisig 2009). Since topoisomerase II is also present in plants and performs exactly the same function of DNA copying and cell division, quinolone and naphthyridine treatment leads to the same kind of DNA and chromosomal aberrations in plant cells (Fukata et al. 1986; Reddy et al. 1999). Additionally fluoroquinolones are reported to cause varying levels of oxidative stress in bacteria (Becerra and Albesa 2002) and a number of eukaryotes (Pouzaud et al. 2004). Induction of oxidative stress by fluoroquinolones accompanies a series of severe DNA damages (Halliwell 1990). Inhibition of topoisomerase II enzyme and the oxidative damages especially breakdown of DNA strands by these compounds may induce the formation of micronuclei.

1.2.7 Dyes

Dyes constitute a heterogeneous group of chemicals having wide range industrial and domestic applications. Earlier people used to get dyes from a wide range of natural sources like the flowers of forest fire to colour their clothes. Some other dyes of plant origin include indigo, logwood and madder. However, dyes like Tyrian purple, kermes, cochineal and many others are obtained from animals. All these natural dyes are easily biodegradable and hence were not polluting the environment. Unfortunately, in view of non-availability and expensive rates of natural dyes, synthetic dyes which are relatively cheaper and easily available find a large-scale use at industrial and domestic scales, but at the same time are resistant to biodegradation and pollution causing.

Most of the synthetic dyes are known to have enormous genotoxic effects in plants. Azo dyes (containing the Azo functional group, -N=N-) are the principal synthetic textile colouring agents studied with respect to their genotoxic consequences in plants (Balakrishnan et al. 2016). Some classes of these Azo dyes, containing the Azo functional group have the tendency of releasing carcinogenic amines which are highly genotoxic. An important example of an Azo dye releasing genotoxic agent, benzidine, is Acid Red 85. Azo compounds are reduced to free aromatic amines by anaerobic microbes of the gut and azo-reductases present in the liver and intestines of mammals.

Huge quantities of dyes are released into the environment on a daily basis along with the effluents food, cosmetic, drug and textile industries. The chemicals coming out of textile and dyeing industries are immensely coloured and their drainage into the water bodies adversely impacts their well-being and aesthetic beauty. Besides, the salts and other heavy metals in the effluents of dyeing industries were reported to have many disastrous impacts on the aquatic vegetation of the receiving water bodies

(Wells et al. 1994). Additionally a disproportionate fraction of dyeing stuff and chemicals used in textile industries are highly tolerant to degradation by both physical and biological agents (Ogawa and Aiba 1981; Seshadri et al. 1994; Suzuki et al. 2007). They are hard to decompose by biological agents due to their tremendously ordered polymeric nature (Neppolian et al. 1999). In view of this enormous stability and non-biodegradable nature, synthetic dyes pollute a wide range of natural resources including water, soil and progressively find their way into plants, animals and ultimately into humans.

The environmental degradation and the toxic effects of non-biodegradable dyes coming out of textile industries are concerning globally. Besides imparting a persistent colour, they altogether change the water quality parameters and render it unfit for agriculture and domestic uses. Dye and allied textile industries are, therefore, a consistent source of enormously harmful genotoxic agents. According to a report on mutagenic potential of different wastes, Houk (1992) placed textile and dyeing related wastes as moderately mutagenic. Many types of chromosome damages and other mutations are suggested to be induced by the dyes present in textile industry wastewaters.

1.2.8 Industrial Waste

Recent development in the industrial and allied fields has seriously impacted the life of almost every living organism through disturbances of ecological and ecosystem dynamics (Iqbal et al. 2019). Unabated discharge of untreated wastewaters from different industrial units into the river ecosystems has tremendously disturbed the ecological balance and deteriorated the water quality of these freshwater ecosystems (Salles et al. 2016). Long-term exposure of organisms to the hazardous chemicals contained in wastewaters causes various chromosomal aberrations with strong follow-up genotoxic effects, reflected in humans as well (Mazzeo et al. 2018). A number of plant species including *Allium cepa* (onion), *Vicia faba* (broad bean), *Tradescantia* (spiderwort), *Pisum sativum* (pea), *Hordeum vulgare* (barley), *Zea mays* (corn), *Crepis capillaries* (smooth hawksbeard) and *Nicotiana tabacum* (tobacco) were appropriately utilized as genetic models to emphasize the toxicity of industrial effluents (Iqbal and Nisar 2015; Bhat et al. 2017). Amongst all these genetic models, the bioassays done on *Allium cepa* and *Vicia faba* are strongly recommended biomonitoring devices to evaluate the genotoxicity of industrial effluents (Mazzeo et al. 2018). These tests are preferred due to the detection of different end points with a good focus on revealing phytotoxicity (effect on length of roots and germination index), cytotoxicity (related to mitotic index), genotoxicity (chromosome alterations) and mutagenicity (micronucleus formation) (Mazzeo et al. 2018; Iqbal et al. 2019). A number of other plant-based genotoxicity tests were applied to assess the toxicity of wastewaters and sludges coming from various sources like dyeing and paper mills (Grover and Kaur 1999), silk industries (Sudhakar et al. 2001), domestic sewage (Srivastava et al. 2005), Azo dyes contaminated waters (Carita and Marin-Morales 2008) among many others.

Furthermore it has been reported that these toxic industrial wastewaters not only affect the flora and fauna of aquatic ecosystems, but their effects are well transmitted to humans through the food chains. In the biomonitoring of textile wastewaters by Grover and Kaur (1999) using *Allium cepa*, it has been shown that the effluent, besides causing anaphase abnormalities, induces the formation of micronuclei. Furthermore with the increase in the concentration and time of silk effluent exposure, the authors reported a significant decrease in the mitotic index.

To understand the cytotoxic and genotoxic influence of textile industry effluents, Samuel et al. (2010) employed *Allium cepa* biomonitoring assay and found significant DNA aberrations in its root cells. Additionally other chromosomal abnormalities in the form of vagrants, bridges, fragments and adhesive chromosomes have been observed in this plant species. The test samples showed considerable decline in their mitotic index values (9.42%) compared to the controls (11.68%) when exposed to textile wastes containing dyes. Furthermore, another study by Okoro and Okoro (2011) showed that exposure of root tip cells of *A. cepa* to textile effluents induces micronuclei formation, causes aberrations in chromosome and DNA structure.

The wastewaters of paper and pulp industries are largely a mixture of different endocrine and DNA altering substances (Balabanič et al. 2017). These toxic substances in the wastewaters of paper and pulp mills disrupt the ecological stability of aquatic habitats by reducing both the population density and species richness (Pokhrel and Viraraghavan 2004). Numerous attempts aimed at understanding the toxicity of paper and pulp mill effluents on the environment were performed by employing varied bioassays (Chaparro and Pires 2011, 2015; Haq et al. 2016, 2017). Grant et al. (1992), for instance, determined the genotoxic nature of pulp and paper mill wastewaters by means of *Tradescantia* and *V. faba* biomonitoring assays.

The large build-up of tremendous amounts of wastes in open lands from sugar mills in developing and underdeveloped countries is a serious issue due to its harmful effects on soil quality and pollution of water bodies. For the assessment and evaluation of toxicity of sugar mill effluents, Ozkara et al. (2011) employed *Hordeum vulgare* biomonitoring device. They reported that the sugar mill effluents significantly downsized the germination rate, declined root extension and reduced mitotic index of exposed seedlings of *H. Vulgare* in contrast to control. In addition a number of chromosome anomalies including c-mitosis, lagging chromosomes, multipolar anaphases and bridged chromosomes were observed in *H. Vulgare* root cells treated with sugar mill effluents in comparison with controls. One more study to examine the genotoxic effects of sugar mill wastewaters was done by using *A. cepa* bioassay (Bhat et al. 2014). The effluents were found to have detrimental effects on both the root cell extension and mitotic index of *A. Cepa*.

It is well known that the vermicomposited pressmud sludge causes numerous irregularities in the cytology and chromosome structure of plant cells among which anaphase interruption, C-mitosis, laggards, vagrants, bridge formations, sticky and severed chromosomes are extensively studied. The effect of vermicompost on detoxifying the sugar beet pulp wastewater was studied by Bhat et al. (2018) using the *A. cepa* bioassay. A considerable increase in the root length and mitotic index

values of *A. cepa* after being exposed to the vermicomposited pulp suggests that vermicompositing reduces the toxicity of sugar beet pulp to a considerable degree. Furthermore it has been found that vermicomposited sugar beet pulp's ability of causing chromosome abnormalities got declined by almost 34–62% as compared to the raw pulp. It has been speculated that the earthworm *Eisenia fetida* detoxifies the sugar beet pulp during the process of vermicompositing. The toxic effects of sugar mill wastes have also been studied by Anacleto et al. (2017) who separately examined the negative consequences of 6 months and 3 months vermicomposited sugar mill filter cake sludge on *A. cepa*. The mitotic index of *A. cepa* roots exposed to primary (non-vermicomposited) sugar mill filter cake sludge samples got reduced while the structural alterations in chromosomes got scaled up to an appreciable degree. Most importantly, it has been acknowledged that sugar mill pulp samples vermicomposited for 6 months got notably reduced in their influences on cell integrity, genome structure and the associated mutational implications. Similarly the toxicity of sugarcane vinasse was comprehensively studied by Garcia et al. (2017) using *A. cepa* biomonitoring device. Numerous genomic defects like bridging of chromosomes during anaphase, loss and frequent cuts were reported in bioassays treated with sugarcane vinasse wastes. Besides, the mutagenic potential of sugarcane vinasse extracts also got concretely supported by the presence of micronuclei in various bioassays.

1.3 Conclusion

Recent progress in industrialization and other related human developments in agro-technology and allied fields have contributed huge loads of disastrous heavy metals in the environment. Besides toxic heavy metals, there are numerous other environmental stresses which significantly reduce the performance and yield of crop plants. The effects of environmental stresses are by and large effectuated at biochemical and physiological levels, compromising the stability of membranes, curtailing production of photosynthetic pigments, reducing biomass production, causing DNA replication and transcription setbacks. To cope up with these negative impacts of stress-causing agents, plants in due course of time have evolved an array of counteracting and scavenging pathways which better equip them to reduce the incidences of stress-induced production and fitness losses. For a way forward this study is aimed to highlight and comprehend the recent advancements in our understanding of how plants resist and, in some cases, tolerate the negative effects of environmental factors. How strongly these pathways contribute to the fitness and performance elevation of plants growing under natural field conditions is still an open question and merits some substantial future investigations.

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Genotoxicity and DNA Damage Induced by Herbicides and Toxins in Plants

2

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Abstract

Genetic toxicology is a multidisciplinary field of research that explores the detection of harmful and defensive DNA compounds, the understanding of DNA disruption's biological effects, and its molecular modes of action that lead to the modification and repair of genetic material. The damage to the genetic material is caused by the genotoxic substance's interaction with the structure and sequence of deoxyribonucleic acid of plants. These genotoxic substances function at a specific position or base sequence of the structure of DNA, causing disruption, fracturing, fusion, deletion, mis-segregation or non-disjunction, resulting damage, and mutation. Many herbicides use inactivation "target proteins" (usually enzymes) that are necessary for important functions such as chemicals or other plant-specific pathways of synthesis. Since crops usually use competing weeds to share these cycles, most herbicides are non-selective. Others are used mostly by collection of resistant species, primarily due to a differential absorption or metabolism of the herbicides or to a certain position. Another provides protection against herbicides of wide-spectrum. This could encourage the use and choice of these different compounds to be environmentally responsible and non-toxic. A plant can reduce the translocation of herbicides on several pathways. In modern years, plants were genetically engineered to fight the lethal effects of herbicides. The resistance of the natural herbicides in plants is responsible for different forms, the target site insensitivity, and the toxic herbicide

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degradation of the toxic by-products are noteworthy. Both these pathways have been simulated in genetically engineered plants either by excessive expression of the target enzymes or by developing foreign defence products that could easily detoxify the herbicides.

Keywords

Genotoxicity · Herbicides · Phytotoxicity · 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase · Metabolic detoxification

2.1 Introduction

Genotoxicity may be a word employed in biological science that describes the possession of a substance that has harmful results on the genetic material and integrity of the (Phillips and Arlt 2009). Genotoxicity is a word utilized in hereditary qualities that portray the ownership of a substance that destructively affects the hereditary material of the cell along these lines influencing the uprightness of the cell. Genotoxicity could be a word utilized in biological science that describes the possession of a substance that incorporates a harmful result on the genetic material of the cell therefore touching the integrity of the cell. Genotoxins are mutagens that may cause genotoxicity resulting DNA damage or body material therefore inflicting mutation. Genetic pharmacological medicine is that the branch of science that deals with the study of agents or substances that may harm the cell's DNA and chromosomes. It is noted that always genotoxicity is confused with mutagenicity. All mutagens are genotoxic but all genotoxic substances are not agents (De Flora and Izzotti 2007).

Pesticides together with herbicides and fungicides are used extensively to boost crop yields, and as a result they accumulate within the surroundings. More than 2.5 million tons of pesticides and herbicides are applied per annum to agricultural crops worldwide (Van der Werf 1996). Herbicides tend to be terribly reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA (Crosby 1982). Owing to their biological activity, the employment of herbicides could cause unsought effects to plant health.

Varied use of pesticides in crop production is very important to make sure crop viability by thwarting weeds, insects, fungi, and sickness. Studies purpose to genotoxic risk to agricultural plants, moreover as humans and animals, from chemical agents employed in crop production. Reactions of DNA bases with pesticides or their metabolites alter the structure of the super molecule, and forestall correct replication. This degradation of genetic material ends up in the inferior development of the vascular plant, leaf, roots, fruit, and ultimately to inferior quality or yield of the agricultural product (Boerth et al. 2005).

Herbicides have been widely used for a greater exploitation of various plants in industrial cultivation and landscape turf maintenance. They are successful but have a heavy biological activity against plants. They are produced worldwide for farmers

who have suffered heavy losses due to weeds. The gains, though, accomplish a substantial cost. They are degradation-resistant and bio-accumulated in the ecosystem, impacting higher species and causing adverse secondary effects in plants. They can also be identified by their "site of action" or by the particular biochemical site that the herbicide's impact. Its characteristics improve the chance of shipping, including tolerance to corrosion, and high solubility in water. The fate of herbicides in the ecosystem is determined by the preservation, conversion, transport, and contact mechanisms of all these processes (Spadotto 2011). The repeated and frequently incorrect use of herbicides will lead to pollution of soil, air, groundwater, and surface water, as well as food and living species. In the 1960s, the use of agrochemicals in developed agriculture became popular in developing countries and was characterized as the green Revolution (Bolognesi 2003; Spadotto 2004).

2.2 Mechanism of Genotoxicity

Genetic toxicology is a multidisciplinary area of science that deals with the identification of dangerous and protective DNA substances, the understanding of the biological effects of DNA disruption, and its molecular modes of action that contribute to genetic material alterations and repairs. To detect genotoxic effects, concentration protocols or highly sensitive detection systems that can be used for in situ monitoring are needed. As all the concentration methods currently available lead to the loss of potentially active substances, the second option is preferable. Several plant bioassays that can be used for environmental monitoring in situ have been produced (Uhl et al. 2003).

For several decades, most of the indicator organisms have been used in genetic science and essential characteristics insert the abundance of information on their genomic composition, a limited number of chromosomes suitable for the study of aberrations. The damage to the genetic material is caused by the interactions of the genotoxic substance with the DNA structure and sequence. These genotoxic substances interact at a specific location or base sequence of the DNA structure causing lesions, breakage, fusion, deletion, mis-segregation or non-disjunction leading to damage and mutation. For example, in its high-valent oxidation state, the transition metal chromium interacts with the DNA so that DNA lesions occur leading to carcinogenesis. Researchers have found that the mechanism of damage and base oxidation products for the interaction between DNA and high-valent chromium are relevant to in vivo formation of DNA damage leading to cancer in chromate-exposed human population, thus making high-valent chromium a carcinogen.

It is important for the prevention of DNA changes caused by the environment to understand the biological consequences of DNA damages and their molecular modes of action that lead to repair or alterations of the genetic material. Numerous genotoxicity assay systems have been developed to identify DNA reactive compounds. The available data show that plant bioassays are important tests in the detection of genotoxic contamination in the environment and the establishment of

controlling systems. The plant system can detect a wide range of genetic damage, including gene mutations and chromosome aberrations.

The harm to the genetic material is caused by the interactions of the genotoxic substance with the deoxyribonucleic acid structure and sequence. These genotoxic substances act at a particular location or base sequence of the deoxyribonucleic acid structure inflicting lesions, breakage, fusion, deletion, mis-segregation or non-disjunction resulting in harm and mutation. For instance, in its high-valent number, the transition metal interacts with the deoxyribonucleic acid so that deoxyribonucleic acid lesions occur resulting in carcinogenesis. It is necessary for the interference of deoxyribonucleic acid changes caused by surroundings to know the biological consequences of deoxyribonucleic acid damages and their molecular modes of action that result in repair or alterations of the genetic material, varied genotoxicity assay systems are developed to spot deoxyribonucleic acid reactive compounds. According to the available data, plant bioassays are necessary tests for detecting genotoxic pollution in the environment. The plant system will observe a large variety of genetic harm, together with factor mutations and body aberrations.

2.3 Types of Changes Induced by Herbicides

Chromosomal aberration: Herbicide causes mitotic and meiotic irregularities both in vivo and in vitro in plant cells (Khalatkar and Bhargava 1982). In addition, fractures, erosion, bridges, laggards, micronuclei, chromosomal anomalies like univalent and multivalent at diakinesis, precocious separation, stray chromosomes, stickiness, and polyads have been used as an indicator of reproductive performance in plants for many years and have been correlated with morphological and taxonomic improvements, relationships between fertility and sterility, mutations, and other features of polyploidy and aneuploidy (Fiskesjo et al. 1981; Pavlica et al. 1991; Khan et al. 2009). The herbicide contamination of the soil at the rates of application suggested for agricultural use resulted in a substantial rise in the incidence of aberrant cells. Changes in mitotic function, as well as changes in chromosome and chromatin composition, and even changes during the cell cycle were caused by the herbicide in *Allium* root tips (Geras'Kin et al. 2006). In *Allium cepa* and *Oryza sativa*, mutagenic herbicide activity was tested using cytogenetic, chlorophyll mutation, specific locus, and pollen viability endpoints (Pavlica et al. 1991). The occurrence of aberrations was measured in the *Allium* root-tip experiment. The incidence of aberrant cells has increased with an increase in concentration. In the case of mutation assays, there was associated rise in mutation frequency with increasing concentration. In addition to sterile pollen, very large chlorophyll-deficient and waxy mutants were found (Kumari and Vaidyanath 1989).

Homologous recombination: The transgenic *Arabidopsis thaliana* point mutation and recombination experiments were used by (Filkowski et al. 2003) tracking the genetic effects of the herbicide-associated A— > G mutation. They observed an important impact of the herbicide on the incidence of homologous recombination.

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

Chromosome Stickiness and Clumping: Grant (1978) proposed that chromosome stickiness grows from the chromosome fiber's improper folding into single chromatids and chromosomes. As a consequence, the fibers are mixed and the chromosomes are linked to each other through sub-chromatid bridges. Stickiness of the chromosomes and clumping have been documented after herbicide application.

Chromosome fragmentation: Zeljezic and Garaj-Vrhovac (2004) stated that chromatid and chromosome split, the number of micronuclei, and the number of nuclear buds were increased by herbicides. The findings are from multiple chromosome split in which chromosome identity is damaged. Fragmentations can vary from partial to complete chromosome disintegration. Chromosome fragmentation of plant cells was found in *Allium cepa* root tip cells following herbicide application (Grant 1978).

DNA strand breaks: A single cell gel electrophoresis (SCGE or comet) assay is the only routine genomic DNA damage test currently conducted on plants. By measuring the degree of DNA migration through an agarose gel in an electric field, the alkaline SCGE assay detects single and double stranded DNA breaks and conformation shifts in genomic DNA (Tice et al. 2000). Comet chemical mutagen induction in tobacco leaves and roots was closely associated with leaf mutation induction (Gichner and Plewa 1998; Gichner et al. 1999).

Sister-chromatid exchange (*SCE*): Chromatid modification can result in somatic recombination and sister-chromatid exchanges (SCE) that can affect gene expression through the lack of heterozygosity. Using meristem root-tip cells of garlic (*Allium sativum* L.) 2,4-D, the effect of herbicides at low concentrations on cell cycle length and sister-chromatid exchange (SCE) frequency was tested to induce a pronounced prolongation of the cell cycle. The Genotoxic potential of several herbicides in plant cells is summarized in Table 2.1 (Enan 2009).

2.4 Herbicides and their Effects on Plant

Herbicides, as the name implies, are compounds designed to kill plants. Most of them do a respectable job. They remove unwanted herbs while leaving the desirable ones. Every so often, one turns crazy and harms alluring plants. They kill or suppress plants by interfering with essential plant processes such as photosynthesis. The entirety of the collaborations between an herbicide and a plant from application to the last impact are alluded to as the method of activity. Understanding the method of activity of an herbicide is basic in choosing the correct herbicide, diagnosing herbicide injury side effects, forestalling herbicide opposition issues, and staying away from non-target natural effects. The terms method of activity and component of activity are frequently utilized conversely. The mechanism of action refers to the plant's specific biological process that is ceased by the herbicide, whereas the mode of action is a general term referring to all of the plant–herbicide interactions.

Table 2.1 Genotoxic potential of herbicides in plant cells (Mohamed R. Enan 2009)

S. No.	Plant	Type of changes	References
1	*Allium cepa*	Chromosomal aberrations	Mohandas and Grant (1972)
		Chromosome fragmentation	Kumari and Vaidyanath (1989)
		Chromosome stickiness and clumping	Grant (1978)
2	*Allium sativum*	Chromosomal aberrations Clastogenicity	Ateeq et al. (2002)
		Sister-chromatid exchange	
		Mitotic index	Doležel et al. (1987)
3	*Arabidopsis thaliana*	Homologous recombination	Filkowski et al. (2003)
		(A--- > G mutation)	
4	*Oryza sativa*	Chromosomal aberrations	Kumari and Vaidyanath (1989)
5	*Pisum sativum*	Chromosomal aberrations in meiosis	Grant and Owens (2001)
		C-mitosis	
		DNA damage	
6	*Secale cereale*	Endoreduplication	Grant (1978)
7	*Triticum aestivum*	Sister-chromatid exchange	Murata (1989)
8	*Sorghum*	Meiotic chromosome aberrations	Plewa (1978)
9	*Hordeum vulgare*	Mitotic chromosome aberrations	Geras'kin et al. (2006)

Glyphosate is one of arguably the most commonly commercialized herbicides (Vivancos et al. 2011). It is known as a systemic herbicide inhibitor of EPSPS (Enzyme-5 enolpyruvyl-shikimate-3-phosphate synthase) inactivated by soil material that can regulate most annual and perennial plants. EPSPS is an enzyme that is only present in micro-organisms and plants. By inhibiting the synthesis of aromatic amino acids such as tryptophan and tyrosine needed for protein formation in susceptible plants, it regulates the weeds (Pipke et al. 1987). Multiple biochemical pathways are also impaired and if these effects are taken into consideration, they can be significant in the ultimate lethal activity of glyphosate.

Atrazine is listed under the triazine class used in various crops such as corn, sorghum, sugarcane, and to some degree in landscape vegetation to avoid pre and post-emergence broadleaf weeds. After glyphosate, atrazine was the second most commonly used herbicide found in the rural area in the USA. It does not take place spontaneously. When treated sequentially with ethylamine and isopropylamine, it is prepared from cyanuric chloride. Atrazine can be consumed by plants from the roots or by the leaves. When ingested, it accumulates and prevents photosynthesis in susceptible plant species in the growing tips and new leaves of the plant.

Several experiments have been undertaken to research the effect of herbicides such as cyanazine, gespax, goltix, aventox, and atrazine on large-scale mitotic

activity, chromosomes, and nucleic acids in root tip cells of various plants (Wu and Grant 1966; Liang et al. 1967; Stroev 1970; Hakeem and Shehab 1972; Liang and Liang 1972; Dryanovska and Petkov 1980; Badr 1983, 1986; Mousa 1982; Tomaskova and Mydilova 1986; Airapetyan et al. 1984; Papes et al. 1989; Haliem 1990; Ashour and Abdou 1990). The previous authors suggested that both herbicides prevented the division of cells and caused chromosomal defects, and that the inhibition was integrated with nucleic acid reduction in some cases.

2.5 The Mode of Action of Herbicides Involves

2.5.1 Contact and Absorption

Herbicides must contact the plant surface to be powerful. Herbicides with restricted portability that are powerful at the site where they contact the plant are known as contact herbicides. Herbicides that must be consumed and moved to the site of activity to be viable are called foundational herbicides. Contact herbicides ordinarily influence just the bit of the plant with which they come into actual contact. These are quick-acting, and injury indications can show up inside long stretches of use. On the other hand, injury side effects from fundamental herbicides can take from a few days to weeks to show up, yet the whole plant may, at last, be killed. Soil-applied herbicides are applied to the best couple of crawls of the soil, and at last ingested through root tissue, though foliar-applied herbicides are applied to leaves or stems. Most contact herbicides are foliar-applied, though fundamental herbicides can be either soil or foliar-applied.

Picking the fitting herbicide relies on track species science, herbicide selectivity, application technique, and site conditions. It is imperative to comprehend these components to guarantee that a powerful herbicide is chosen. For instance, contact herbicides are best against yearly obtrusive plants and in circumstances in which plant regrowth is not a worry. On the other hand, foundational herbicides are more successful in enduring intrusive plants and can restrict the recovery of treated plants. Soil-applied herbicides are most effective on seedlings or germinating plants prior to their emergence above the soil. The set up plants may require a foliar-applied herbicide for successful control. Develop plant tissues ingest herbicides less effectively than youthful plant tissues because of the thickening of the external tissues in more established plants.

2.5.2 Translocation

Systemic herbicides move, or translocate, from the point of application to the site of action through either the phloem (tissue that transports sugars from the leaves to the roots), xylem (tissue that transports water from the roots to the leaves), or through both. A few herbicides move more effectively inside plants than others.

2.5.3 Site of Action

To be effective, an herbicide must arrive at the site of activity. Herbicide ties to a particular area inside the plant, commonly a solitary protein, and accordingly, upsets a physiological cycle basic for ordinary plant development and improvement.

2.5.4 Mechanism of Action

Herbicides can influence different locales of activity inside plants, and they are frequently arranged into various systems of activity dependent on how they work and the injury side effects they produce. The different classes of herbicides follow up on by various systems of activity, viz. photosynthetic inhibitors, color inhibitors, lipid union inhibitors, amino corrosive combination inhibitors, development controllers, cell layer disrupters, and breathe inhibitors.

2.6 Effect on the Target Plant

Photosynthesis inhibitors block the light responses of photosynthesis where plants convert the energy from sunlight into the synthetic structures needed for plant digestion. The photosynthesis inhibitors incorporate the accompanying herbicide families', viz. triazines, phenyl ureas, uracils, benzothiadiazole, and nitriles. Photosynthesis inhibitors shut down the photosynthetic (food delivering) measure in defenceless plants by authoritative to explicit locales inside the plant's chloroplasts. Hindrance of photosynthesis could bring about moderate starvation of the plant; in any case, the plant encounters a more quick demise that is accepted to be because of the creation of auxiliary poisonous substances. Injury manifestations incorporate yellowing (chlorosis) of leaf tissue followed by death (putrefaction) of the tissue. Three herbicide families (triazines, phenyl ureas, and uracils) are taken up into the plant through the roots or foliage and move to plant leaves through xylem. Thus, injury side effects will initially show up on the more seasoned leaves, along the leaf edge. After foliar application, triazine, phenyl ureas, and uracil herbicides are less portable and do not move out of the leaf tissue. The nitrile and benzothiadiazole herbicide families are not versatile in plants and are named post-rise contact herbicides. Contact herbicides should altogether cover a vulnerable plant's foliage if full oversight is to be accomplished. Photosynthetic inhibitors may control yearly or perpetual grass or broadleaf weeds.

The growth regulators types of herbicides include the following herbicide families' viz. phenoxy acetic acids, benzoic acids and the pyridines. Growth regulator herbicides can act at multiple sites in a plant to disrupt hormone balance and protein synthesis and there by cause a variety of plant growth abnormalities. These herbicides specifically execute broadleaf weeds; notwithstanding, they are fit for harming grass crops. Herbicide take-up is essentially through the foliage yet root

take-up is conceivable. Injury side effects are generally clear on recently creating leaves.

The seedling development inhibitors incorporate the accompanying herbicide families: dinitroanilines, acetanilides, and thiocarbamates. Seedling development inhibitors meddle with new plant development, along these lines lessening the capacity of seedlings to grow typically in the soil. Herbicides in these families must be soil-applied. Plants can take up these herbicides after sprouting until the seedling rises out of the soil surface. Accordingly, these herbicides are just successful in seedling yearly or lasting weeds. Seedling development inhibitors are dynamic at two fundamental destinations, the creating shoot and the root. Substantially more is thought about how seedling root hindering herbicides work than about how seedling shoot inhibitors work. The root inhibitors prevent plant cells from separating, which hinders shoot prolongation and parallel root development. Take-up is through creating roots and shoots. Since herbicide development inside the plant is restricted, herbicide injury is kept fundamentally to plant roots and shoots. Shoot inhibiting herbicides are taken up by developing roots and shoots and can move via the xylem to areas of new growth. There is proof to propose that these herbicides can influence different locales inside a plant, fundamentally meddling with lipid and protein combination.

The lipid union inhibitors incorporate the accompanying herbicide families: aryloxyphenoxypropionates and cyclohexanediones. These herbicides forestall the arrangement of unsaturated fats, parts basic for the creation of plant lipids. Lipids are fundamental to the uprightness of cell films and new plant development. The lipid blend inhibitor herbicides repress a solitary key compound engaged with unsaturated fat biosynthesis. Broadleaf plants are open-minded to these herbicide families, notwithstanding, practically all perpetual and yearly grasses are defenseless. Injury indications are delayed to create and show up first on new leaves arising out of the whorl of the grass plant. These herbicides are taken up by the foliage and move in the phloem to territories of new development.

2.7 Effect on Non-Target Plants

Beside the fact, the purpose in utilizing herbicides is to kill undesirable plants to empower food harvests or ornamentals to flourish, now and then the utilization of herbicides has the unintended outcome, when applied improperly, of harming non-target plants. Herbicides can likewise have unintended ramifications for non-target plant species, species structure, and plant species lavishness and variety. For instance, herbicides, for example, picloram that is specific for broadleave plants can control broadleave obtrusive plants, for example, spotted knapweed (*Centaurea maculosa*) and sulfur cinquefoil (*Potentilla recta*) and advance recolonization of local grasses. In any case, due to this selectivity for broadleaved species, these herbicides can advance intrusion by obtrusive grass species and contrarily sway local broadleave plants, diminishing local species wealth and variety.

Herbicide harm on non-target plants may make slight genuine injury indications and can sporadically cause financial harm also. Herbicide science and actual properties for the most part decide how herbicides cooperate with the organic and actual frameworks of the plant. Elements deciding herbicide viability and yield well-being are intricate and incorporate plant species, plant size, phase of development, soil substance and actual properties, soil dampness, temperature, and relative moistness. Post-rise herbicide take-up and adequacy can be influenced by shower added substances that improve the presentation of the herbicide, however, may likewise expand the danger of yield injury.

Herbicide indications differ contingent upon the herbicide, the pace of utilization, phase of development, sort of presentation, and the plant species receptor included. All in all, herbicides with a similar method of activity produce comparative injury indications, because the outward appearance of an injury is a component of herbicide impact on the plant at the cell level. Consequently, it is a lot simpler to analyze manifestations having a place with various herbicide methods of activity than herbicides inside similar methods of activity. In addition, diagnosing herbicide symptoms can be difficult because herbicide symptoms may look very similar to symptoms caused by diseases, nutrient deficiencies, environmental stress, and soil compaction.

While at times it is preposterous, by visual perception alone, to figure out what specific herbicide from a similar method of activity may have caused plant harm, it is conceivable to do as such with some different methods of activity. For instance, five kinds of herbicide that restrain acetolactate synthase. Herbicide sciences, and the individual herbicides inside them, may have diverse physicochemical properties, natural exercises, weed control ranges, soil exercises, and half-carries on with, however, all, for the most part, produce comparable injury indications on non-focused on plants. Then again, 11 sorts of herbicide hinder photosynthesis; notwithstanding, a portion of these herbicides may cause explicit indications that can be recognized. Moreover, herbicides from a similar method of activity or science may cause various side effects and injury on similar species. For instance, pyridine carboxylic corrosive herbicide picloram causes various side effects on cotton contrasted with other pyridine carboxylic acids, for example, clopyralid and triclopyr.

When all is done, yearly plants that quickly move herbicide are more vulnerable to herbicide harm and may show more injury indications. Alternately, enduring plants will in general move herbicide slower than yearly plants and are likewise ready to weaken herbicide in bigger biomass frameworks, bringing about less injury. Moreover, enduring plants may have a greater capacity to breakdown herbicide and recuperate from injury side effects. It is not phenomenal for plants influenced by herbicide to recuperate from side effects, even with the event of significant dieback. This is especially obvious with trees and other woody plants that can store starches, and secured meristems in lethargic buds. Trees have an exceptional capacity to endure and recuperate from herbicide injury.

Herbicides can harm foliage, shoots, blossoms, and organic products. On the off chance that injury is sufficiently serious, either from one episode or rehashed

introduction, it might diminish yield, produce helpless natural product quality, mutilate fancy or nursery plants, and sometimes cause plant passing. Herbicide indications might be obvious for a couple of days to quite a long while relying upon the herbicide in question, plant species, stage and pace of development, ecological and soil conditions, and social practices. Moreover, herbicides may diminish non-target plant life, increment defenselessness to sickness, and abbreviate the existence pattern of a plant. Herbicide injury to non-target plants additionally may bring about unlawful build-ups on the uncovered yield. In decorative nursery plants, even slight herbicide manifestations may influence the attractiveness of harmed plants.

A few herbicide injury side effects, for example, general and interveinal chlorosis, mottled chlorosis, yellow spotting, purpling of the leaves, putrefaction, and stem dieback, may result from causes other than herbicide introduction. On the off chance that herbicide harm is suspected, the movement of indications and the investigation of herbicide symptomology completely are basic. Examination at a few colleges, including the university of California, shows that numerous side effects from biotic and abiotic stresses imitate some herbicide manifestations and can be hard to recognize for the undeveloped onlooker.

Precisely diagnosing plants that show herbicide injury manifestations is troublesome. As a rule, other biotic and abiotic causes might be included or it could be hazy what herbicides were applied. Prepared analysts, be that as it may, might have the option to affirm or limit the chance of herbicide injury by looking at plant manifestations, injury movement, and contemplating other data, for example, kind of herbicides utilized and history, herbicide rates and application timing, injury designs, plant species influenced, climate information, and soil conditions. In any case, positive affirmation of herbicide indications requires lab testing of the live plant tissue as well as the soil while the compound is as yet present at recognizable levels. In cases researching herbicide indications, it is simpler to precisely analyze these side effects from tainted tanks, soil remainder, misapplication, or sprayer covering than from herbicide float.

2.8 Herbicides Phytotoxicity and Manifestation

An herbicide is a phytotoxic synthetic specialist by and large known as a weed executioner. These are the substances used to control undesirable vegetations by killing, hindering, or smothering their development, for example, home-grown or horticultural weeds and obtrusive species. A more limited term, called weedicide is utilized for executing weeds. The overall transformation in horticulture set off by the principal fruitful disclosure of 2, 4-D particular herbicide by Templeman and Marmoy (1940), Zimdahl (2007a).

Because of selectivity, herbicides can be explicit to certain plants or particular and vague or non-specific. Particular herbicides control or smother some particular sort of plants without influencing the existing pattern of other plant species, which might be because of movement, differential ingestion, or physical (morphological) or

physiological changes between plant species. By and large, all the pre-development, just as post-rise herbicides that are applied to handle crops are specific. For instance, herbicides, for example, 2, 4-D, mecoprop, and dicamba control numerous broadleaf weeds, however, stay inadequate against grasses while non-particular herbicides are vague in acting against specific plant-animal groups that kill all the plants regardless of their inclination with which they come into contact. Such herbicides are utilized to clear non-edited zones, for example, modern destinations, squander ground, side of the road, railroads, and rail route dikes. For instance, paraquat, diquat, glufosinate, glyphosate, and so on.

In light of the hour of utilization, herbicides can be pre-plant joined, pre-development, and post-rise. The pre-plant joining herbicides are commonly non-particular, and applied to the soil before the planting of yields. These herbicides are precisely joined into the soil to forestall misfortune through photodecomposition and instability. Agrarian yields, for example, tomato, maize, soybean, and strawberry are commonly filled in soil treated with pre-plant herbicides. Fluchloralin, trifluralin, EPTC, and other soil fumigants like metam-sodium, dazomet are being used as pre-plant herbicides. Pre-emergence herbicides are applied after the planting of yields, however, before the rise of harvests. They do not keep the weeds from growing, however, they kill weeds as they develop through the herbicide applied zone by influencing the cell division in the creating seedling. Pendimethalin, atrazine, alachlor, butachlor, dithiopyr, and so forth, are pre-development herbicides. Weeds that have just arisen before the use of herbicides are not influenced by pre-herbicides as their prime developing stage got away from the treatment. Post-emergence herbicides are applied after the harvest just as weed seedlings have arisen through the soil surface. These herbicides can be foliar or soil ingested, specific or non-particular, and contact or foundational. Models are 2, 4-D, glyphosate, isoproturon, metasulfuron, and so forth.

The herbicides can be consolidated into the soil during furrowing or ploughing or can be legitimately applied to the foliage. Herbicides applied to the soil are normally taken up by the foundation of the arising seedlings and are utilized as pre-plant or pre-rise treatment. Herbicide adsorption to soil colloids or natural issues regularly decreases its sum accessible for weed retention. The situating of the herbicide in the right layer of soil is significant that can be accomplished precisely and by precipitation. Herbicides on the soil surface area exposed to a few cycles that diminish their accessibility. Instability and photolysis are two regular cycles that decrease the accessibility of herbicides. Many soil-applied herbicides are assimilated through plant shoots while they are still underground prompting their passing or injury. EPTC and trifluralin are soil-applied herbicides. Foliar herbicides are applied to a part of the plant over the ground and are consumed by uncovered tissues. These are by and large post-emergence herbicides and can either be moved (foundational) all through the plant or stay at a particular site (contact). Outside hindrances of plants like fingernail skin, waxes, cell divider, and so forth influence herbicide ingestion and activity. Glyphosate, 2, 4-D, and dicamba are foliar-applied herbicide (Anwar et al. 2013).

Table 2.2 Mode of actions and mechanism of major herbicides within plant

	Microtubules assembly inhibitors	Pendimethalin, Asulam, Barban
	Mitosis inhibitors	Propachlor, Bromoxynil
Cell division inhibitors	Cell division inhibitors	2,4-D
Photosynthesis(PS) inhibitors	PS-II inhibitors	Atrazine, simazine, Metribuzin
	PS-I inhibitors	Paraquat, Diquat
Respiration inhibitors	Uncoupling of oxidative phosphorylation	Dinoseb, DNOC, Bromoxynil
	Inhibitory uncoupling	Dinoseb, DNOC, Bromoxynil
Nucleic acid, amino acid, and protein biosynthesis inhibitors	Shikimate pathway inhibitors	Glyphosate
	Branched chain amino acid synthesis inhibitors	Bispyribac Na, Sulfosulfuron
	Glutamine synthesis inhibitors	Glufosinate ammonium
Lipid biosynthesis inhibitors	Acetyl co-A carboxylase inhibitors	Clodinafop-propargyl, Sethoxydim
Carotenoids biosynthesis inhibitors	Phytoene desaturase inhibitors	Fluridone, norflurazon
	4-hydroxyphenylpyruvate dioxygenase(4-HPPD) inhibitors	Isoxaflutole, Sulcotrione
	Protoporphyrinogen oxidase (PPO) inhibitors	Oxadizon

Herbicides are having different modes of action as well as mechanism of action. Below given the examples of some of the major herbicides with their mechanism of action within plant body in Table 2.2 (Das 2008; Zimdahl 2007b). Except some of the non-selective or total killer herbicides, the herbicides are generally used to kill some plants specifically termed as weeds within a group of plants. So it is very essential to follow the exact dosage and time of application for herbicides to ensure lesser crop toxicity. After using many precautions also, it is not always possible to keep the crop unaffected. Many times the crop plants suffer toxicity from mild to mediocre to severe or acute category. Hence, it is very essential to know the toxic effects of herbicides on crop plants.

Toxicity can be described as the ability of a chemical to cause damage to a plant or organism. Generally, it depends upon the higher dosage along with the wrong timing of herbicide application. The herbicide toxicity symptoms in crop plants may be seen as chlorosis followed by necrosis, epinasty, wilting, leaf burning that leads to the stunted growth of plants, and in severe cases the plants may die (Das 2008).

Plants that show phytotoxic responses incorporate the accompanying manifestations:

- Abnormal development: The elevated roots or suckering or maybe the whole plant will encounter inordinate development.
- Chlorosis: Spots or tip edge or leaf yellowing shows up.
- Leaf Distortion: The leaf crinkles, curls, or appears to be cupping.
- Stunting: The plant in its entirety is reduced in size, or specific parts such as the fruit, roots, or flowers may look smaller in comparison to the rest of the plant.
- Wilting: Happens because of spillage of cell sap.
- Leaf consuming: due to the use of non-selective herbicides causing photosynthesis disruption.
- Altered cell division, cell prolongation, and tissue separation.
- Excessive tillering because of heavy apical damages.
- Multiple shoots from a single node.
- Poor seedling elongation.
- Improper secondary root elongation.
- Ear head abnormality/malformation.
- Albinism because of demolition of chloroplast.

Although using herbicides leads to killing of unwanted plants and weeds enabling the survival of food grain crops or maintaining the purity of harvested seeds, sometimes the inappropriate use of herbicides has the adverse consequence of injuring the non-targeted plants. This inadvertent injury from herbicides could occur from herbicide drift, herbicide misapplication, herbicide-contaminated tank, and carryover from previous crops.

Herbicide drift is defined as physical movement of a herbicide through air either at the time of application or soon afterward, to any place other than that proposed. Three ways by which the herbicides can move to the non-targeted areas are physical spray particle drift, vapor drift, and herbicide-contaminated soil.

Herbicide misapplication occurs when a particular herbicide that is applied to soil or a crop was not planned to be applied on. Such mistakes can be prevented if special attention is given during tank mixes preparation or when spraying was done to ensure correct herbicide application. Symptoms of treated plants with inappropriate herbicides are usually uniform throughout the area and need to be destroyed due to illegal herbicide residues as well as significant injury on the plants.

Herbicide-contaminated tank occurs in case the sprayer is not properly washed after the previous herbicide application. This can be problematic in highly diversified cropping systems and cause severe damage to the crops. Herbicide residue remains on the side of the spray tank, spray lines, sumps, pump, filters, and nozzles that can be a potential source of contamination.

Sometimes, herbicide residues may persist in the soil and severely damage the susceptible crops for one or more years following application. Thus the injury from herbicide residue in the soil is may not be due to herbicide application in the previous year but can also from the herbicide applied to burn down weeds just before sowing. Symptoms of this can vary from minimal damage to complete death of the crops. The conditions favorable for herbicide carryover are drought and increased soil pH.

In wheat, crop injury occurs due to the late application of 2, 4-dichlorophenoxy acetic acid (2, 4-D). Susceptibility to 2, 4-D herbicide on wheat is mainly observed from emergence to the four-leaf stage and from jointing to the soft dough stage of growth. It results in yield loss due to less tillers, unfolded leaves, spike distortion or malformation, and shorter stems (Kumar and Singh 2010). In addition to this, the application of isoproturon in wheat crop results in oxidative stress and chlorophyll reduction even at very low concentration. Symptoms of chlorosis, necrosis, plant deformations, discoloration, withering of leaves and growth retardation get visible (Varshney et al. 2012).

Due to the residual nature of 2, 4-D herbicide (up to 3–4 weeks), and carryover injury is seen in rotational crops, such as soybean crop. The key symptoms of this injury are striped leaves due to chlorotic or necrotic veins on the leaves. Some other herbicides, such as atrazine and metribuzin also lead to carryover injury in soybean. High soil pH makes the conditions even worse for the crop. The eroded knolls in the field are clearly seen at the sites of injury (Hartzler 2017).

Herbicides also have unintended consequences for non-target plant species, species composition, and plant species richness and diversity. Herbicides such as picloram that is selective for broadleaves plants can control broadleaves invasive plants, such as spotted knapweed (*Centaurea maculosa*) and sulfur cinquefoil (*Potentilla recta*) and promote recolonization of native grasses. However, because of this selectivity for broadleaves species, these herbicides can promote invasion by invasive grass species and negatively impact native broadleaves plants, reducing native species richness and diversity (Tyser et al. 1998; Pokorny et al. 2004; Denny and Sheley 2006). Persistent herbicides can stay dynamic in the climate for significant periods, conceivably causing soil and water tainting and unfriendly consequences for non-target life forms. Sometimes, exacerbates that outcome from herbicide degradation may keep on being essentially harmful in the climate.

2.9 Herbicide Toxicity Evaluation at Molecular Level

2.9.1 Microtubule Assembly Inhibitors

The herbicides inhibiting the microtubule assembly are generally dinitroaniline groups, for example, pendimethalin, fluchloralin, trifluralin, etc. During cell division, spindle formation is one of the vital stages of the mitosis during metaphase. The spindles are made out of hollow cylindrical structure of filamentous protein, namely microtubules made up of tubulin, a dimeric protein. According to the dynamic instability theory of microtubule growth, there is two ends in the microtubules, one is '+' or 'A' end where there are tubulin heterodimers are being added up and the other end i.e. '−' or 'B' end where the tubulin subunits are being wasted or depolymerized. This process is well known as "treadmilling." The earlier mentioned herbicide molecules get attached with the tubulin heterodimers which subsequently added up to the "+" end ceasing the further addition of dimer unit. On the other end, the depolymerization process goes on making the spindle shorter and shorter which

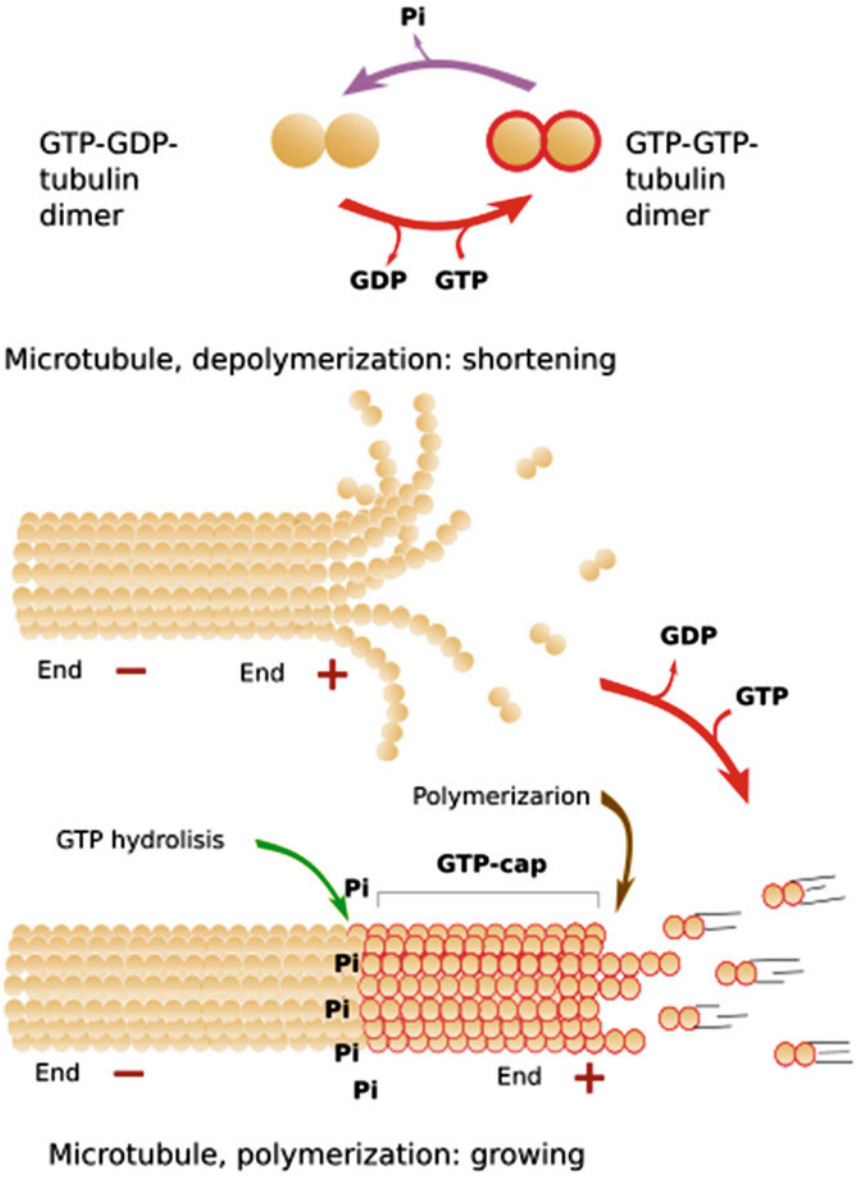

Fig. 2.1 GTP-GTP and GTP-GDP-tubulin dimers are depicted. In the cytosol, GTP-GDP-tubulin dimers are transformed in GTP-GTP-tubulin dimers, whereas GTP hydorlization occurs in the so-called hydrolysis zone of the microtubule wall. A microtubule shrinks when GTP-GDP-tubulin dimers are part of the plus end (there is no GTP cap), and it grows when GTP-GTP-tubulin dimers constitute the plus end (there is GTP cap). (Ohi and Zanic 2016)

eventually leads to the complete loss of microtubules (Das 2008, Fig. 2.1). This resulted in the absence of plane of cell division, ceasation of chromosome movement to their respective poles which in turn affect the proper distribution of genetic material in the susceptible plant.

2.9.2 Photosynthesis Inhibitors

2.9.2.1 PS-II Inhibitors

This is also known as electron transport inhibition and this effect is shown by maximum categories of herbicide like traizines, phenylureas, acetanilides, phenylcarbamates. In the Z-scheme of photosynthetic pathway (Fig. 2.2), when the electron excites from the reaction center of PS-II (P-680), it is accepted by quinone (Q) or plastoquinone A (PQ-A) subsequently pass on to plastoquinone B (PQ-B). Here, there also two proteins, namely D1 and D2, where the PQ-B and PQ-A get attached, respectively. In the niches of D1 protein, PQ-B is attached via two hydrogen bonds, one with serine-264 and the other with histidine-215. When PQ-B accepts two electrons from PQ/PQ-A, it gets reduced and the hydrogen bond gets broken down and two unreduced fresh PQ-B take that place (Radosevich et al. 1997). But, when the herbicides interfere in this aspect by acting as the non-reducible analogue of PQ-B and get attached to D1 protein via two hydrogen bonds via serine-264 and phenylalanine 265 with a greater affinity than PQ-B. As the herbicide molecule is non-reducible, it does not accept electrons from PQ-A any further by

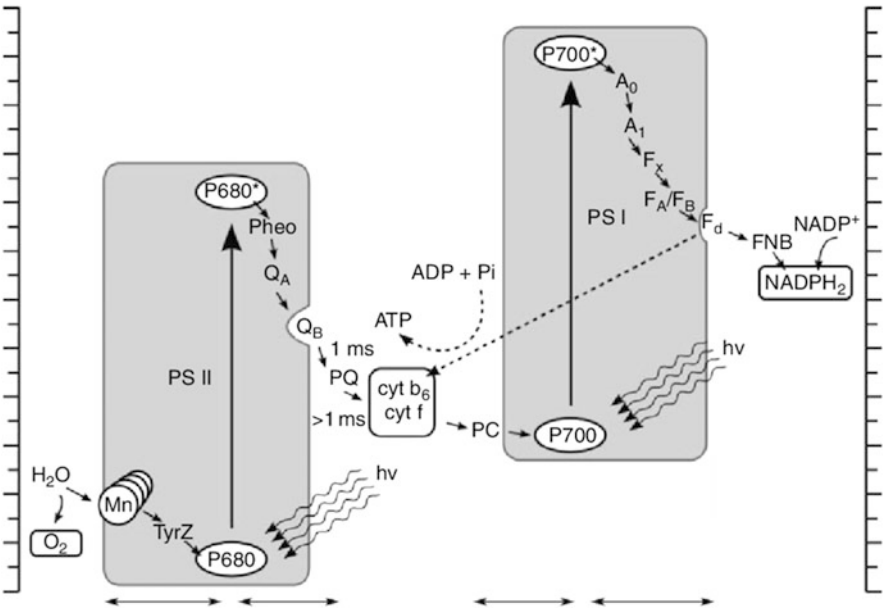

Fig. 2.2 Z-scheme photosynthetic pathway (Masojídek et al. 2013)

gradually stopping the electron transport and in turn the photosynthesis in the susceptible plants.

2.9.2.2 PS-I Inhibitors

This is done by bipyridilium group of herbicides like paraquat, diquat, cypermequat, and also known as electron diverters. This is so because it diverts the electron that was excited by the reaction center of PS-1 (P-700), and is supposed to be accepted by Fe-S complex and ferredoxin. The herbicide molecule takes up the electrons and forms their respective free radicals. The free radicals undergo auto oxidation subsequently to yield superoxide ion and hydrogen peroxide, that react with each other to form highly toxic free hydroxyl radical (OH). This radical destroys the integrity of the cell membrane and deactivates the cell metabolism rapidly leading to rapid bleaching action and death of susceptible plants (Dodge 1990). However, some of the plants like *Conyza bonariensis* and *Lolium perenne* show resistant to this bipyridilium group of herbicides by detoxification of free radicals, with the help of scavenging enzymes like superoxide dismutase, ascorbate peroxidases, catalases, peroxidases, glutathione reductase. This detoxification pathway is known as "Halliwell-Asada system" (Shaaltiel et al. 1988).

2.9.3 Shikimate Pathways Inhibition

In shikimate pathway, the enzyme 5-enolpyruvyl shikimate-3-phosphate synthase (EPSP synthase) catalyzes the biosynthesis of three aromatic amino acid, namely tryptophan, tyrosine, and phenylalanine (Fig. 2.3). Glyphosate, a systematic total killer generally utilized all through the world, inhibit the action of EPSP synthase and subsequently inhibit the production of the above mentioned amino acids. These amino acids are liable for the production of auxin, anthocyanin, flavonoids which cannot produce in the susceptible plant system.

2.9.4 Glutamine Synthesis Inhibition

Ammonia is produced by different metabolic pathways like nitrate reduction, amino acid metabolism in the plant cell and also gets utilized for the production of different amino acids like glutamine. The process undergoes the action of enzyme glutamine synthetase on glutamic acid and ammonia (Fig. 2.4). Herbicides like glufosinate ammonium inhibit the enzymes leading to the accumulation of ammonia in the plant that is very toxic in its effect and cause lots of cell damage, bleaching action, and finally complete death of the plant in the susceptible plant species.

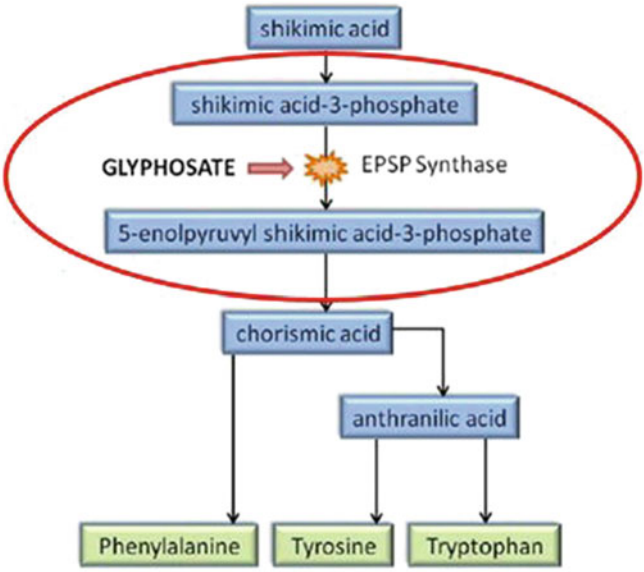

Fig. 2.3 Shikimate pathway (Balbuena et al. 2015)

Fig. 2.4 Glutamine synthesis (Das 2008)

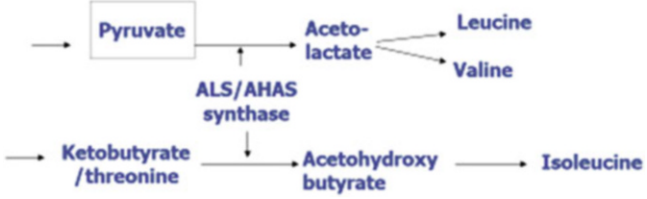

Fig. 2.5 AHAS and ALS inhibition (Das 2008)

2.9.5 Acetohydroxy Acid Synthase (AHAS) and Acetolactate Synthase (ALS) Inhibition

The herbicide group like sulfonylureas, imidazolinones, pyrimidinylthiobenzoates inhibit the AHAS and ALS enzyme, which catalyze the synthesis of three branched chain amino acids, namely leucine, isoleucine, and valine (Fig. 2.5). The enzymes are located in the plastid. The enzymes get similarly attached to the enzymes n to that of PS-II inhibitor (Dekker and Duke 1995).

2.9.6 Phyteone Desaturase (PDS) Inhibition

Carotenoids pigments like alpha-carotene, beta-carotene, xanthophyll, violaxanthin do not have a direct role in the photosynthesis process but have a significant effect in the safeguarding of chlorophyll molecule from the detrimental photo-oxidation process and check the formation of toxic triplet chlorophyll and singlet oxygen. The enzyme phyteone desaturase has a vital role in the production of carotenoids pigments like lutein and zeaxanthin from phyteone via the cyclization process. The herbicides, namely norflurazon, fluridone, flumeturon inhibit the PDS enzymes which in turn inhibit the biosynthesis of carotenoids pigments leading to hampering to chlorophyll pigment and photosynthesis process in the susceptible plant groups.

2.10 Engineering of Herbicide Resistance in Plants

Many herbicides exert their effects by inactivating "target proteins" (usually enzymes) essential for important functions like the chemical action or different synthesis pathways distinctive to plants. As a result of crop plants typically share these processes with competitive weeds, several herbicides are non-selective. Others are often used by selection on tolerant crops, in the main as a consequence of a differential uptake or metabolism of the weed killer or by a particular localization of the weed killer application. Another is to confer resistance to crops against broad-spectrum herbicides. This might allow the employment of such herbicides and choice from this variety of compounds of these that are environmentally acceptable and non-toxic.

Two approaches are discovered for the engineering of chemical resistance in plants: (1) the modification of the accelerator or different target for herbicidal action within the plants to render it insensitive to the chemical or by causing the production of the unqualified target super molecule, therefore allowing traditional metabolism to occur despite the presence of the chemical; (2) the introduction of associate in nursing accelerator or accelerator system to degrade and/or detoxify the herbicide within the plant before it will act. Plants changed in these ways in which could also be obtained either selectively for resistance against the chemical or by applying sequence transfer techniques. Genetic engineering of plants may be brought about by the use of gene transfer vectors derived from the soil bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* or by direct gene transfer of DNA into plant cells or protoplasts (Botterman and Leemans 1988).

Herbicides are synthetic substances that act with specific molecular targets during a plant, leading to disruption of traditional metabolic processes. As an outcome, the plant cannot endure and in the long run perishes (Dhingra and Daniell 2004). Herbicides are currently a significant piece of present-day agribusiness and are utilized basically for three significant reasons (Schulz et al. 1990).

- Elimination of weeds lessens the opposition for light, water, and supplements.
- They lessen the peril of cross-disease from bugs and microbes from weeds to crops.
- Use of Herbicide benefits crops, especially during harvesting time by increasing the yield.

Herbicide resistance is the inherited ability of a plant to survive and reproduce the following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis (WSSA 1998). Herbicide resistance mechanisms are broadly divided into target site and non-target site mechanisms. However, within each category, there are distinct resistance mechanisms.

2.10.1 Target Site Resistance Mechanism

Target site pathways include a modification in the protein that binds to the herbicide, leading to a loss of biochemical pathway inhibition. The most noticeable where the binding of the herbicide is decreased or removed by a mutation within the target protein. This is the classic mutation of the target site that is usually seen to provide the herbicide with virtual immunity (Preston 1994). That is not always the case, tough and weak target site mutations, as well as strong target site mutations, are probable. Due to single point mutations in the underlying DNA that affect an amino acid in the protein, target site mutations occur. This modified amino acid can either delete the bond needed to bind the herbicide or modify the binding pocket shape.

Target site mutations are common in weeds with resistance to Group A, Group B, and triazine Group C herbicides, but also occur with resistance to Group D and Group M herbicides. It is normal for cross-resistance to other herbicides with the same herbicide mode of action to be obtained with target site resistance. There is more than one potential mutation that will have resistance to herbicides at most target sites. Different mutations provide different degrees of resistance and cross-resistance patterns in certain situations. Resistance selected by herbicides of sulfonylurea does not result in resistance to herbicides of imidazolinone. Our evidence shows that cross-resistance to imidazolinone herbicides exists with broadleaf weeds approximately 30% of the time and with grass weeds 50% of the time. This is variable among species. Within the ALS protein, there are eight distinct amino acids where mutations are believed to result in herbicide resistance. Of these, four give strong resistance to herbicides containing sulfonylurea and six give strong resistance to herbicides containing imidazolinone. Thus, exposure to both classes of herbicides is given by just some of the mutations. The reasons for this are that the various herbicide classes bind differently in the binding pouch so that only one or both herbicide forms are affected by different mutations (Fig. 2.6).

For Group A herbicides, a different condition existed. There are seven amino acids inside the protein for ACCase where mutants are considered to have herbicide

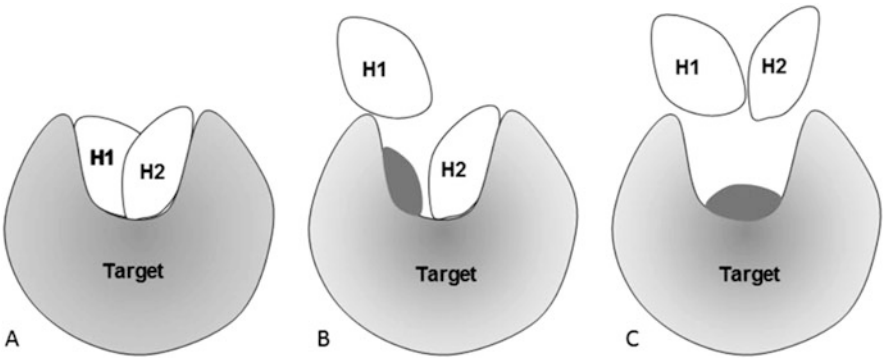

Fig. 2.6 Target A has no mutation to the target site so both herbicide 1 and herbicide 2 can bind to the target. Target B has a mutation to one side of the target site which prevents herbicide 1 from binding to the target site but does not affect herbicide 2 from binding. Target C has a mutation to the target site which prevents both herbicide 1 and herbicide 2 from binding to the target site (Preston 2014)

resistance. Most of these are resistant to fop herbicides, but only three are resistant to clethodim, with only one alone having a high degree of resistance. The bulk of target site mutants selected by fop herbicides can therefore be controlled by clethodim. This has helped farmers in southern Australia to first exploit fop herbicides to control ryegrass and use clethodim to control ryegrass after those herbicides had failed. Higher rates were used until clethodim began to fail, as there was only one mutation that provided high resistance to clethodim.

The other type of resistance to target sites is where the target site has far more copies than would usually be present. The extra target sites behave like a sponge that soaks up the herbicide in this form of resistance. So far this mechanism has only been seen with weeds resistant to glyphosate. If this type of mechanism was to occur for another herbicide target site it would be expected to provide resistance to every herbicide in that mode of action.

2.10.2 Non-Target Site Resistance Mechanism

Mechanisms of non-target site resistance allow plants to withstand herbicide application by not allowing sufficient herbicide to enter the target site. The weed can initially be harmed by the application of the herbicide but will survive and seed set. The most common example of non-target site resistance is due to increased herbicide detoxification. There is more gradual degradation of the herbicide within the plant for this resistance mechanism, and less active herbicide enters the target site to destroy the plant. The species would start with some capacity to metabolize the herbicide with this mechanism, but in the resistant individuals that becomes greatly enhanced. For this cause, increased metabolism is usually found in herbicides, such as groups A, B, C, D, and I, which can be used selectively in the crop.

The precise form of the mutation that results in resistance due to accelerated degradation of herbicides has not been established. However, evidence points to the elevated activity rather than of a single enzyme of many enzymes. One consequence of this type of process is that it also contributes to the cross-resistance of multiple modes of action against herbicides. This makes resistance prevention of herbicides seriously difficult. Cross-resistance trends appear to be extremely complex and volatile, meaning that many forms of improved detoxification of herbicides are occurring. Reduced herbicide activation is a variant of herbicide detoxification. As pro-herbicides, some herbicides are added and depend on the plant to metabolize them to the active compound. The herbicide will not work if the plant fails to do this. Changes to the translocation of herbicides within the plant require a second non-target site mechanism. The herbicide becomes trapped in the leaf tips in this system and reduced concentrations are found in the meristem and other portions of the plant. To bring down the plant, where the herbicide needs to be present in the developing tissue, decreasing translocation would decrease the herbicide concentration in these core tissues at the target site. This sort of mechanism is predominant in weeds resistant to herbicides of Group L and Group M, but has also been seen in weeds resistant to herbicides of Group A.

There are many pathways whereby plants can minimize herbicide translocation. The key method tends to be to inject the herbicide into the vacuole of the cell. As this requires particular herbicide transporters, resistance usually only applies to a single herbicide. Resistance to paraquat is the case where cross-resistance to diquat often exists. An alternate method of reduced translocation, when tissues are shed from the plant is stuck in the herbicide. This resistance to "rapid necrosis" resembles the plant reaction to pathogen invasion, but on a large scale where the whole leaves die and fall off easily taking the herbicide with them. This form of resistance to glyphosate has been found elsewhere. Theoretically, two other kinds of non-target site resistance are probable but have not been well established. Reducing the uptake into the plant of the herbicide would decrease the herbicide concentration at the target site. It is only possible that such a mechanism would be successful with herbicides that are ingested only or predominantly by leaf tissue. The other form of process is where the plant usually by improved ability to cope with oxygen radicals can prevent the adverse impact of the herbicide action. For example, this was suggested as a paraquat resistance mechanism but is only a realistic mechanism if the plant itself has the potential to extract the herbicide from the target site easily.

More recently, to counter the deadly effects of herbicides, plants have been genetically modified. There are several pathways responsible for plant natural herbicide resistance, notable is insensitivity to the target site and non-toxic by-products degradation of the toxic herbicide. In genetically modified crops, all these pathways have been simulated either by over-expression of the target enzymes or by designing foreign proteins that can detoxify the herbicides easily (Freyssinet 2003). Besides, Freyssinet 2003, noted that for functional and economic purposes, resistance to herbicides was among the first characteristics to be genetically engineered into crops. Around 75% of transgenic crops are engineered for herbicide resistance globally (Castle et al. 2004).

Table 2.3 List of chloroplast specific enzymes, biosynthetic pathways, herbicides, and resistance mechanisms

Enzyme/gene	Pathway	Herbicide/active ingredient	Resistance mechanism or gene
5-enol-Pyruvylshikimate-3-phosphate synthase (EPSPS)	Shikimic acid	Glyphosate	Mutant EPSPS, CP4 EPSPS, Petunia EPSPS, aroA-M1, goxA, gat
Acetolactate synthase (ALS)	Branched chain amino acid	Sulfonylureas, Imidazolinones, Triazolopyrimidine sulfonamides	csr1–1 imr-1
Glutamine synthetase (GS)	Glutamine biosynthesis	Methionine sulfoximine, Phosphinothricin (glufosinate), and Tabtoxinine-β-lactam	Bar, pat
Acetyl co-A carboxylase (ACCase)	Lipid biosynthesis	Cyclohexanediones, Aryloxyphenoxy propionates	
D-1 polypeptide	Photosynthesis (PS-II)	Substituted ureas, s-triazines, and phenols Bromoxyni	Mutant psbA, oxy
Photosystem I	Photosynthesis	Paraquat and Diquat	Glutathione reductase, copper/zinc chloroplast superoxide dismutase
Protoporphyrinogen oxidase (Protox)	Tetrapyrrole biosynthesis	Diphenyl ethers	Protox (*A. thaliana*), Protox (*B. subtilis*)
Phytoene desaturase (PDS)	Carotenoid biosynthesis	Pyridazinones metflurazon, norflurazon	crt1, pds
4-Hydroxyphenylpyruvate dioxygenase (HPPD)	Prenylquinone pathway	Isoxaflutole, Sulcotrione, NTBC	HPPD
Dihydroxypteroate synthase (DHPS)	Folate biosynthesis	Asulam	Sul1

Any of the herbicides that exist inside the chloroplast target critical pathways. Goal site amplification or expression of insensitive/mutant target enzymes/proteins is one of the pathways for conferring herbicide resistance. In Table 2.3, an illustration of the proteins targeted by herbicides, the mechanisms in which those enzymes participate, the herbicides targeted by those proteins, and the tolerance or resistance mechanism are encapsulated.

2.11 Herbicide Insensitive Enzymes Approach

2.11.1 Photosynthesis

Photosynthesis is a crucial step of metabolism conducted by plants. In the chloroplast, all the light reactions of this process are carried out and photosystem II and photosystem I are two main participants in the process. Since the 1950s, herbicides causing photosynthesis have been detected. Some herbicides work on photosystem II's electron flow and others act by diverting the electron flow to photosystem I (Fig. 2.7).

2.11.1.1 D-1 (Q_B) Protein

The D-1 protein is a 32-kDa polypeptide of the Photosystem II encoded by the psbA gene in the chloroplast genome and has a high rate of turnover that is light dependent. The D-1 protein functions as a QB apoprotein, a specialized type of plastoquinone that mediates electron flow inside the thylakoid membrane to the plastoquinone reservoir (Dodge 1991).

In particular, substituted ureas, s-triazines, and phenols attack the D-1 polypeptide and block the transport of plastoquinone, a mobile electron carrier (Devine et al. 1993). Electron flow disruption between the two photosystems results in the blocking of the photosynthetic production of the reducing by-products used for carbon fixation. The apparent reduction in beta-carotene that exposes chloroplasts to oxidative damage is a side effect of this.

In some crop plants, resistance mechanisms against PSII inhibitors are already active, regulated by mitochondrial detoxification or reduced absorption and translocation. A mutation in the psbA gene mediates the most common resistance mechanism (Golden and Haselkorn 1985). A Ser264 to Gly mutation confers resistance to herbicides in most cases (Shukla and Devine 2000). This is a mutation that affects

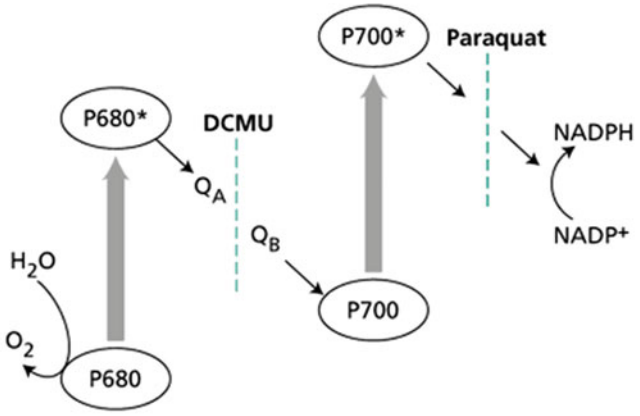

Fig. 2.7 Mechanism of action of photosynthetic inhibitor herbicides (Taiz et al. 2015)

the protein's stromal side and reduces the binding of herbicides to s-triazine. This mutation also results in reduced photosynthesis and development of plants resulting weak plant vigor (McCloskey and Holt 1990). Alternatively, genes for metabolic herbicide detoxification may be used to engineer the mechanism of resistance.

2.11.1.2 Photosystem I (PS-I)

Photosystem I acts as a light-driven plastocyanin-ferredoxin oxidoreductase composed of many intermediate redox components that tend to be aligned with a seven-polypeptide PS-I core complex.

The natural electron flow is redirected between iron sulfur centers A, B, and NADP+ by the bipyridinium herbicides paraquat and diquat (Zweig et al. 1965). The bipyridyl radical contributes to toxic forming with the molecular oxygen present in the grana and is essentially responsible for cell destruction. Natural resistance to PS-I specific herbicides is documented, but it remains elusive to elucidate the exact molecular mechanism. Resistance is indicated to be either due to the activation of the enzyme involved in the detoxification of oxygen radicals or the sequestration away from the chloroplast of the harmful chemical compound (Preston 1994). Resistance to PSI-specific herbicides was engineered by expressing glutathione reductase in model system tobacco from E. Coli and a pea dismutase copper/zinc chloroplast superoxide (Aono et al. 1993; Gupta et al. 1993).

2.11.2 Enolpyruvylshikimate Phosphate Synthase (EPSPS)

Enolpyruvylshikimate phosphate synthase (EPSPS) is a shikimic acid pathway enzyme that links the reduction of photosynthetic carbon to the synthesis of tyrosine, phenylalanine & tryptophan aromatic amino acids and many other secondary products in plants. In addition to protein synthesis, aromatic amino acids are important for hormone synthesis, the production of energy-transduction compounds such as plastoquinone, the development of cell walls and protection against pathogens and insects (Duke 1988).

EPSPS is directly activated by glyphosate (Fig. 2.8), which binds the enzyme in a complex way, leading to a confirmation transition that in turn inhibits phosphoenolpyruvate (PEP), one of the two EPSPS substrates, from binding (Sikorski and Gruys 1997). Inhibition of EPSPS by glyphosate results in aromatic amino acid degradation and shikimic acid hyper accumulation (Hoagland et al. 1978; Amrhein et al. 1980).

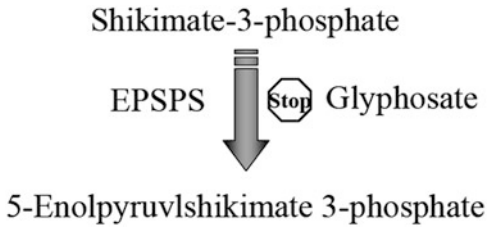

Fig. 2.8 Shikimic pathway (Dhingra and Daniell 2004)

In major crops, glyphosate resistance has been successfully engineered by the engineering of modified EPSPS enzymes. Original efforts centered on the discovery of glyphosate-resistant mutant forms of EPSPS in bacteria from mutagenesis screens. The aroA mutant gene isolated from *Salmonella typhimurium* coding for insensitive EPSPS (Comai et al. 1983) was engineered into the nuclear genome of tobacco and tomatoes (Comai et al. 1985; Fillatti et al. 1987).

The transgenic plants did not have an agronomically beneficial herbicide tolerance level, primarily because the enzyme was not aimed at the chloroplast and its PEP binding ability was impaired (Mousedale and Coggins 1985; Padgette et al. 1996). Through developing a mutant EPSPS derived from petunia, the first active glyphosate-resistant transgenic plant was obtained. Transgenic petunia cells and plants that express the insensitive EPSPS are glyphosate resistant (Shah et al. 1986). A naturally occurring EPSPS from *Agrobacterium* sp. later on Strain CP4 was shown to have all the favorable properties for ideal gene resistance to glyphosate (Barry et al. 1992). This gene has been used successfully in the engineering of soybean and cotton resistance to glyphosate (Padgette et al. 1995; Nida et al. 1996). This gene was not successful for maize where a double mutant of maize EPSPSS conferred commercial level immunity to glyphosate (Lebrun et al. 1997). Mutated variants of the aroA gene have been produced with the aid of novel techniques that confer enhanced glyphosate resistance. Using the aroA genes from *Salmonella typhimurium* and *Escherichia coli*, the phased extension process culminated in the development of four randomly mutated and recombined versions of the two genes. Three of these carried de novo mutations hitherto unknown in mediating glyphosate tolerance. Increased resistance was attributed to a 2–ten-fold rise in particular activity, a 0.4–eight-fold decrease in glyphosate affinity, and a 2.5–19-fold decrease in phosphoenolpyruvate Km (He et al. 2001). One of these aroA-M1 mutants was recently engineered into tobacco and it was shown that up to 0.8 mM of glyphosate could live in transgenic plants (Wang et al. 2003).

Two lines of glyphosate-resistant rapeseed are commercially cultivated in the USA. The EPSPS and gox gene are expressed in both lines; the former gene in both cases is derived from *Agrobacterium*, but the gox gene is derived from *Agrobacterium* and *Achromobacter*. Similarly, three lines of glyphosate-resistant maize are grown that in combination with gox, express EPSPS alone or EPSPS. The gene for EPSPS is derived either from *Agrobacterium* or from maize itself. Every line of cotton and soybean resistant to herbicides is also commercially produced, expressing EPSPS derived from *Agrobacterium*.

2.11.3 Acetyl co-a Carboxylase (ACCase)

The very first step of de novo fatty acid biosynthesis in plants is catalysed by acetyl Co-A carboxylase. The reaction takes place within the chloroplast and results in acetyl Co-A and bicarbonate to form malonyl-Co-A.

Two classes of herbicides, cyclohexanediones (CHD) and aryloxyphenoxy propionates (AOPP), are blocked by ACCase (Fig. 2.9). It is understood that two

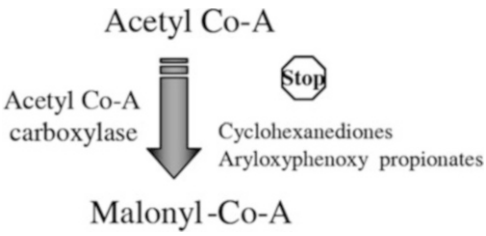

Fig. 2.9 Lipid biosynthesis pathway step catalyzed by Acetyl CO-A carboxylase (Dhingra and Daniell 2004)

types of ACCase occur in dicots. One is known as a heteromeric prokaryotic form and the other is known as a homomeric eukaryotic form. Although the eukaryotic homomeric form is susceptible, the prokaryotic heteromeric form is immune to herbicides. The prokaryotic type is absent from grasses, rendering them susceptible to herbicides since members of the graminae family lack the plastid accD gene which codes for one subunits of ACCase. In a range of large leaf and cereal crops, this ensures selectivity for grass weed control (Konishi and Sasaki 1994).

Mutations in the 400 amino acid fragment inside the carboxyltransferase β-subdomain impart susceptibility to CHD and AOPP herbicides (Nikolskaya et al. 1999). Several such mutations will give these herbicides unusual cross-resistance patterns. In the USA, out of six field trials, four for maize and two for rapeseed, where the crops express the ACCase gene (ISB 2004).

2.11.4 Phytoene Desaturase (PDS)

Phytoene desaturase is an enzyme involved in carotenoid biosynthesis that protects plants against photo-oxidative harm. Inhibition of PDS contributes to the deposition of phytoene that has short chromophores, which cannot guard against photooxidation leading to plant death by light and oxygen.

Herbicide pyridazinones, metflurazon and norflurazon inhibit carotenoid biosynthesis by specifically blocking desaturation that results in the accumulation of phytoene. In certain crops such as cotton, red beet, and soybean there is a fair degree of natural resistance to PDS inhibitors and decreased absorption and translocation of the resistance are assured. Bacterial PDS is insensitive to typical herbicides blanking. One such PDS encoded with the crtI gene was deleted and translated into a tobacco nuclear genome by *Erwinia uredovora*. The protein was targeted against chloroplast, in which carotenoid biosynthesis happens and may provide the herbicides with a strong tolerance. The new cyanobactreal desaturase gene mutated with the synechococcus PCC 7942, mutant NFZ4, has recently transformed tobacco. Transgenic plants have 58 times greater tolerance to norflurazon than wild plants, and transgenic plants preserved a higher degree of photosystem 2 protein D1 that indicates less likely to be photooxidized while norflurazon is involved. (Wagner et al. 2002).

2.12 Metabolic Detoxification Approach

Another mechanism for engineering herbicide resistance is to express genes that code for enzymes which detoxify the toxic herbicides.

2.12.1 Bromoxynil Tolerance

Bromoxynil herbicide is used as an inhibitor and uncoupler in photosynthetic electron transfer. Dicots do not withstand well but effectively kills large leaf weeds in wheat fields. Gene-encoding oxygen of nitrilase used as a medium in bromoxynil was isolated from the ozenae (Stalker et al. 1988). Bromoxynil is detoxified into non-toxic benzoic acid by this enzyme. Tobacco, cotton, potato, and oilseed rape successfully conveyed the oxygen where a high resistance was seen (Freyssinet et al. 1989). Even before the nitrilase is achieved in the chloroplast, bromoxynil is detoxified in the cytosol.

2.12.2 Glyphosate Tolerance

A glyphosate resistance can also be conferred through the expression of suppressed variants of the EPSPS enzyme, as well as enzymes deliberately disrupting the herbicide. Glyphosate detoxification gox, detected from a glyphosate waste source, encodes for glyphosate oxidoreductase and supports the LBAA bacterium of *Achromobacter* spp. (Barry et al. 1992). Gox is not used alone, but in tandem with C4 EPSPS and the potency of the conferred immunity improves as all plastids are attacked (Mannerlof et al. 1997). Transgenic canola and maize are also widely cultivated (Saroha et al. 1998).

Another process for detoxification is N-acetylation of glyphosate, which transforms it into N-acetylglyphosate in its non-toxic type. *Bacillus* sps have recently discovered enzymes that exhibit glyphosate N-acetyltransferase activity. One such gene gat from *Bacillus licheniformis* was subjected to repeated DNA shuffling to obtain a gene that provides increased glyphosate tolerance when engineered into *E. coli, Arabidopsis*, tobacco and maize (Castle et al. 2004). While a 1000 fold increase in Kcat/Km in DNA shuffling relative to the parental enzyme, the gene conferred only mild glyphosate resistance in transgenic plants. This may be due to the cytosolic location of the enzyme instead of plastids.

2.12.3 Glufosinate Tolerance

Glufosinate blocks the synthesis of glutamine and the metabolism of nitrogen. The expression of the pat and bar genes isolated from two *Streptomyses* spp. has been developed to promote glufosinate tolerance in more than 36 plant species (Strauch et al. 1988). The first plant designed for tolerance to glufosinate was tobacco where

expression of the bar gene gave successful glufosinate tolerance without deleterious effects on flowering or setting of seeds. Several dicot and monocot crops have since developed glufosinate tolerance.

In more than eight countries worldwide, glufosinate-resistant plants are cultivated commercially. The cultures contain nine rows of *Zea mays* and eight rows of *Brassica napus*, four rows of *Glycine max* and one row of sugar beet, *Brassica rapa* and chicory.

2.13 Conclusion

The vigor, fertility, and yield of the exposed plant can be negatively impacted by chromosomal abnormalities. In such activity, herbicides may also modify the genetic constitution of the seed, resulting in a very dangerous mutational alteration. Hence for crop plants in the environment, the highest concentration of both herbicides can become genotoxic, chromotoxic, and clastogenic. Therefore the higher dosage, in particular the herbicide glyphosate, is not suggestive of all persons because its toxicity is so large.

Several results show that the cytotoxic effect of glyphosate based on cytological analysis was higher than that of atrazine. Therefore, where a greater concentration of herbicides is found in the atmosphere and ingested by plants, the genetic system can be adversely affected, causing chromosome damage in crops. A possible hazard to the genetic structure of crops and livestock is the regular application of herbicides in farming practices. Judicial uses of these herbicides are thus, important. As far as possible, indiscriminate herbicide use should be prevented. Instead, bio-herbicides and bio-control agents that do not pose harmful threats to both crops and the environment should be substituted.

So, if a higher concentration of herbicides is present in the environment and absorbed by the plants, it may adversely affect the genetic system causing damage to the chromosome in crop plants. Regular uses of herbicides in agricultural practices are a potential threat to the genetic constitution of crop plants and animals. Therefore, the judicial uses of these herbicides are essential. An indiscriminate use of herbicide should be discouraged as far as practicable. Rather, it should be replaced with bio-herbicides and bio-control agents which do not pose adverse risks to crops as well as the ecosystem.

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Role of Physical Agents in Inducing Genotoxicity and Oxidative Stress in Plants

3

Girjesh Kumar, Shefali Singh, Rajani Singh, and Radha Mishra

Abstract

Physical agents such as soil salinity, drought, low and high temperatures, solar UV radiations, gamma radiation and others either directly or indirectly via the induction of oxidative stress and overproduction of reactive oxygen species (ROS) frequently perturb the chemical or physical structures of DNA and induce both cytotoxic and genotoxic stress. This genomic instability eventually affects biochemical properties and the morphological characteristics of the plant. The impact of these physical agents damages enzymatic and non-enzymatic components of the plant cell, recurrently resulting in loss of cell viability therefore resulting retarded plant growth and development. There are different sites for the production of ROS such as chloroplast, mitochondria, peroxisome, apoplast and cell wall. The ROS comprises both free radicals ($O2^-$ superoxide radicals; OH^- hydroxyl radical; HO_2^- perhydroxy radical and RO^- alkoxy radicals) and non-radicals (molecular) forms ($H_2O_2^-$ hydrogen peroxide and $^1O_2^-$ singlet oxygen). In chloroplasts, photosystem I and II (PSI and PSII) are the major sites for the production of $^1O_2$ and O_2^-. Plants have developed a highly proficient mechanism for stress tolerance via the rapid change in the expression of the responsive genes at the transcriptional level. The antioxidant defense machinery protects plants against oxidative stress damages. Plants possess very efficient non-enzymatic (ascorbic acid (ASH), glutathione (GSH), tocopherols, carotenoid, proline) and enzymatic superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione peroxidase (GPX) and glutathione-S- transferase (GST) and antioxidant defense systems that work in recital

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to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS.

Keywords

Antioxidant defense · Environmental stress · Genotoxicity · Oxidative damage · Physical agents

Abbreviation

APX	Ascorbate peroxidase
ASH	Ascorbic acid
CAM	Crassulacean acid metabolism
CAT	Catalase
DHAR	Dehydroascorbate reductase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione-S- transferase
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
MDHAR	Monodehydroascorbate reductase
NADP	Nicotinamide adenine dinucleotide phosphate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase

3.1 Introduction

Plants are sessile organism and happen to be the most astonishing living entities on which all other living beings are dependent, be it directly or indirectly. Unlike animal, plants are immovable forms and have to live often in exquisitely unfavourable environmental conditions where they are prone to myriad of physical agents. Terrestrial biome has plethora of challenging attributes and the plants are constantly exposed to these challenges in their surroundings that range from facing limitations of essential resources (water and nutrients) to being at the receiving end of biotic, abiotic and anthropogenic elements. It can be contemplated that the terrestrial environment is more dynamic than the aquatic environment where life originated initially. Thus, it has arisen several questions how plants are affected by these prevailing physical factors? How their metabolic responses are affected towards these physical agents as well as in what ways the cytological cues are switched on? Plants are extremely sensitive and receptive to these agents and these

physical variables implicit their effects on innumerable facets of plant growth. Even then plants have established themselves successfully to this scheme and have supported all other living beings by trapping solar energy and translating it into the usable energy sources. To answer the lingering quest and to have an articulated concept on plant responses to these physical agents, this chapter has been written.

Plants are highly responsive to the physical factors of their vicinity. Prominent external physical factors that impart potential effects on plants morphogenesis and reproductive biology are high intensity solar radiation such as ionizing rays, non-ionizing rays, infrared, cosmic and other radiations of different wavelengths, abiotic factors such as high salinity, drought, flooding, chilling injury, nutrient imbalances, biotic factors such as exposure to bacterial and fungal pathogens and metabolic by-products of endogenous processes represent some of the frequent stress factors for plants. According to Wang et al. (2006), physical stress has been described as a complicated stress factor whose properties can be separated into several physical aspects, which would induce certain specific or non-specific responses in plant growth. These physical agents exert a spectrum of physical stress responses that tends to disturb the highly sensitive oxidant-antioxidant ratio. Free radicals, reactive nitrogen species (RNS) and other reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide are the complete sets of most reactive species derived intrinsically from the normal metabolism of oxygen or exogenous physical factors and agents. Free radicals are molecular species containing an unpaired electron in an atomic orbital due to which these molecules become highly unstable and reactive. The action of free radicals leads to homeostatic disruption by attacking important macromolecules leading to cell damage. Stress response of plants also varies to the stress level that the plant can withstand. On this basis, Lichtenthaler (1998) elucidated the idea of eu-stress and dis-stress in plants. Plant growth may be positively activated and promoted by eu-stress, but negatively affected by dis-stress. This concept of opposites implied the existence of a certain threshold of stressor, under which the stress is mild and may activate cell metabolism, increase the physiological activity of a plant and not cause any irreversible acute damage. In contrast, a stress exceeding this threshold limit will cause both local damage and general senescence to the plant, finally leading to death if the stressor is imposed for a longer period. Any modification in the somatic cells is shown the alteration in gametes, as plants get deficient in conserved germline and meiotic cells are produced in delayed development (Walbot and Evans 2003). Plant subjected to environmental stress can reform not just in the genome but also in the epigenome modifications to the later may be abundant in organisms (Grossniklaus et al. 2013). For instance, *Mesembryanthemum crystallinum* which is the halophytic species under drought conditions can change metabolic processes from C_3-photosynthesis to crassulacean acid metabolism (CAM). These modifications in plants trigger several questions to plants 'biochemical plasticity'. These changes in the pathway are associated with profound alternation in the activity of enzymes that are referred by modulation in DNA methylation as witnessed in *Pinus sylvestris* that subjected to highly stress condition, have hypermethylated DNA after chernobyl disaster (Kovalchuk et al. 2003). The seeds

grown in contaminated soil of chernobyl for six generations have shown slightly increased level of DNA methylation in its rootlets (Georgieva et al. 2017).

Besides, plants support biological diversity and ecological balance while these also foster bountiful of agricultural and horticultural yield. These significant facts enforce for designing a thorough study on some vital physical agents and their course of action on plants metabolic responses with special reference to the complex mechanism of oxidative stress and genotoxicity. Henceforth, this piece of work has been conceptualized elucidating the plants inherent ability against the stress condition.

3.2 Types of Abiotic Physical Agents

3.2.1 Drought

Drought is an environmental condition and can be defined as duration having insignificant rain. Normally this stress condition takes place when the soil moisture is already low and increased transpiration rate further decreases the amount of moisture in the soil. In the present scenario, water deficiency is becoming a world-wide problem for abidance of farming and sustainable food production (Jaleel et al. 2008). Water deficiency caused by key limiting factors such as soil acidity and soil dryness is often faced by plants that ultimately cause significant loss in productivity. Forbearance to abiotic stress is a complex process, due to interactions of different stress factors as well as plant physiological phenomena. Plant response drought stresses in two ways, the first one is when moisture availability to root is insufficient and secondly when the rate of transpiration is more, i.e., beyond the threshold limit (Anjum et al. 2011; Jaleel et al. 2009). Besides, drought stress also regulates the water conduction in plants via limiting the gaseous exchange through the stomatal opening/closing process. Water scarcity or severe water stress and shrinkage of cell may result in the arrest of the cell cycle, photosynthesis, disturbance of metabolism and finally cell death of plant (Jaleel et al. 2008). The various effects of drought stress that impede plant growth are presented in Fig. 3.1.

3.2.2 Salinity

Salinity is a second major abiotic stress limiting the growth and fecundity of plants worldwide due to injudicious use of irrigation facility as well as chemical fertilizers. Plants based on adaptive evolution can be categorized basically into halophytes (Plant that can survive in salinity) and glaucophytes (Plants that cannot survive in salinity). The majority of crop species are glycophytes. Thus, it can be concluded that it is one of the critical environmental stresses which lowers the crop productivity globally. Plants experience salinity stress in two ways, Firstly, higher absorption of Na in root creates osmotic stress, that has resulted into decrease in water potential while on the other hand it also creates in disturbance of nutrient balance leading to

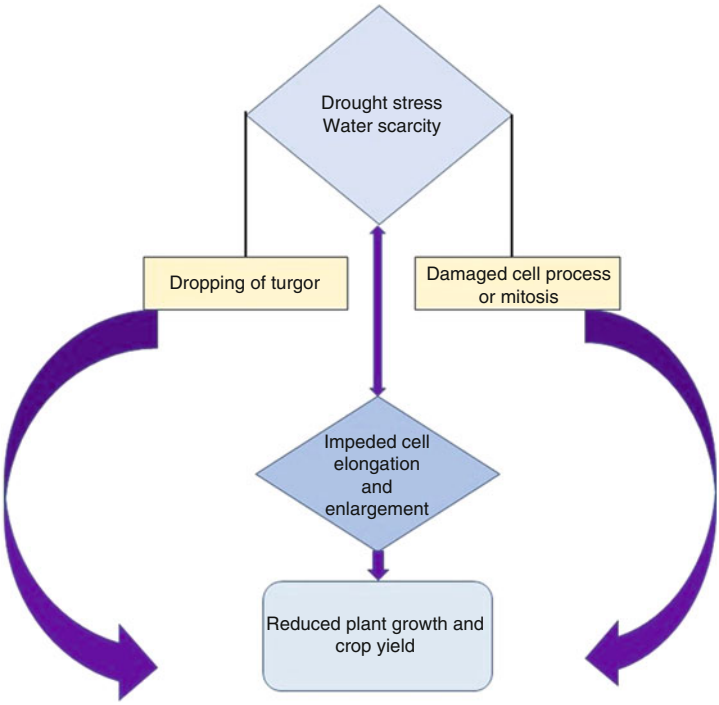

Fig. 3.1 Impacts of plant response against the drought stress

ionic stress (Munns and Tester 2008) (Fig. 3.2). An adequate quality of Na^+ and Cl^- in the soil solution may conquer the availability of essential nutrients via forming the most extreme ratio of $Na^+/C\ a^{+2}$, Na^+/K^+, Ca^+/Mg^+, and Cl^+/NO_3^- (Munns and Tester 2008). The increased level of salinity in plants has been portrayed to fall in the different physiological process, such as obtrusion of membranes, the disparity in nutrient balance, despair the cell cycle, and also decreased photosynthetic activity.

3.2.3 Temperature/Heat Stress

Under natural environments, crops are often exposed to various abiotic stresses concurrently throughout their life which hampers the viability and productivity of plants. Out of all the environmental conditions, extreme temperature conditions are the main environmental threat to crop growth and productivity and finally to the food security in climate. It is defined as the increase of temperature beyond the threshold limit for a particular period of time that causes permanent damage to plant growth and development. Pei et al. (1998) reported that temperature stress may occur in plants due to the insignificant water availability to check the transpirational loss meeting the evaporative demand, whereas Wahid et al. (2007) stated that heat stress

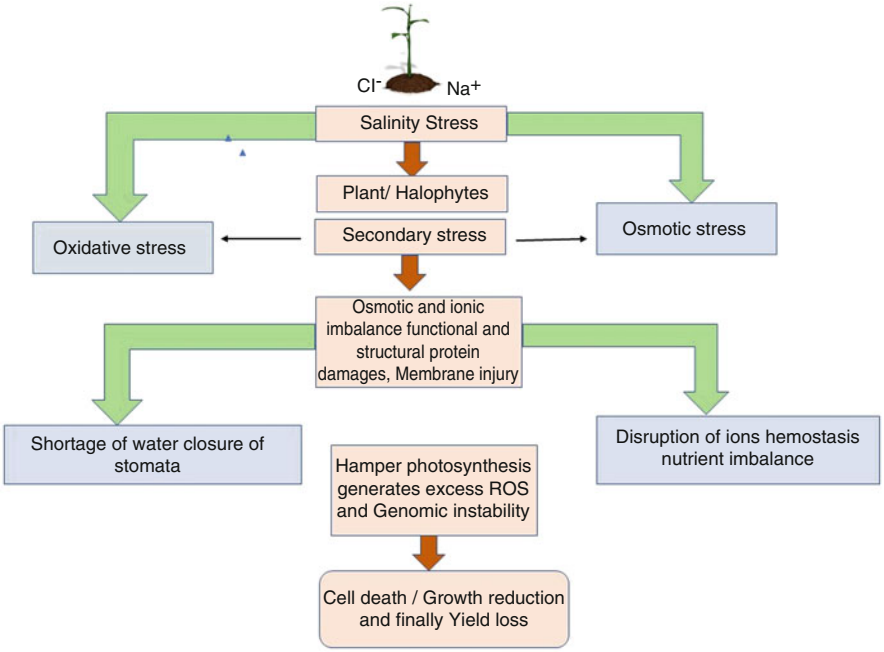

Fig. 3.2 Mechanism of salinity stress against plant responses

is a corollary to drought due to excessive transpiration that leads to the deficiency of water to decrease nutrient uptake and photosynthetic efficiency of plants. Temperature stress causes a significant reduction in biomass and leaf area that hamper to decline in root/shoot length, protein content, photosynthetic pigment content as well as modulate various other physiological, biochemical and cytological parameters in numerous plants. Heat stress causes denaturation of proteins and in turn improper functioning of cells (Khan and Shahwar 2020). In response to this, plants have also capabilities to cope up with the temperature stress as plants have the mechanism to create—a chain of enzymatic and non-enzymatic detoxification system to counter—action of AOC (antioxidant capacity), thereby protecting the cell from oxidative damages as well as stockpiling of certain organic substances of low molecular mass, normally called as compatible osmolytes (Sakamoto and Murata 2002). A piece of evidence shows that temperature stress considerably affects the vital process of cell division and cell elongation impacts on both mitosis and meiosis, which leads to decline length and weight of plant and creates chromosomal aberrations. Shah et al. (2011) show that heat stress prominently influences more or less every plant growth from very beginning, i.e., emergence stage to ripening as well as harvesting stages. Heat stress causes a deteriorating impact on seed germination, growth and morphology, reproductive development, cell membrane, photosynthesis as well as other vital processes in plant. That can be summarized in Fig. 3.3.

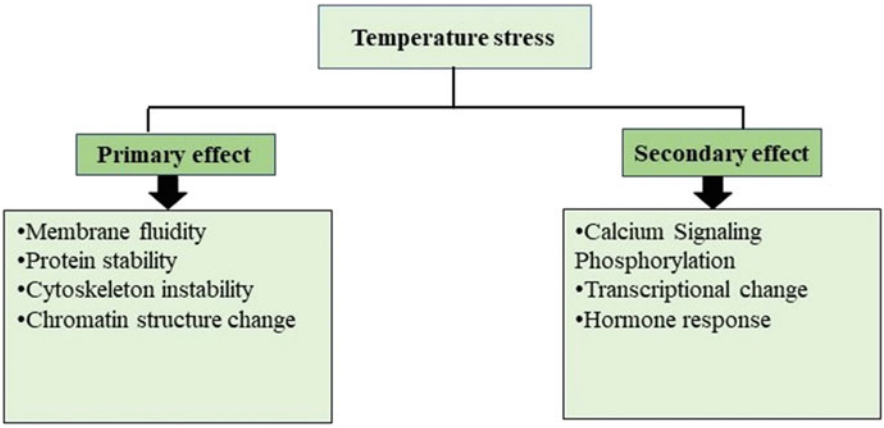

Fig. 3.3 Primary and secondary responses against the temperature stress

3.2.4 Chilling

Typical harsh conditions faced by the plants in their natural habitat creates some drastice changes in their growth, development and production. Chilling is one of them and can be defined as an excessive cold condition. Plants experience period of utmost low temperature in different geographical regions worldwide (Ruelland et al. 2009; Wang and Apel 2016). In general, chilling stress thermodynamically decreases the kinetics of much physiological as well as metabolic process taking place in plants (Ruelland et al. 2009; Hossain and Dietz 2016). Due to the delay in the physiological process which hampers crop production, chilling stress may lead to necrotic lesions on leaves, delayed leaf development, prolong cell cycle with decreased cell growth, induce wilting, increase vulnerability to pathogen and decrease survivability (Rymen et al. 2007). The effect of the cold stress depends on the low temperature sensitivity of every plant species.

Chilling stress causes injury in plants and its symptoms can be investigated at the cell level and demonstrates the large impact of low temperature on subcellular ultra-structure. The chilling stress caused by low temperature is summarized in given diagram (Fig. 3.4).

3.2.5 Ultraviolet Stress

Plants are obligated to be presented to different abiotic stress factors throughout their lifetime but some of the stress can adjust to changing ecological parameters by various morphological, physiological and other abiotic substances (Diaz et al. 2007). Due to anthropogenic activities the stratospheric ozone layer is continually being depleted primarily due to contamination of anthropogenic chlorofluorocarbon resulting diminishing of stratospheric ozone layer that causes the expansion of UV

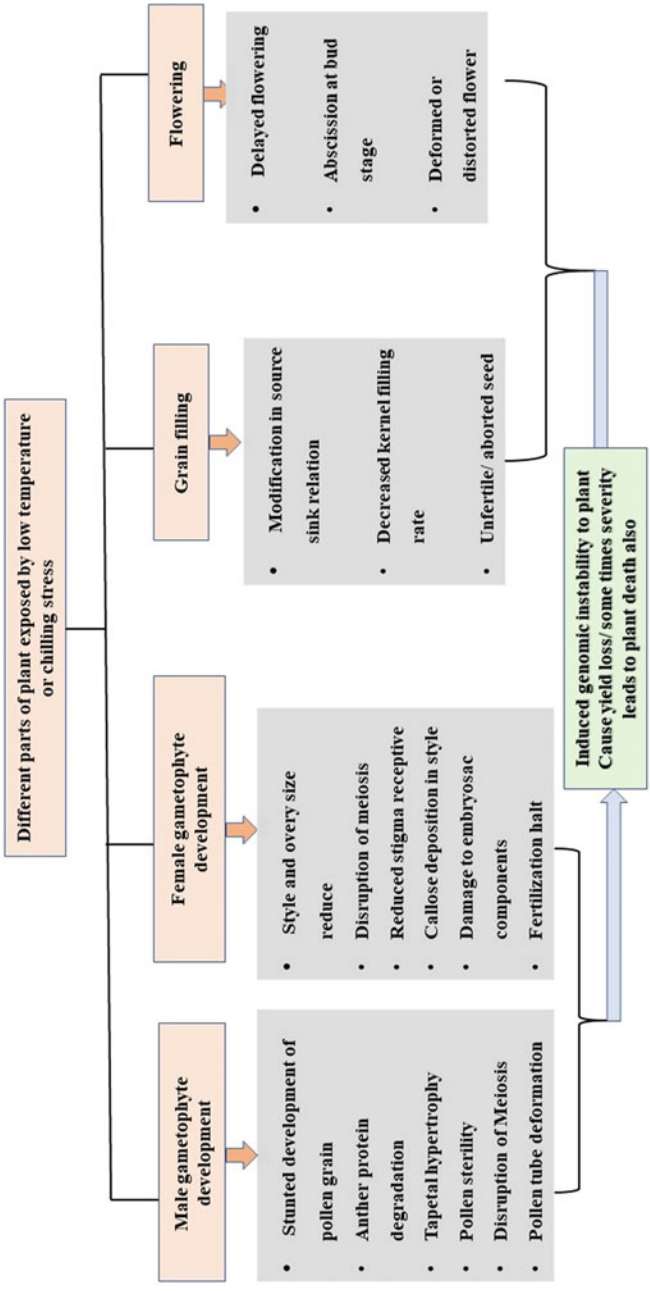

Fig. 3.4 Impact of chilling stress on various parts of plant

radiation intensity. Traditionally UV-rays are categorized into three wavelength ranges: UV-C (200–280 nm) is greatly damaging to organisms but not pertinence under natural conditions of solar irradiation, UV-A (320–400 nm) represents approximately 6.3% of the insulation and damaging part of UV radiation (Hollosoy 2002). UV-B (280–320 nm) is of particular interest because this wavelength represents only 1.5% of the total spectrum but can induce a variety of damaging effects in plants. The depletion of the ozone layer leads to the increase in ultraviolet radiation reaching to the earth surface. Increase of UV radiation acts as environmental stress (abiotic factor) on plants which finally causes the slowing of plant growth, damages the photosynthetic pigments and also hampers the biomass production of plants and reduces the productivity (Tevini and Termura 1989). Various diverse flora response differently to UV-B, some can tolerate this stress and however some become sensitive that cannot tolerate the stress condition. These plants acquire different physiological ways to avoid the stress condition. The UV-B mainly manifests its effect in reduced germination of plants. The various manifestation of UV-B effect could be due to DNA damage, disturbance in number and structure of chromatin. There are many plants that acclimatize them according to various stresses, but some are sensitive to such radiation resulting from array of physiological, morphological changes to even survival threat of plant (Fig. 3.5).

3.2.6 Gamma Radiation

Electromagnetic radiations are among the most vigorous physical agents to elicit their effects on plants and among these electromagnetic spectra the most significant is gamma rays that consist of ionizing rays. Gamma rays contain high energy from 10 to 100 KeV which ensure that the most penetrating and energetic potential among other radiations that earth receives from the solar rays. These ionizing rays consist of high energy photons that are capable of interacting with atoms and molecules and lead to the formation of free radicals. The scenario in which plants first emerged to terrestrial life was substantially different as the proportion of IR (Infrared radiation) was particularly more than at present and therefore it becomes highly significant to investigate the evolutionary background of plants (Gensel 2008). According to Siasou et al. (2017), studies on IR might help in unearthing adaptive evolution of living beings, for instance, at DNA repair level, that has been critical in plant establishment. Furthermore, it might also help to describe the present phenomenon of radio assistance and sometimes even capability to adjust lower levels of irradiation. Direct effects of background radiations on DNA must have been very significant in high background areas since antiquity; they take part in the evolution of both genetic construction and DNA guidance process of life (Caplin and Willey 2018).

Higher doses of gamma rays exert severe genotoxicity to the plants as higher doses promote cell cycle arrest during G_2/M phase and apprehended growth during cell division (Preussa and Britta 2003). Alterations in DNA during ionizing radiation induced mutagenesis can be of three types: intragenic (point mutations within a gene sequence), intergenic (inversions, deletions, duplications, translocations of DNA)

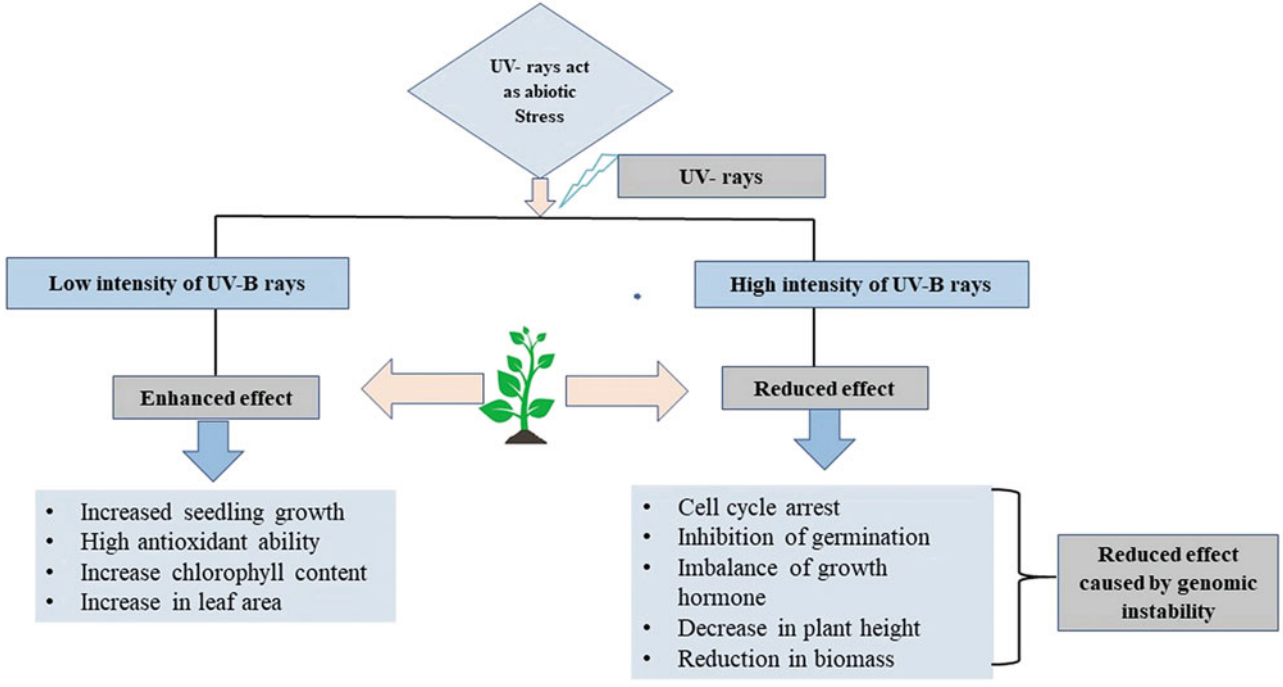

Fig. 3.5 Action of UV-rays as abiotic stress and their effects on plant

and changes in chromosome number (Oladosu et al. 2016). Unrepaired lesions in DNA, viz. strand breaks consequents in chromosomal aberrations like fragments, dicentrics and chromosomes with damaged kinetochore following division appear as micronuclei in the daughter cells (Rao et al. 2006a). These aberrations are cytogenetical markers for DNA damage at the chromosomal level. Soehendi et al. (2007) deciphered that gamma rays act on leaf canopy and seed yield of mung bean; particularly those having a larger area of leaf are highly exposed to photosynthesis resulting in the enhancement of yield rate. Rashed et al. (1994) explained that gamma rays alter the motif of protein in the protein band. The radiation harms the pigment for photosynthesis as an outcome of disturbed thylakoid and chloroplast and induces disorganization in the structure of grana and thylakoid (Kiong et al. 2008; Ali et al. 2016). A very important report was pointed by Kurimoto et al. (2010) as their work suggests that mature plants are extra tolerant towards these irradiations as they are completely equipped pertaining to amendments in the internal organization and biomass when irradiated. The higher doses have inhibitory effects on germination and growth of young seedlings that may be attributed to gamma rays induced mutations in DNA that synthesize DNA at the interphase leading to plant bud disruption resulting interruption of cell differentiation (Ali et al. 2016) or might be due to an increase in production of active radicals responsible for seed lethality.

Ionization of the vital biomolecules results in radiolysis of the most predominant water molecules in plants, with a cascade of reactive molecules that damage lipids, proteins and DNA within plant cells (Moghaddam et al. 2011). A large number of molecules play an essential role in the activity of biological systems and their reactivity not only make them useful in signaling and defense but also to induce injury in biomolecules (Foyer and Noctor 2016). Enhanced activities of antioxidant enzymes following gamma irradiation were reported by Aly and El-Beltagi (2010). It was reported that enhancement in the concentration of the osmolytes and proline in *Psoralea corylifolia* considered as a policy to keep away oxidative damage that led to modulation of certain metabolic and defensive process that is one of the protective mechanisms in the synthesis of osmolytes which is essential to plant growth and development (Esfandiari et al. 2008; Kiong et al. 2008; Jan et al. 2012). Furthermore, differential radioresistance of *P. corylifolia* L. following higher doses of gamma rays may be attributed to the increased production of psoralen as well as activation or over expression of ascorbate peroxidase and glutathione reductase activities. Psoralen is known for its antioxidative and photosensitizing properties hence the radiobiological tolerance may be correlated with the content of anti-oxidant substances at different developmental stages (Frank et al. 1998) (Fig. 3.6).

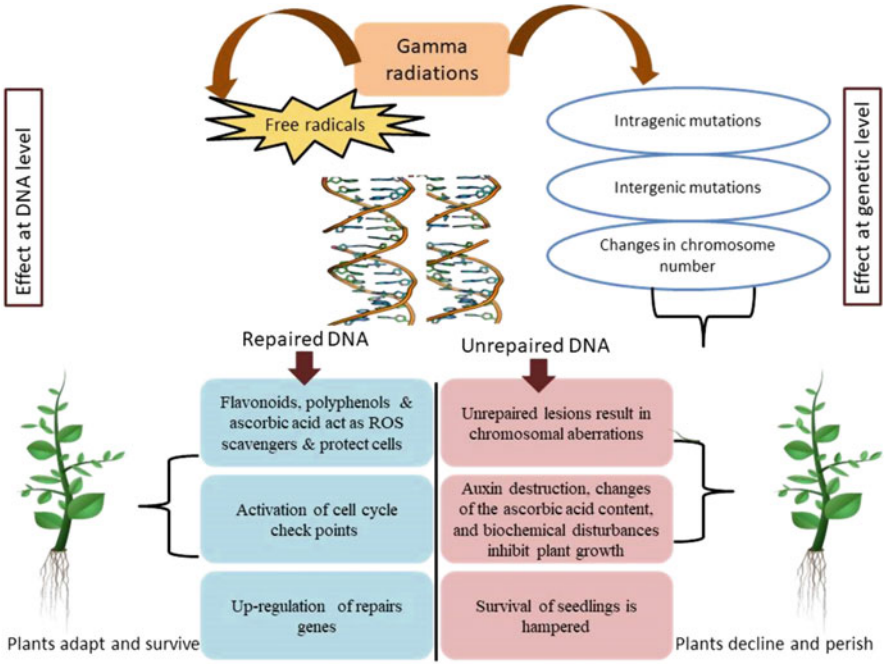

Fig. 3.6 Effect of gamma radiation at genetic and mutational level, Gamma irradiation production of free radicals leads to strand breaks in DNA which might get repaired or remain unrepaired, leading to different consequences (Marcu et al. 2013; Ali et al. 2016)

3.3 Genomic Instability of Plant Under Stress Condition

Maintenance of genome integrity is essential in all living organisms. It is required for proper growth and constant transmission of genetic information from one generation to other. Being sessile organisms, plants are constantly exposed to stress conditions that can also damage their DNA. DNA repair mechanisms help reverse oxidative adducts and other chemical changes to occur in higher plants that are necessary for the repair of DNA strand breaks (Hu et al. 2016). Plant cells generally possess greater stability and resistance towards the production of double strand breaks by physical agents and also they repair them more quickly than animal cells, as explained by Yokota et al. (2005). Mutations of double strand breaks repair proteins in plants tending just to reduce biomass production rather than change fundamental aspects of development, contrary to the multicellular animals (Manova and Gruszka 2015).

Abiotic factors frequently induce genotoxic stress and eventually result in the formation of various forms of lesions in DNA double helix. Unrepaired damages in the DNA strand lead to genomic instability, therefore influencing plant health and productivity. Different abiotic stresses have common and peculiar impacts on plant

species growth and development like photosynthetic declination, osmotic stress, etc. In recent years, an increased number of anthropogenic activities, rapid urbanization, and advanced agriculture practices, had contributed of heavy metal effluents and different kinds of abiotic stresses. Unbalanced dose may induce both cytotoxic and genotoxic effects and thus genomic instability in plants. Several studies had demonstrated abiotic stress mediated plant growth inhibition, explicitly pronounced during seed germination, and at early stages of seedling growth (Zhang et al. 2003; Li et al. 2019). In the stress condition plant exerts strategies for avoidance of stress condition. These avoidance strategies show few changes in plant that can be seen morphologically, biochemically and cytologically. These changes induce inhibitory effects on DNA replication, gene expression and cell division, depending on the level of stress, varieties of stress responses induced in the plant cells. In response to this, plants have developed a sophisticated regulatory mechanism to acclimatize and survive under stressful conditions. However extreme conditions cause genotoxicity which may severely impact plant health, sometimes eventually causing cell death. Plant response to environmental stresses involves complex metabolic webbing that generates various morphological changes, and these changes depend on highly sensitive signaling events inside cell. Various cellular parts contribute to the stress response by giving proper defense strategies that necessarily require enzyme and molecules, but DNA repair stands as the special barrier to conserve the genetic information. The reaction of plant cell to genotoxic stress is dependent on the activity of multiple DNA repair pathway which share distinctive feature to the plant kingdom. Abiotic stresses such as UV-light, temperature, heat, chilling, heavy metal show severe injury affecting plant development and crop yield (Tuteja et al. 2009). These abiotic stresses are critical constraints to crop productivity as well as to maintain quality of crop. The impact of genomic instability resulting from adverse environmental conditions on the plant defense machinery represents a key issue that has not been investigated properly till date. Several empirical studies of the impacts of genotoxic stress highlighted the involvement of different kind of chromosomal aberrations in cells, chromosomal defects in nucleus and cell cycle arrest which are essential information regarding genomic instability. The genomic instability leads to an array of negative impacts on both the quality and quantity of plant product and sometimes even proved fatal to plant. Genomic instability may lead to far and wide impact from biodiversity to food diversity ultimately leading to food insecurity in starving developing nations like India (Fig. 3.7).

3.4 Impact of Physical Agents on Plant Oxidative Stress

Higher plants are sessile therefore are continuously exposed to diverse environmental stress factors like drought, salinity, heavy metals, nutritional disorders, radiation without any defense. Increased contamination in the environment during the past years due to anthropogenic activities, rapid industrialization and modern agricultural practices acts as a major cause of stress. Most of those stresses produce certain common effects on plants, like induced oxidative stress by overproduction of

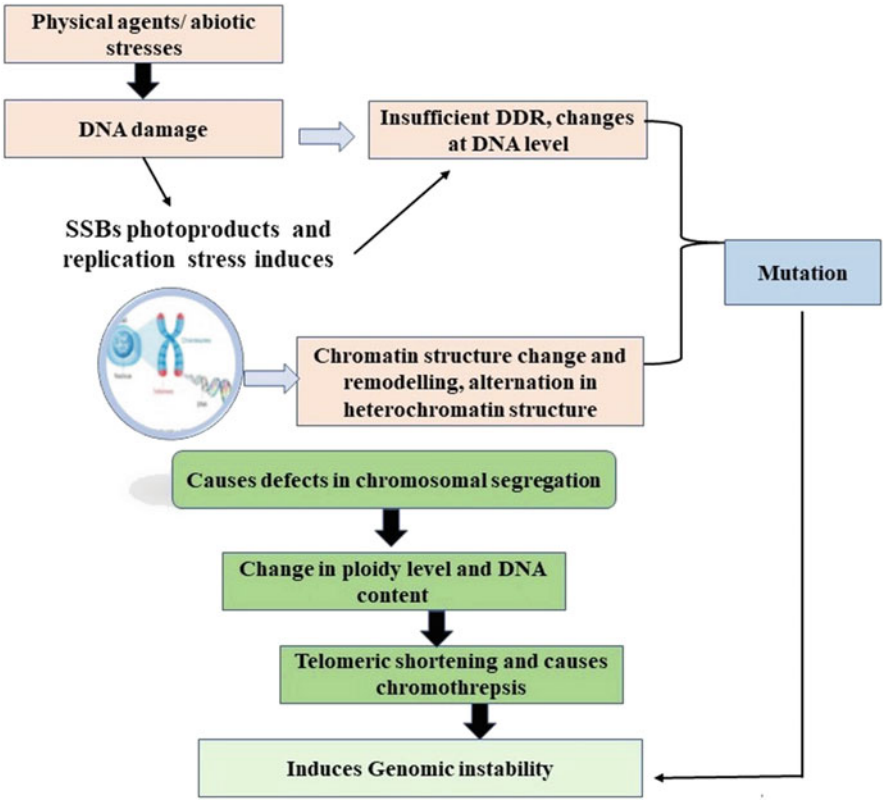

Fig. 3.7 Mechanism of genomic instability under abiotic stress condition

reactive oxygen species (ROS) besides their specific effects (Rao et al. 2006b). Thus, plants have developed their specific responses against each of stress. The plant cell will be in a stress condition as state of "oxidative stress" if the ROS level extents than the inside defense mechanisms (Fig. 3.8).

It then reveals growth regression under oxidative stress including plant height retardation, delay in seed germination, deliberate cell growth, elevated lignin biosynthesis in cell wall and cell senescence. Photosynthesizing plants are mainly at the danger of oxidative damage, due to their oxygenic conditions and therefore the abundance of the photosensitizers and polyunsaturated fatty acids (PUFA) within the chloroplast envelope. These disturbances in equilibrium cause an explosion in the intracellular levels of ROS which may cause significant damage to cell structures and it has been estimated that 1–2% of O_2 consumption results in the formation of ROS in plant tissues (Bhattacharjee 2005). Plants introduced to saline conditions display osmotic stress leading to inhibition of cell expansion and cellular division, changes within the stomatal closure, reduction of cell turgor and changes in homeostasis of cells (Miller et al. 2010). Likewise, drought and heat stress could lead on over

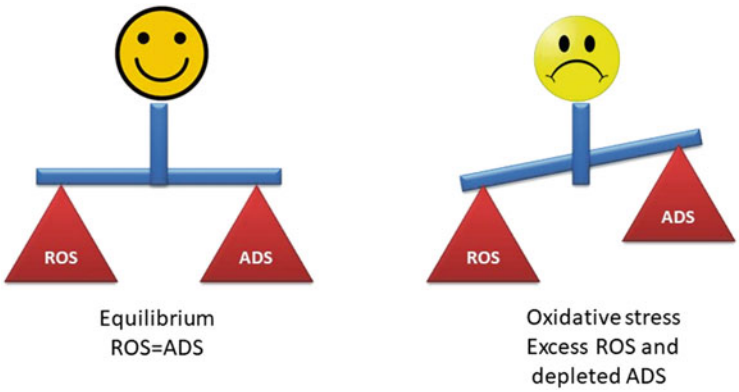

Fig. 3.8 Behaviour of ROS AND AOX under stress condition

production of ROS that deteriorate photosynthetic components in plant. Moreover, under these adverse conditions, plants generate reactive oxygen species (ROS) like superoxide ($O2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Hossain and Dietz 2016). To scavenge this ROS, plants are enabled to synthesize different types of antioxidants.

3.4.1 Production Sites of ROS

Production of ROS is an energy dependent process, it might be formed by an inevitable leakage of the electron from electron transport activities of chloroplasts, peroxisomes, mitochondria, plasma membranes, endoplasmic reticulum (ER), apoplasts and cell wall or as a by-product of various metabolic pathways localized in several cellular compartments (Navrot et al. 2007; Sharma et al. 2012; Das and Roychoudhury 2014; Saed-Moucheshi et al. 2014; Xia et al. 2015; Corpas et al. 2015) (Fig. 4.1). In light, the chloroplasts and peroxisomes are the indisputable source of ROS generation (Foyer et al. 1994). Mitochondria were found to be the main source of ROS producers in the darkness. It has been estimated that 1–5% of the O_2 consumption of isolated mitochondria leads to ROS production (Moller 2001).

In the oxidative phosphorylation an intermediary product ubisemiquinone formed at complex I and III transfers electrons to oxygen and creates $O_2^{\bullet -}$ to be reduced as H_2O_2 (Huang et al. 2016). Peroxisomes are important sites for ROS production, mainly via photorespiration and fatty acid β-oxidation pathways (Corpas 2015). In the generation of $O_2^{\bullet -}$many different enzymes play an important role like nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NADH/NADPH) placed within the peroxisomal membrane and xanthine oxidoreductase (XOD/XDH) and uricase ubicated in peroxisome matrix (García-Caparrós et al. 2019). In plants apoplast is the space between cells where solutes are often

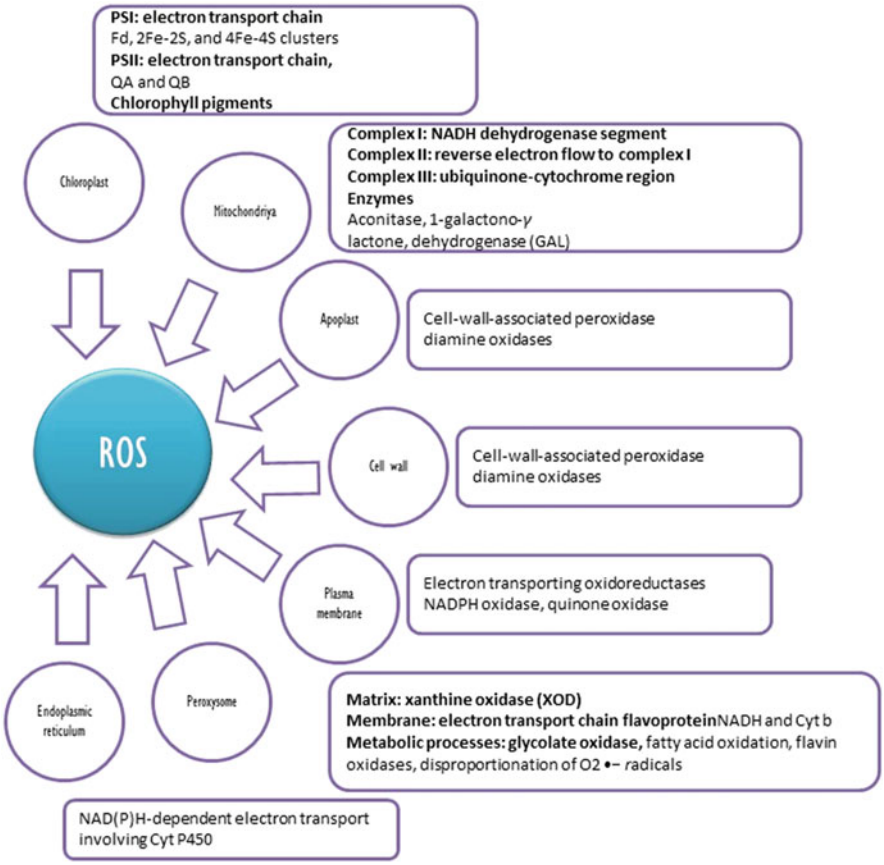

Fig. 3.9 Different production sites of ROS

transported from one cell to other cell. Usually, apoplast is known for a reduced antioxidant capacity and has acidic environment than the cytoplasm which is responsible for the reduction of cysteine and low molecular weight antioxidants like glutathione and ascorbate (Qi et al. 2017). Under stressed conditions, enzymes like NADPH oxidases, class III cell wall peroxidases and amino oxidases are responsible for the production of ROS in apoplast (García-Caparrós et al. 2019). Cell walls are also considered as active sites for ROS production. The role of cell wall associated peroxidase in H_2O_2 generation has been shown in Fig. 3.9. In horseradish malate dehydrogenase was found to be the only candidate for providing NADH (Gross 1977) in isolated cell walls creating H_2O_2 and this reaction was catalysed by peroxidase. Due to the attack of bacterium *Xanthomonas campestris* pv. malvacearum in cotton a hypersensitive response activated which causes the generation of ROS in the cell wall (Martinez et al. 1998).

3.4.2 Types of ROS and Damages

The main ROS in plants is superoxide radical, singlet oxygen, hydroxyl radical and hydrogen peroxide (Fig. 3.10). Superoxide radical ($O_2^{\bullet -}$) is involved in the proliferation of other species associated with oxidative stress. Mehler reaction is responsible for the assembly of superoxide radical in chloroplast where electrons from the photosynthetic electron placed in the transport chain reduce O_2. Even so, the existence of $O_2^{\bullet -}$ is together with the activity of Cu, Zn (Superoxide dismutase) which alters $O_2^{\bullet -}$ to H_2O_2 in chloroplasts (Takagi et al. 2016).

Singlet oxygen ($^1O_2$) symbolizes a high reactivity power and is an unavoidable by-product of oxygenic photosynthesis due to the reaction of chlorophyll triplet state (Telfer 2014). Furthermore, the generation of this ROS explains eminent effect in both photosystems (PS I and PS II) (Wang and Apel 2016). Hydroxyl radical ($^{\bullet}OH$) deciphers high reactivity, especially towards $^3O_2$. Hydrogen peroxide (H_2O_2) attributes a moderate capacity of reactivity and has no unpaired electrons. H_2O_2 is produced in the cells under normal conditions also on as a wide selection of stressful conditions like drought, chilling, UV irradiation, exposure to intense light, wounding, etc. This ROS is generated in several organelles like chloroplast, nucleus,

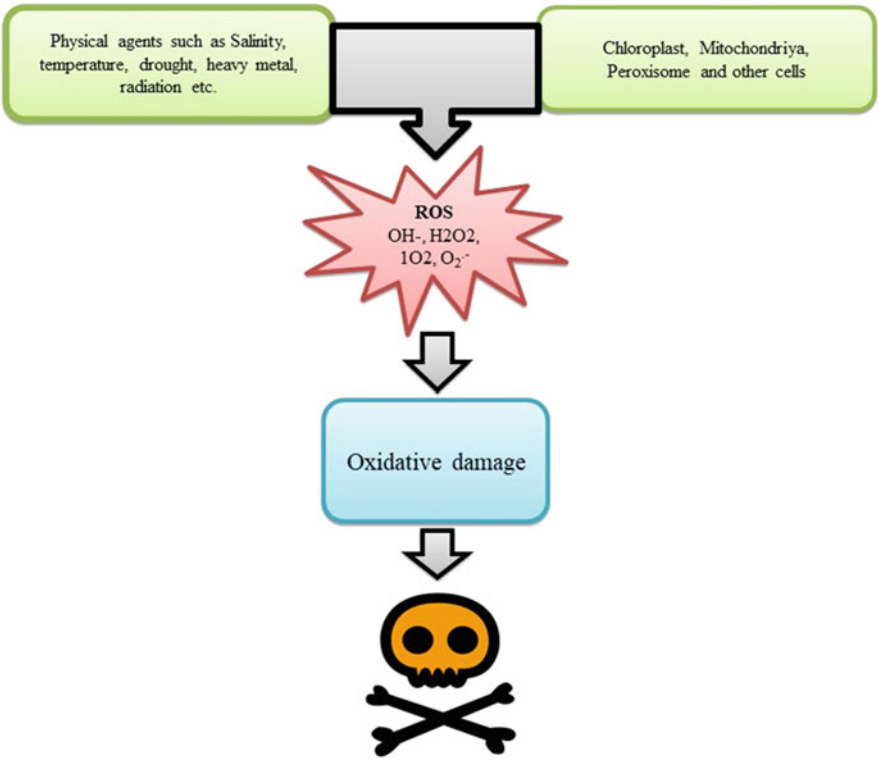

Fig. 3.10 Induced ROS production and cell death

plasma membranes, endoplasmic reticulum and mitochondria especially in the respiratory electron transport chain and peroxisomes especially in the photosynthetic carbon oxidation cycle (Miller et al. 2010). Photooxidation reactions, NADPH oxidase as well as xanthine oxidase (XOD) also contribute to H_2O_2 production in plants. Through transition metals, like Fe and Cu, further reduction of H_2O_2 breaks into OH^- and OH·. -OH· potentially reacts with all biological molecules, such as DNA, proteins and lipids. If these overproduced hydroxyl radicals are not removed by enzymatic or non-enzymatic defense mechanisms, it can cause cell death (Desikan et al. 2005; Gill and Tuteja 2010).

Several modern studies have confirmed that H_2O_2 is involved in stress signal transduction pathways, which may activate multiple acclamatory responses that emphasize resistance to several biotic and abiotic stressors. Overexpression of pepper (*Capsicum annuum*) *CaWRKY41* in *Arabidopsis* specifies that it weakens Cd tolerance, enhances Cd levels through activating Zn transporters and accelerates H_2O_2 accumulation. On the opposite, *CaWRKY41* silenced via VIGS in pepper plants displayed increased Cd tolerance and reduced H_2O_2 levels (Lie et al. 2019). Mutations of *Cu/Zn- SOD1* (*csd1*), *csd2and sodx* led to enhanced resistance to *Magnaporthe oryzae* and increased H_2O_2 accumulation in rice. Further studies exposed that they altered the expression of CSDs and other SOD family members, causing an increase in total SOD enzyme activity and higher H_2O_2 production compared to WT (Dang et al. 2019). These transgenic studies pronounced the role of H_2O_2 in the formation of plant tolerance to different biotic and abiotic stress.

Rising levels of ROS production cause cellular damages, such as oxidative degradation of lipid peroxidation, protein oxidation and DNA damage (Fig. 3.11.)

Lipid peroxidation involves three different stages known as initiation, progression and termination. In the first stage, breakage of membrane associated polyunsaturated fatty acids (PUFA) by hydrogen abstraction or addition of an oxygen radical increases the presence of radicals like $O_2^{\bullet -}$ and $^{\bullet}OH$. In the second stage, these ROS react with the methylene groups of the PUFA originating conjugated dienes, lipid peroxyl radical and hydroperoxides. Finally, in the very last step termination occurs through the generation of different lipid dimers provoke by different lipid-derived radicals. A further result of ROS-attack in cells is an increase in protein oxidations. Oracz et al. (2007) explained various mechanisms that might lead to protein oxidation, such as the formation of disulphide cross-links and glycol oxidation adducts nitration of tyrosine residues and carbonylation of specific amino acid residues. The spectrophotometric measurement of protein carbonyl with dinitrophenylhydrazine (DNPH) process is widely used marker for detection of protein oxidation in biological organisms.

3.4.3 Effect of Various Physical Agents on the Production of ROS

In a wild, plants are exposed to many harsh and unavoidable environmental stress conditions, which affect their developmental, physiological, biochemical, morphological and molecular integrity. The above-mentioned physical agents like drought,

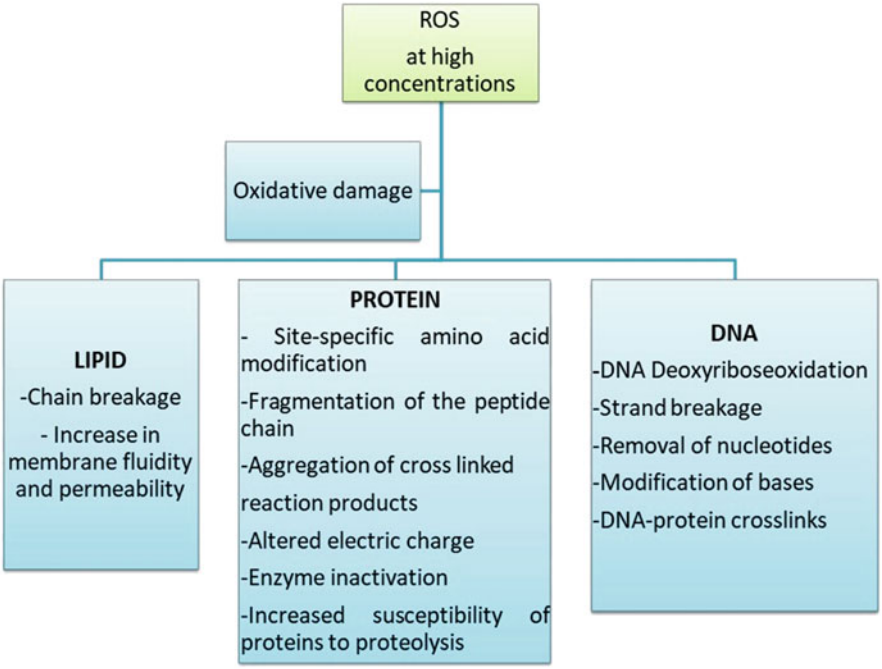

Fig. 3.11 ROS-induced oxidative damage to lipid, protein and DNA

salinity, high light, UV and gamma radiation and extreme temperatures (heat shock, chilling) show various effect on plants. (Reddy et al. 2004) Further, we will see how various physical agents affect plant differently.

3.4.3.1 Drought

Virtually, draught stress is seen in all the plants however, its extent varying between the species (Reddy et al. 2004) via Mehler reaction -ROS produced which leads to higher leakage of electrons to O_2 in drought condition due to inhibition of CO_2 assimilation resulting in irregular photosynthetic activity (Smirnoff 1998). Drought stress causes stomatal closure which leads to reduced $NADP^+$ regeneration through the Calvin cycle. Biehler and Fock (1996) mentioned in their report that 50% more leakage of photosynthetic electrons to the Mehler reaction occurred in drought stressed wheat plants, compared to unstressed plants. Dissipation of excess light energy in the PSII core and antenna leads to generation of ROS that are potentially dangerous under drought stress conditions. Noctor et al. (2002a) reported that under drought stress, the photorespiratory pathway is also enhanced, especially, when RUBP oxygenation is maximal due to limitations in CO_2 fixation. Under drought stress conditions, production of $O_2^{\cdot-}$ and H_2O_2 in wheat chloroplast get amplified (Sairam et al. 2005). On the other hand, some ROS-dependent changes are interconnected in plants and often unified with drought stress, like lipid peroxidation

and changes in antioxidant levels (Oztetik 2011). Lipid peroxidation increases in wheat (Sairam et al. 1998) and *Brassica napus* (Aziz and Larher 1998) tissues during drought. All these effects act differently in various plants.

3.4.3.2 Salinity

Among the environmental stresses, salinity in the soil is one of the factors which may limit growth and productivity of plants (Allakhverdiev et al. 2000). It reduces water potential and causes disturbances in osmotic and ionic homeostasis and toxicity. When the salt concentration increases than the normal requirement of the plant, it causes guard cells to become hypopolarized resulting in stomatal closure, which reduces CO_2 concentration in the leaves and inhibits carbon fixation. Concomitantly causes unbalanced impairment of electron transport system in chloroplast and mitochondria resulting in enhanced generation of ROS and induced oxidative stress. Continuously reducing CO_2 favours photorespiration resulting to increased production of ROS, such as H_2O_2 (Hernandez et al. 2000). Elevated CO_2 increases the oxidative stress caused by salinity, involving lower ROS generation and better maintenance of redox homeostasis as a consequence of higher assimilation rates and lower photorespiration (Perez-Lopez et al. 2009). Salinity-induced ROS disrupts normal metabolism through lipid peroxidation, denaturing proteins and nucleic acids in several plant species (Tanou et al. 2009; Hernandez et al. 2000; Karray-Bouraoui et al.; 2011). During the last 30 years, research on salinity in plants has produced a huge number of literatures. Some of the salt stress, its physiological traits and plant responses to high salinity have been discussed (Rains et al. 1980; Munns 2002), while in others molecular aspects and genetic information related to the salt stress have been analysed (Hasegawa et al. 2000; Xiong and Zhu 2002).

3.4.3.3 Temperature Stress

Exposure of plants to high temperature leads to damage and inhibition of photosynthetic machinery. The inhibition caused by damage of PSII results production of hydroxyl radical ($OH^{\bullet}$). It is formed due to the reaction of H_2O_2 with $O_2^{\bullet-}$ (Haber–Weiss reaction), reactions of H_2O_2 with Fe^{2+} (Fenton reaction) and decomposition of O_3 in apoplastic space (Halliwell 2006; Moller et al. 2007). Singlet oxygen ($^1O_2$) is also formed during photoinhibition and PS II electron transfer reactions in chloroplasts. This radical directly oxidizes protein, polyunsaturated fatty acids and DNA (Karuppanapandian et al. 2011a, b). The drastic increase in lipid peroxidation due to HT stress was reported by many researchers (Hasanuzzaman et al. 2012; Wu et al. 2010). Several studies indicated that under heat stress conditions, malondialdehyde (MDA), a product of peroxidation of unsaturated fatty acids, has been used as a good marker of free radical damage to cell membranes (Suzuki and Mittler 2006; Tommasino et al. 2012). In wheat seedlings, gradual increase in the accumulation of H_2O_2 was observed (0.5, 0.58, 0.78 and 1.1μmol g^{-1} FW) in response to differential heat shock treatment of 22, 30, 35 and 40 °C for 2 h (Kumar et al. 2012).

Low temperature also proves to be lethal for the plants as it results in the overproduction of ROS by aggravating imbalance between light absorption and

light use by inhibiting the Calvin-–Benson cycle activity (Logan et al. 2006), enhancing photosynthetic electron flux of O^2 and causing over reduction of respiratory ETC (Hu et al. 2008). Chilling stress also causes significant reductions in *rbc*L and *rbc*S transcripts, RUBISCO content and initial RUBISCO activity, leading to higher electron flux of O^2 (Zhou et al. 2006). Accumulation of H_2O_2 in chloroplast was negatively correlated with the initial RUBISCO activity and photosynthetic rate (Zhou et al. 2006), also chilling injury led to lipid peroxidation and protein oxidation in plants (Freyer et al. 1998; Prasad 1997). Protein carbonyl content, an indicator of oxidative damage, increased twofold in maize seedlings when exposed to chilling temperatures (Prasad 1997). According to Freyer et al. (1998) in chill stressed maize leaves lipoxygenase activity as well as lipid peroxidation was increased suggesting that lipoxygenase mediated peroxidation of membrane lipids contributes to the oxidative damage.

3.4.3.4 UV-B Radiation

Due to the depletion of ozone layer UV-B concentration enhanced significantly which inhibits net photosynthetic rate. A significant decrease in the light saturated rate of CO_2 assimilation was observed in UV-B treated plants accompanied by decrease in carboxylation velocity and RUBISCO content and activity (Allen et al. 1997). It is reported that limited CO_2 assimilation due to UV-B leads to excessive production of ROS that causes oxidative damage in plants (Strid et al. 1994). Rao et al. (1996) suggested that UV-B exposure generates activated oxygen species by increasing NADPH oxidase activity. These reactive oxygen species lead to severe damage of membrane lipids, nucleic acid and protein (McKersie and Leshem 1994; Imlay and Linn 1988). A recent study observed that almost higher exposure of UV-B depicted special interference with meiotic pollen mother cells and pollen grains cause genotoxic effect in *Vicia faba* L. (Abdel Haliem et al. 2013). An important component of root RUS1/RUS2 complex, involved in UV-B-sensing pathway regulates the seedling morphogenesis and development at early stages in *Arabidopsis*. Its destruction interferes the development of seedling due to increased signal generated from photoreceptors after the perception of UV-B (Leasure et al. 2009). Plants must adapt to the harmful effects of UV-B radiation because they are reliant on sunlight for photosynthesis and therefore cannot avoid exposure to UV-B radiation.

3.4.3.5 Gamma Radiation

Gamma radiation that belongs to ionizing radiation can react directly with macromolecules causing immediate cellular damage such as DNA strand breaks, lipid oxidation and protein inactivation. Cellular damage can also be initiated by reactive oxygen species (ROS) that are generated during the radiolysis of water, an important pathway under gamma irradiation (Ward 1988). ROS have both positive and negative effect as both toxic by-products of aerobic metabolism and key regulators of biological processes, such as growth, cell cycle and response mechanisms to various stress situations (Miller et al. 2010). As plasma membrane related superoxide producing NADPH oxidases can be a possible source for ROS

during this oxidative burst. Gene expression levels for several NADPH oxidase isoforms were investigated for irradiated *Arabidopsis thaliana* leaves and roots but no alterations were observed, suggesting that ROS production under ionizing radiation stress is probably due to water radiolysis and not via an oxidative burst at the plasma membrane level. Lipid peroxidation can be initiated via immediate interaction with ionizing radiation, but also indirectly by interaction with various ROS, produced under stress conditions. MDA is the end product of lipid peroxidation, ordinarily indicating the level of lipid peroxidation and reflecting the membrane deleterious (Wang et al. 2010). This process proceeds by a free radical chain-reaction mechanism, thereby consequent into overproduction of ROS such as hydroxyl radicals, superoxide radical and hydrogen peroxide. Interaction of ROS molecules with almost all structural and functional organic molecules causes substantial interruption of cellular metabolism (Noreen and Ashraf 2009).

3.4.4 Antioxidant Effect

Plants have efficient complex enzymatic and non-enzymatic antioxidant defense systems to avoid the toxic effects of free radicals (Fig. 3.12). These inhibitors of oxidation of biomolecules can be sorted into two categories: enzymatic and non-enzymatic. The common enzymatic antioxidants include superoxide dismutase,

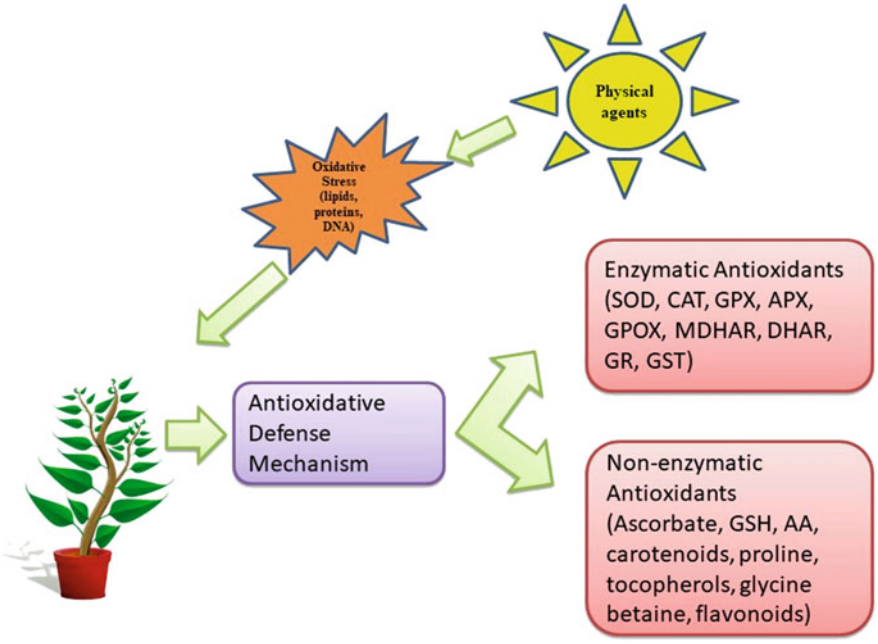

Fig. 3.12 Physical agents induced ROS generation and antioxidative defense

catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase. The non-enzymatic antioxidants comprise reduced glutathione, ascorbic acid, carotenoids, tocopherols and flavonoids (Ashraf 2009). Enzymatic systems include SOD, catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) while non-enzymatic systems consist of low molecular weight antioxidants such as ascorbic acid, glutathione, proline, carotenoids, phenolic acids, flavonoids and high molecular weight secondary metabolites such as tannins. Alscher et al. (1997) reported that glutathione and ascorbate are synthesized in plants within the chloroplast stroma and cytosol using NADPH as the ultimate electron donor. These low molecular weight antioxidants function as redox buffers and interact with cellular components thereby affecting plant growth and development by modulating processes from mitosis and cell elongation to senescence and death (Foyer and Noctor 2005). Plants also synthesize and accumulate a range of low and high molecular weight secondary metabolites that play important roles in ROS metabolism and avoidance of uncontrolled oxidation of essential biomolecules. These metabolites are also important for adaptation of plants to environmental fluctuations (Baier and Dietz 2005).

3.4.4.1 Non-Enzymatic Components of Antioxidative Defense System

Nonenzymic components of the antioxidative machinery comprised of cellular redox buffers, such as ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH), tocopherol, carotenoids and phenolic compounds. They interact with numerous cellular components and in addition to crucial roles in defense and as enzyme cofactors, these antioxidants also influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and cell death (De Pinto and De Gara 2004). Mutants with decreased nonenzymic antioxidant contents have been shown to be hypersensitive to stress (Semchuk et al. 2009).

Ascorbate (AsA)

Ascorbate is the most abundant antioxidant ensuing pivotal role in defense against oxidative stress which is incurred due to enhanced ROS level. Efficiency of AsA is its ability to donate electrons in many enzymatic and non-enzymatic reactions and therefore it is considered a powerful antioxidant. Several pivotal roles played by ascorbate in physiological processes include the regulation of sulphate transport, signal transduction, detoxification of xenobiotics, conjugation of metabolites and the expression of stress-responsive genes. Both of them are also the main components of the Halliwell–Asada Cycle (Gill and Tuteja 2010). AsA is also synthesized via uronic acid intermediates, such as D-galacturonic acid (Isherwood et al. 1954). Most of AsA, approximately 90%, is localized in cytoplasm, but unlike other soluble antioxidants a substantial portion is exported to the apoplast, where it is present in millimolar concentration. Apoplastic AsA is believed to represent the first line of defense against potentially damaging external oxidants (Barnes et al. 2002). AsA mostly exists in reduced state in chloroplast where it also acts as a cofactor of violaxanthin de-epoxidase, thus sustaining dissipation of excess excitation energy

(Smirnoff 2000). It provides membrane protection by directly reacting with $O_2^{\bullet -}$, H_2O_2 through oxidation process. Oxidation of AsA occurs in two sequential steps, first producing monodehydroascorbate (MDHA) and subsequently dehydroascorbate (DHA). In the AsA-GSH cycle, two molecules of AsA are utilized by APX to reduce H_2O_2 to water with concomitant generation of MDHA.

Glutathione

Tripeptide glutathione (γ-glutamyl cysteinyl-glycine, GSH) is one of the crucial low molecular weight non-protein thiol that performs an important role in intracellular defense against ROS-induced oxidative damage. Glutathione is detected virtually in various organelles, viz. cytosol, chloroplasts, endoplasmic reticulum, vacuoles and mitochondria (Foyer and Noctor 2003). Cytosolic and chloroplasts GSH level is synthesized by compartment of specific isoforms of γ-glutamyl-cysteinyl synthetase (γ-ECS) and glutathione synthetase (GS). Cellular redox state is stabilized by the maintenance of the balance between the GSH and glutathione disulphide (GSSG). Due to its reducing power, synthesis of phytochelatins for metal chelation, detoxification of xenobiotics and the expression of the stress-responsive genes happen (Foyer et al. 1997). GSH, as an antioxidant, has multitude functions. It can function directly as a free radical scavenger for $O_2^{\bullet -}$, $^{\bullet}OH$, H_2O_2 thereby protecting macromolecules (i.e., proteins, lipids, DNA) either by the formation of adducts directly with reactive electrophiles (glutathiolation) or by acting as proton donor in the presence of ROS or organic free radicals, yielding GSSG. It also participates in regenerating AsA, that itself an efficient antioxidant through the AsA-GSH cycle. GSH recycles AsA from its oxidized form to reduced form via enzyme DHAR. GSH can also reduce DHA by a nonenzymic mechanism at pH >7 and at GSH concentrations greater than 1 mM. The role of GSH in the antioxidative defense system provides a rationale for its use as a stress marker. Tausz et al. (2004) in their study observed that when apple trees were subjected to progressive drought, the initial response was a little oxidation of the GSH pool, followed by increased GSH concentrations. When the stress increased, GSH concentrations dropped and redox state became more oxidized, which marked the degradation of the system. Similar to drought stress, the altered ratio of GSH/GSSG has also been reported in plants under various stress like salinity (Hefny and Abdel-Kader 2009) and metal toxicity (Maheshwari and Dubey 2009; Tanou et al. 2009; Mishra et al. 2011).

Tocopherol

Tocopherols, a lipid soluble antioxidant, are considered as potential scavengers of ROS and lipid radicals in bio-membranes, where they play both antioxidant and non-antioxidant functions. Tocopherols as antioxidants establish membrane stability and quench ROS like $^1O_2$. The antioxidant is localized in plants in the thylakoid membrane of chloroplasts. Out of four isomers of tocopherols present in plants (α-, β-, γ-, δ-), α-tocopherol has the highest antioxidative activity due to the presence of three methyl groups in its molecular structure (Kamal-Eldin and Appelqvist 1996). It is synthesized from γ-tocopherol in chloroplasts by γ-tocopherol methyl transferase (g-TMT; VTE4). A high level of α-tocopherol has been found in the leaves of many

plant species including *Arabidopsis* but these are low in γ-tocopherol. Nitration of γ-tocopherol is considered to be an important mechanism for the detoxification of NOx in animal tissues. In plants, in-vivo 5-nitro-γ-tocopherol (5-NgT) was also identified in leaves of the *Arabidopsis* mutant line (vte4). Reduced NOx concentration has been found in the leaves of vte4 mutant than vte1 and WT. Germinating seeds of *Brassica napus, N. tabacum* and *A. thaliana* also showed the presence of 5-NgT. It can be said that γ-tocopherol prolongs early development by reducing NOx concentration (Desel et al. 2007). It has been proved that tocopherols prevent the chain propagation step in lipid autooxidation which makes it an effective free radical trap. Additionally, it has been estimated that one molecule of α-tocopherol can scavenge up to 120 $^1O_2$ molecules by resonance energy transfer (Munné-Bosch 2005). Recently, Gang et al. (2007) proposed that oxidative stress activates the expression of genes responsible for the synthesis of tocopherols in higher plants. Increased levels of α-tocopherol and ASH have been found in tomato following trizole treatment which may help in protecting membranes from oxidative damage and thus chilling tolerance in tomato plants (Shao et al. 2007). Increase in tocopherol during water stress in plants has also been reported by many workers, such as Wu et al. (2010) and Shao et al. (2007).

Carotenoid

Carotenoids are a lipid soluble antioxidant, which are potential scavengers of ROS and lipid radicals. They are known as major antioxidants in biological membranes for the protection of membrane stability against lipid peroxidation, including quenching or scavenging ROS like $^1O_2$. Carotenoids act as energetic antenna, absorb light at wavelength between 400 and 550 nm and transfer it to the chlorophyll. They protect the photosynthetic apparatus by quenching a triplet sensitizer (Chl^3), $^1O_2$ and other harmful free radicals that are naturally formed during photosynthesis. Carotenoids ensue stability to the light harvesting complex proteins as well as thylakoid membrane stabilization (Gill and Tuteja 2010). Carotenoid contents increase under different oxidative stress conditions, as reported in *Citrus lemon* shoots (Helaly and El-Hosieny 2011). Carotenoid content also increased in salt tolerant *Chrysanthemum morifolium* strain, which was improved *using* in vitro selection technique (Hossain et al. 2006). Gomathi and Rakkiyapan (2011) observed that high carotenoid content favours better adaptation of sugarcane plants under saline conditions.

Proline (pro)

Proline is considered as the most important stress indicator in plant. It is potent antioxidant and potential inhibitor of PCD. Therefore, Pro can now be regarded as non-enzymatic antioxidants that various living entities require to mitigate the adverse effects of ROS. The synthesis of L-Pro from L-glutamic acid via D1-pyrroline- 5-carboxylate (P5C) is catalysed by the activities of the enzymes D1-pyrroline-5-carboxylate synthetase (P5CS) and D1-pyrroline- 5-carboxylate reductase (P5CR) in plants (Verbruggen and Hermans 2008). On the other hand, mitochondrial enzymes Pro dehydrogenase (oxidase) (ProDH) and P5C

dehydrogenase (P5CDH) metabolize L-Pro into L-Glu via P5C. In case of stress such as salt, drought and metal stress, there is a dramatic accumulation of Pro. Free Pro has been proposed to act as an osmoprotectant, a protein stabilizer, a metal chelator, an inhibitor of LPO and OH^- and $^1O_2$ scavenger (Ashraf and Foolad 2007; Trovato et al. 2008). Smirnoff and Cumbes (1989) tested sorbitol, mannitol, myo-inositol and Pro for OH scavenging capacity and documented that Pro appeared as an effective scavenger of OH^-. Therefore, Pro is not only an important molecule in redox signaling but also an effective quencher of ROS formed under salt, metal and dehydration stress conditions in all plants, including algae (Alia and Matysik 2001). Furthermore, it has also been noted that Pro also protected the yeast cells from herbicide MV. It was suggested that the ability of Pro to scavenge ROS and the ability to inhibit ROS-mediated apoptosis can be an important function in response to cellular stress. Increased accumulation of Pro has been correlated with improved tolerance towards abiotic stress especially salt and drought. Enhanced synthesis of Pro under drought or salt stress has been implicated as a mechanism to alleviate cytoplasmic acidosis and maintain NADPþ: NADPH at values compatible with metabolism (Hare and Criss 1997). An additional advantage of the refilling of NADPþ supply by Pro synthesis may be to support redox cycling, which is especially important in plant antioxidant defense mechanisms during stress (Babiychuk et al. 1995).

3.4.4.2 Enzymatic Components of Antioxidative Defense System

Superoxide Dismutase (SOD)

Metalloenzyme SOD is the most effective intracellular enzymatic antioxidant which is ubiquitous in all aerobic organisms and in all subcellular compartments prone to ROS-mediated oxidative stress. It is well established that various environmental stresses often lead to the increased generation of ROS, where SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS. The SODs remove O_2^- by catalysing its dismutation, one O_2^- being reduced to H_2O_2 and another oxidized to O_2. It removes O_2^- and hence decreases the risk of OH^- formation via the metal catalysed Haber–Weiss-type reaction that occurs 10,000-fold faster than spontaneous dismutation. SODs are classified by their metal cofactors into three known types: copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD) and iron (Fe-SOD), which are localized in different cellular compartments. In *A. thaliana* genome, three FeSOD genes (FSD1, FSD2 and FSD3), three Cu/ZnSOD genes (CSD1, CSD2 and CSD3) and one MnSOD gene (MSD1) have been reported (Kliebenstein et al. 1999). A significant increase in SOD activity under salt stress has been observed in various plants, viz. *Morus alba* (Harinasut et al. 2003), *Cicer arietinum* (Kukreja et al. 2005) and *Lycopersicon esculentum* (Gapinska et al. 2008). Eyidogan and Oz (2007) noted three SOD activity bands (MnSOD, FeSOD and Cu/ZnSOD) in *C. arietinum* under salt stress. Furthermore, significant increase in the activities of Cu/ZnSOD and MnSOD isozymes under salt stress was observed. Pan et al. (2006) studied the effect of salt and drought stress on *Glycyrrhiza uralensis* Fisch and found significantly

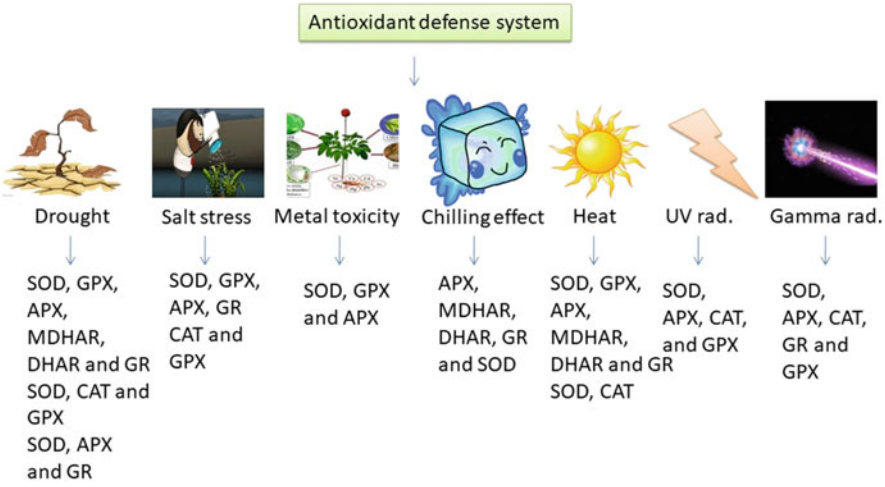

Fig. 3.13 Various Enzymatic antioxidants produced against physical agents

increased SOD activity (Fig. 3.13) but an additional MnSOD isoenzyme was detected under only salt stress. Moreover, increased SOD activity has also been detected following cadmium treatment in *Hordeum vulgare* (Guo et al. 2004), *Brassica juncea* (Mobin and Khan 2007), *Vigna mungo* (Singh et al. 2008), *Cicer arietinum* (Hasan et al. 2008). An increment in SOD activity was noted in three cultivars of *Phaseolus vulgaris* (Zlatev et al. 2006), *Alternanthera philoxeroides* (Wang et al. 2008) and *Oryza* sativa (Sharma and Dubey 2005) following drought stress.

Catalase (CAT)

Among antioxidant enzymes, catalase was the first enzyme to be discovered and characterized. It is a ubiquitous tetrameric heme-containing enzyme that catalyses the dismutation of two molecules of H_2O_2 into water and oxygen. It is highly specific for H_2O_2, but weak against organic peroxides. Plants contain several types of H_2O_2-degrading enzymes; however, CATs are unique as they do not require cellular reducing equivalent. CATs have a very fast turnover rate, but a much lower affinity for H_2O_2 than APX. The peroxisomes are major sites of H_2O_2 production. CAT scavenges H_2O_2 generated in this organelle during photorespiratory oxidation, β-oxidation of fatty acids and other enzyme systems such as XOD coupled to SOD (Yin et al. 2010). All angiosperm species contain three CAT genes till date. Ushimaru et al. (2006) proposed a classification of CAT based on the expression profile of the tobacco genes. Class I CATs are expressed in photosynthetic tissues and are regulated by light. Class II CATs are expressed at high levels in vascular tissues, whereas Class III CATs are highly abundant in seeds and young seedlings. Overexpression of a CAT gene from *Brassica juncea* introduced into tobacco enhanced its tolerance to Cd induced oxidative stress (Dixon et al. 2002).

Ascorbate Peroxidase (APX)

APX is thought to play the most essential role in the scavenging ROS and protecting cells in higher plants, algae and other organisms. APX is involved in the scavenging of H_2O_2 in water-water and ASH-GSH cycles and utilizes ASH as the electron donor. The APX family consists of at least five different isoforms including thylakoid (tAPX) and glyoxysome membrane forms (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Noctor and Foyer 1997). APX has a higher affinity for H_2O_2 (mM range) than CAT and POD (mM range) and it might play more crucial role in the management of ROS during stress. Enhanced expression of APX in plants has been demonstrated during different stress conditions. An increased leaf APX activity under Cd stress has been reported in *Ceratophyllum demersum* (Arvind and Prasad 2003), *Brassica juncea* (Mobin and Khan 2007), *Triticum aestivum* (Khan et al. 2007) and *Vigna mungo* (Singh et al. 2008). Srivastava et al. (2005) reported enhancement in APXactivity in salt stressed *Anabaena doliolum*. A significant increase in APX activity was noted under water stress in three cultivars of *Picea asperata* (Yang et al. 2008). Sharma and Dubey (2005) found that mild drought stressed plants had higher chloroplastic-APX activity than control grown plants, but the activity declined at the higher level of drought stress. The findings of Koussevitzky et al. (2008) suggest that cytosolic APX1 plays a key role in protection of plants to a combination of drought and heat stress.

Monodehydroascorbate Reductase (MDHAR)

MDHAR is a flavin adenine dinucleotide (FAD) enzyme that is present as chloroplastic and cytosolic isozymes. MDHAR exhibits a high specificity for monodehydroascorbate (MDHA) as the electron acceptor, preferring NADH rather than NADPH as the electron donor. Asada (1999) studied the multi-step reduction of FAD in detail. The first step is the reduction of the enzyme-FAD to form a charge transfer complex. The reduced enzyme donates electrons successively to MDHA, producing two molecules of ascorbate via a semiquinone form [E-FAD-NADP(P)þ]. It is well established that the disproportionate by photoreduced ferredoxin (redFd) in the thylakoids is of great importance. Since redFd can reduce MDHA more effectively than NADPþ, MDHAR cannot participate in the reduction of MDHA in the thylakoidal scavenging system. Therefore, MDHAR only functions in the presence of NAD(P)H, whereas redFd not (Asada 1999). Accompanying APX, MDHAR is also located in peroxisomes and mitochondria, where it scavenges H_2O_2 (Del Río et al. 2002). Sharma and Dubey (2005) reported that the activities of enzymes involved in the regeneration of ASH, i.e., MDHAR, DHAR and GR were higher in drought stressed rice seedlings. The increased MDAR activity contributes towards chilling tolerance in tomato fruit (Stevens et al. 2008). Overexpression of MDAR in transgenic tobacco increased tolerance against salt and osmotic stress (Eltayeb et al. 2007).

Dehydroascorbate Reductase (DHAR)

DHAR regenerates ASH from the oxidized state and regulates the cellular ASH redox state which is crucial for tolerance to various abiotic stresses leading to the

production of ROS. It has also been found that DHAR overexpression also enhances plant tolerance against various abiotic stresses. In a study, under Al stress, the role of MDAR or DHAR in ASH regeneration has been studied in transgenic tobacco plants overexpressing cytosolic DHAR (DHAR-OX) or MDAR (MDAR-OX). It was found that DHAR-OX transgenic plants showed higher levels of ASH with or without Al, whereas MDAR-OX plants only showed higher ASH level in the absence of Al in comparison to WT. Significantly higher levels of ASH and APX in DHAR-OX plants showed better tolerance under Al stress but not MDAR-OX plants. It is clear that plants overexpressing DHAR showed tolerance to Al stress by maintaining high ASH level (Yin et al. 2010). Overexpression of DHAR increased salt tolerance in *Arabidopsis* (Ushimaru et al. 2006).

Glutathione S-Transferases (GST)

Glutathione transferases, also known as glutathione S-transferases, catalyse the conjugation of electrophilic xenobiotic substrates with the tripeptide glutathione (GSH; g-glu-cys-gly). Plant GSTs have diverse function in plant cells as in herbicide detoxification, hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification, regulation of apoptosis (Dixon et al. 2010). Noctor et al. (2002a) reported that GSTs probably remove the damaging cytotoxic or genotoxic compounds of DNA, RNA and proteins. Plant GSTgene families are large and highly diverse with 25 members reported in soybean, 42 in maize and 54 in *Arabidopsis* (Sappl et al. 2004). These are generally cytoplasmic proteins, but microsomal, plastidic, nuclear and apoplastic isoforms have also been reported (Frova 2003). GST covers more than 1% of soluble proteins in plant cells (Edwards et al. 2000). An increased GST activity was found in leaves and roots of Cd-exposed *Pisum sativum* plants (Dixit et al. 2001) and in roots of *Oryza sativa* and *Phragmites australis* plants (Moons 2003). Gapinska et al. (2008) noted increased GST activity in *Lycopersicon esculentum* roots under salinity stress. Considerably higher activities of GST and CAT were found in drought tolerant (M35–1) and drought sensitive (SPV-839) *Sorghum* varieties displayed efficient H_2O_2 scavenging mechanisms which were subjected to 150 mM NaCl for 72 h.

Glutathione Peroxidase (GPX)

GPXs belong to a family of diverse isozymes, used in aiding the plant cells from oxidative stress by using GSH to reduce H_2O_2, organic and lipid hydroperoxides (Noctor et al. 2002b). Millar et al. (2003) recognized a family of seven related proteins in cytosol, chloroplast, mitochondria and endoplasmic reticulum, named AtGPX1-AtGPX7 in *Arabidopsis*. Stress enlarges GPX activity in cultivars of *Cuminum annuum* plants but diminishes in roots and causes no significant change in the leaves of Cd-exposed *Pisum sativum* plants (Dixit et al. 2001). Gapinska et al. (2008) reported that 150mMNaCl stress significantly increased the GPX activity in *Lycopersicon esculentum* Mill. cv 'Perkoz' roots. Leisinger et al. (2001) reported the up-regulation of a GPX homologous gene (Gpxh gene) in *Chlamydomonas reinhardtii* following oxidative stress. It was noted that Gpxh gene showed high stimulation by the $^1O_2$ generating photosensitizers neutral red, methylene blue and

rose Bengal. When Gpxh promoter bind with the arylsulfatase reporter gene resulting activation of Gpxh genes which fight against $^1O_2$ photosensitizers (Leisinger et al. 2001). It was noted that GPX activity in transgenic *G. hirsutum* seedlings was 30–60% higher under normal conditions but was not unlike than GPX activity in WT seedlings under salt stress environment (Light et al. 2005).

3.5 Conclusion

A perusal of the diverse aspects pertaining to physical agents has helped in elucidating facts that plants have well developed enzymatic and non-enzymatic detoxifying systems continuously involved in cellular protection against ROS arising from both the environment and the cell metabolism. The effects of ionizing radiation and UV-B rays on higher plants are of interest to agriculture, horticulture, ecology and space science and recent findings have elucidated that lower doses elicit mutagenic behaviour and bring in several significant modifications. Plants are more efficient to rectify the errors in genome such as strand breaks and have much prompt cellular checkpoints and this reflects their better perpetuation than animals and this is also a probable reason for the plasticity of plants to the physical agents of low magnitude.

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Plant Responses to Induced Genotoxicity and Oxidative Stress by Chemicals

4

Abdelfattah Badr, Hanaa H. El-Shazly, and Heba I. Mohamed

Abstract

In recent decades, the use of chemicals of anthropogenic activities such as agrochemicals, industrial and environmental chemicals, and nanoparticle has been growing with great benefits for food production and human health and welfare. However, hazards imposed by different categories of chemicals on plants and the whole ecosystem have been widely reported. Plants under stress suffer a rapid and transient overproduction of reactive oxygen species (ROS) that lead to DNA damage. Two of the major impacts of chemicals, not only in plants but also in all living organisms, are genotoxicity and oxidative stress. Oxidative stress may trigger some reactions that can be involved in stimulating genotoxicity in plants by inducing DNA damage that results in a variety of impairments to cell division and chromosomes. Genotoxicity assays have been developed in the last few decades to test permanent DNA-damage indicators as cellular responses, such as cell division abnormalities and chromosomal aberrations, sister chromatid exchange, nucleus malformation, and micronucleus formation. Recently molecular markers have been also applied as genotoxicity indications. In this chapter, plant-based genotoxicity assays are outlined and the oxidative stress and genotoxicity of agrochemicals, chemical mutagens, industrial and heavy metals, nanoparticles, and nanomaterials, and the response of plants to mitigate the stress imposed by the oxidative stress and genotoxicity.

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Keywords

Genotoxicity · Oxidative stress · DNA damage · Chemicals · Plants

Abbreviations

5-BU	5-Bromouracil
AgNPs	Silver nanoparticles
BrdU	Bromo-2`-Deoxyuridine
DMS	Dimethyl sulfate
DNA	Deoxyribonucleic acid
EMS	Ethyl methanesulfonate
EPA	Environmental Protection Agency
ISSRs	Inter-Simple Sequence Repeats
MMS	Methyl methanesulfonate
PCR	Polymerase chain reaction
RAPD	Random Amplified Polymorphic DNA
ROS	Reactive Oxygen Species
SCE	Sister Chromatid Exchange
SSRs	Simple Sequence Repeats
UV Radiation	Ultraviolet Radiation

4.1 Introduction

The benefits of chemical substances contribute significantly to human welfare. The consumption of chemicals is essential for improving food production as agrochemicals and for the treatment of diseases as pharmaceutical drugs and as components of different industrial products, etc. However, the environmental and human health toxicity of chemicals is a major concern due to their implications is not sufficiently taken into full account (Udeigwe et al. 2015; EPA Report 2018). The application of chemicals also induced the production of reactive oxygen species (ROS) commonly known as free radicals. ROS can oxidize most types of macromolecules, including lipids, proteins, DNA, and RNA, and cause cellular dysfunctions (Ahmad et al. 2010; Apel and Hirt 2004). The major DNA is a major target of ROS and may undergo oxidation to produce a diverse range of genotoxic endpoints. The DNA damage by ROS is an important causative agent of mutagenesis, and consequently genome instability, resulting in retardation of plant growth and deterioration of crop productivity. Markkanen et al. (2011) described various forms of DNA damaging agents among which 7, 8-dihydro-8-oxoguanine (8-oxo-G) is the most predominant one. This may be due to its prevalence and high mutagenic potential, as one of the most abundant mutagenic oxidative DNA lesions which may lead to genotoxicity.

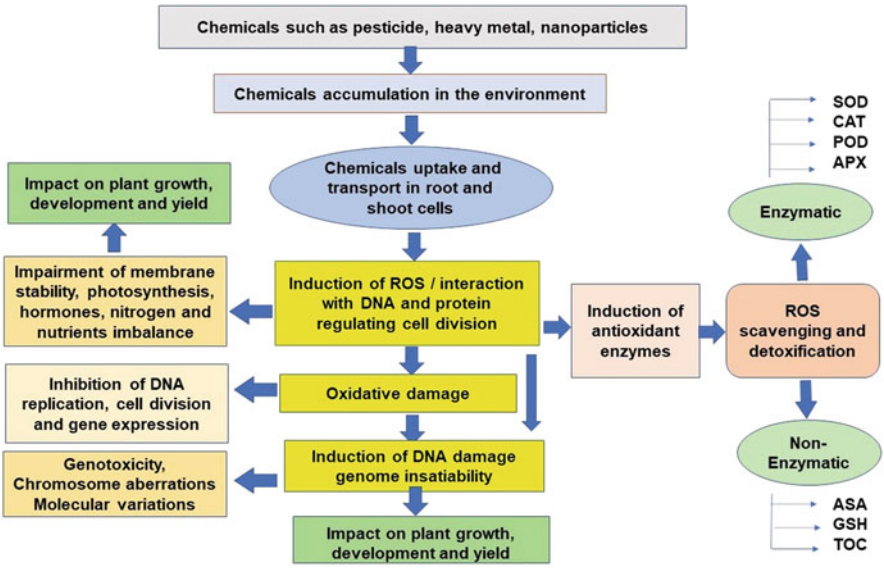

Fig. 4.1 A simplified diagram illustrating the induction of ROS by chemicals as a cause of oxidative stress and genotoxicity in plants and their impact on plant growth

The term genotoxicity is often used to mean mutagenicity, however, all mutagens are genotoxic, but not all genotoxic substances are mutagenic (Grant 1999). Several assays have been developed to assess the genotoxic impacts and their link to changes in plant growth and development. On the other hand, cytotoxicity expressed as disturbance in cell division and chromosomes are considered parts of genotoxicity induced by chemical compounds. The *Allium cepa* has been suggested as a model organism by various environmental organizations but *Vicia faba* assay based on chromosomal and nuclear aberrations induced by various categories of chemicals has been widely used as a plant-based genotoxicity test (Iqbal 2016).

Several lines of evidence have established that a variety of chemicals generates oxidative stress by production of ROS, and the genotoxicity via the ROS mediated DNA damage and genome instability (Fig. 4.1). The ROS attack DNA and induce changes to DNA bases, leading to additional DNA damage, such as single and double-strand breaks in the DNA molecule, which may not be repaired (Roy 2014). DNA double-strand breaks (DSBs) are one of the major forms of DNA damage under ROS stress (Cannan and Pederson 2015) resulting in genome instability and causes DNA fragmentation. Donglin et al. (2014) illustrated that concomitant occurrence of genotoxicity and the generation of ROS are attenuated in the presence of Vitamin C, as a scavenger of cellular of ROS and may contribute to genotoxicity of nitrobenzene. Roy (2014) reported that under heavy metal stress, seed germination is also reduced under oxidative stress due to the generation of ROS. In addition, ROS causes various types of DNA damage such as single and double-strand ruptures and chromosome aberrations (Morales et al. 2016), and chromosomal aberrations

occur preferentially within heterochromatic regions composed mainly of repetitive sequences (Schubert et al. 2004). Fang et al. (2014) showed that Cr (III) interacts with DNA to form a covalent bond with the phosphate backbone and interacts with the DNA base to induce DNA lesion causing cleavage and DNA single/double-strand breakage. These DNA lesions are induced by heavy metal-induced ROS activity which affects genomic stability. The ROS also influence the expression of a number of genes and therefore control many processes in plant life particularly growth and development, cell cycle, and programmed cell death (PCD), in addition to abiotic stress and pathogen responses, and systemic signaling (Gill and Tuteja 2010).

The activation of ROS-scavenging helps to decrease oxidative stress in plants. A large panel of antioxidant molecules and enzymatic pathways control intra-cellular ROS levels in plants (Apel and Hirt 2004). As summarized by Gill and Tuteja (2010), plants possess very efficient enzymatic antioxidant defense systems that work in concert to control the oxidation and protect plant cells from oxidative damage by scavenging of ROS. The enzymes superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione reductase, GR; glutathione peroxidase, GPX; guaiacol peroxidase, GOPX and glutathione-S- transferase, GST. Besides, abundant small molecule antioxidants, for example, glutathione, L-ascorbic acid (Vitamin C, Vc), a-tocopherol (Vitamin E), and carotenoids protect cells from injury by scavenging ROS (Donglin et al. 2014). The roles of enzymatic and non-enzymatic antioxidants in plants during abiotic stress were studied by Ahmad et al. (2010). The present chapter deals with the genotoxicity and oxidative stress caused by various types of chemicals and their potential impacts. Plant-based genotoxicity assays and oxidative stress produced by major categories of chemicals are described and the ways and mechanisms used by plants to mitigate the oxidative stress are described.

4.2 Plant Genotoxicity Assays

As pointed out above, plants exposed to oxidative stress suffer inhibition of cell division and DNA damage resulting in mitotic abnormalities, chromosomal aberrations, and molecular variations. In fact, the DSBs are the critical lesions for the generation of chromosome structural changes by erroneous reciprocal recombination repair. Usually, two DSBs have to interact in cis or trans positions to form a chromosomal aberration (Schubert et al. 2004). Plants have been recognized as excellent indicators of the genotoxicity of chemicals and other genotoxic agents for decades. The advantages of using plant assays for genotoxicity testing are the small number of chromosomes which offer excellent cytogenetic makers with a wide range of genetic endpoints that have been recognized as cytotoxicity and genotoxicity markers. Plant-based assays have been considered reliable bioassays for the testing of genotoxins. The first assay was based on the impairment of cell division and the production of chromosomal aberrations using the *Allium cepa* root

tip cells (Grant 1982, 1999). However, other plant genotoxicity assays have been developed and based on evident and stable changes in the nucleus and chromosome and also assays based on molecular stable variations under genotoxins that could be indicated by molecular markers. Here we briefly outline the principles of the most common assays.

4.2.1 Mitotic Abnormalities and Chromosomal Aberrations

The genotoxicity by chemicals in plants has been manifested as a variety of chromosomal anomalies including mitotic abnormalities due to an action on the spindle apparatus (Deysson 1968) and different types of chromosomal aberrations comprised of chromosome breaks and bridges that were attributed to chromosome breakage and reunion, and fragmentation which is multiple breaks resulting in a loss of chromosome integrity (Grant 1982; Badr 1987) but was also attributed to the stickiness of chromosomes which makes their separation to the cell poles incomplete and thus they remain connected by bridges (Badr 1988). Stickiness was also attributed to changes in nonhistone proteins and DNA induced during chromosome condensation (Gaulden 1987). Jabee and Ansari (2005) suggested that chromosomal breakage may cause stickiness among the chromosomes. Sticky chromosomes indicate a highly toxic effect attributed to the degradation or depolymerization of chromosomal DNA (Badr et al. 2014).

Induction of chromosomal bridges at ana-telophase is firm evidence of clastogenicity which is chromosomal evidence of mutagenicity (Prasad and Kazimierz 2002). Further resolution of such bridges would produce broken ends in the chromosomes that perpetuate bridge formation in ana-telophases of subsequent cell cycles indicating genomic stress (Borboa and De la Torre 1996; Grant 1999). Chromosome bridges are formed as a result of dicentric chromosome formation involving chromosomal breakage (Dizdari and Kopliku 2013). Some plants have been used for scoring the mitotic abnormalities and chromosomal aberrations particularly *Vicia faba* (Kihlman 1975), *Hordeum vulgare* (Constantin and Nilan 1982), and *Crepis capillaris* (Grant and Owens 1998). *Allium cepa* assay is the most widely applied assay and was approved by the United States Environmental Protection Agency (Grant 1999). *Allium* test has been revised by Leme and Marin-Morales (2009) and Bonciu et al. (2018).

4.2.2 Micronucleus Test

The micronucleus test was first developed in *Tradescantia* to indicate genotoxic action through the induction of micronuclei in meiotic or mitotic cells and pink mutations in stamen hairs by Ma (1983). Nevertheless, in many cases, such as environmental pollutants, using *Allium* and *Vicia* root-micronucleus assays for genotoxic assays is sufficient for genotoxicity testing (Grant et al. 1992; Cotelle et al. 1999; Liman et al. 2019, 2020). The micronucleus bioassays like other

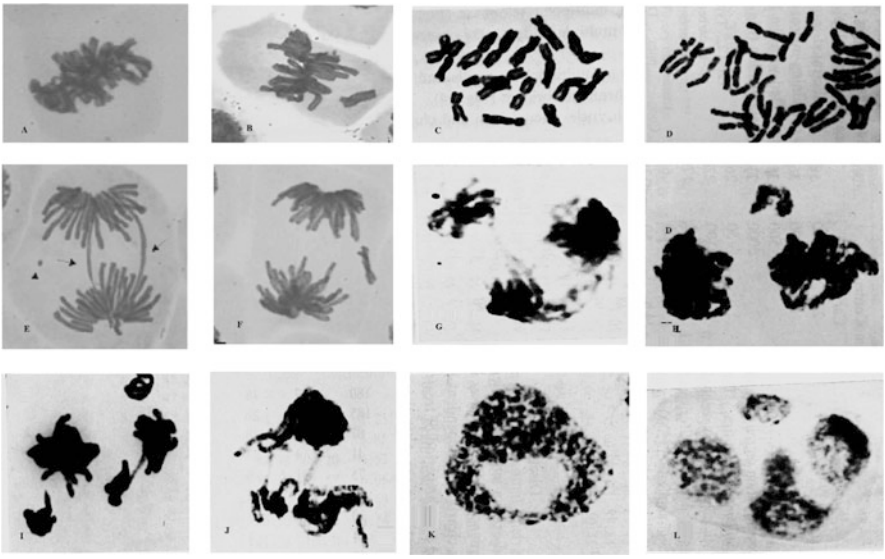

Fig. 4.2 Photographs illustrating chromosomal aberrations and micronucleus induced by the aflatoxin F1 in the root meristem cells of *Allium cepa*. A = chromosome stickiness at metaphase, B = Unoriented chromosomes at metaphase, c = c-metaphase, D = Poyploid cell, E = Anaphase bridges and fragment, F = Lagging chromosome at anaphase, G = Multipolar telophase and bridges, H = Chromosome lagging at telophase, I = Multipolar disturbed telophase, J = Bridges and unoriented chromosome at telophase, K = Nuclear vacuole, L = Multinuclei and micronucleus (Images are taken from AB and HHE collection and publications)

plant-based and non-plant bioassays have been used for more than 40 years in environmental monitoring and are highly sensitive to different groups of mutagens, heavy metals, radionuclides, air pollutants, and certain agrochemicals. Some of these toxins cause negative or only weak effects in bacterial assays and mammalian cells (Mišík et al. 2020). The advantages of the micronucleus test are its simplicity, high sensitivity, and low cost. For these reasons, they are useful components of test batteries for the detection of mutagens in complex environmental mixtures. Examples of the chromosomal aberrations and micronucleus as indicators of genotoxicity are shown in Fig. 4.2. A *Vicia faba* assay based on chromosomal and nuclear aberrations induced by pesticides, metallic compounds, complex mixtures, petroleum derivatives, toxins, nanoparticles, and industrial effluents, was reviewed by Iqbal (2016). A list of many of the observed cytogenetic and mutagenic effects in *Vicia faba* root tip cells as a result of exposure to various classes of environmental concerns are given in Iqbal (2016).

4.2.3 Sister Chromatid Exchange

Another cytogenetic plant assay for testing genotoxicity that provides reliable biomarkers includes the sister chromatid exchange (SCE) test which is well-

known for detecting DNA damage. A model for the production of sister chromatid exchanges was presented by Painter (1980), based on the idea that sister chromatid exchange would be initiated when daughter strands of a duplicated DNA cluster recombine with the parental strands of the partially replicated cluster. The frequency of SCEs per chromosome set increases after treatment with genotoxic/mutagenic agents. Plant species used for SCE test should have a small number of chromosomes and relatively large size such as *Vicia faba* (Cortès and Andersson 1987: Yi and Si 2007), *Allium cepa* (Cortès et al. 1987), and *Crepis capillaris* (Dimitrov 1987). It was not clear whether 5-bromo-2`-deoxyuridine (BrdU) causes DNA damage or influence the DNA repairs after mutagenic treatment (Natarajan et al. 1986). Among the mutagens, BrdU strongly enhances the frequency of the SCEs that were simultaneously induced by UV radiation (Wojcik et al. 2003). The mercuric ions appear to form covalent bonds with DNA and reduce mitotic index and increased frequency of chromosomal aberrations and sister chromatid exchange and in a dose-dependent manner (Beauford et al. 2006).

The SCE assay is based on DNA segregation between two sister chromatids of a duplicating chromosome, which occurs in DNA replication (Maluszynska and Juchimiuk 2005). Buck et al. (2008) used the base analogue 5-ethynyl-2-′-deoxyuridine EdU) incorporation with the click chemical reaction, as alternative to using BrdU antibodies, to examine the DNA replication pattern and to differentiate the sister chromatids and Schubert et al. (2016) incorporated EdU during replication to detect SCE in the holocentric chromosomes of rye. Recently Kwasniewska and Bara (2020) developed a EdU-Based step-by-step method for the detection of sister chromatid exchanges using of Alexa Fluor 488 for application in plant genotoxicity assessment using the two mutagens, maleic acid hydrazide and gamma rays (Fig. 4.3).

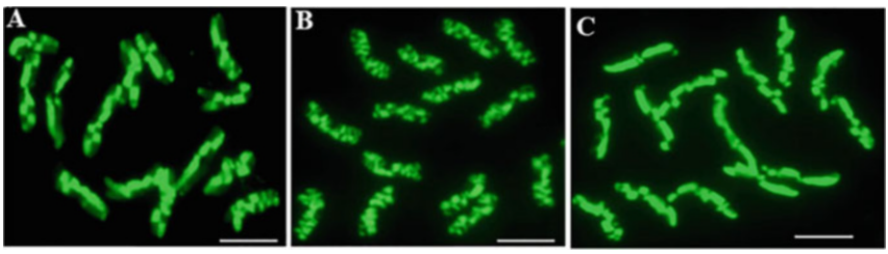

Fig. 4.3 Metaphase chromosomes in root meristematic cells of barley (2n = 14) showing SCE using EdU: A = Control, B = After seed treatment with MH, C = After seed irradiation with Gamma ray. Reconstructed from Fig. 1 in Kwasniewska and Bara. Front. Plant Sci. 11:1146, 24 July 2020. Bar represents 5 μm

4.2.4 Comet Assay

The comet test was introduced, as a new genotoxicity test for DNA strand breaks in *Vicia faba* root cells (Koppen and Verschaeve 1996). The method was later developed for the evaluation of DNA damages and DNA repair capacity at a single-cell level using cell suspensions embedded in agarose on a microscope slide and exposed to lysis by exposure to detergent and high salt solutions (Collins et al. 2008). Using electrophoretic, DNA fragments migrate toward the anode, forming a typical "comet tail." The amount of DNA in the tail represents DNA strand breaks relative to the DNA remaining in the head (Hovhannisyan 2010, Collins et al. 2008). The comet assay is applied to some toxicological in a few plant species for review (Gichner et al. 2009; Ventura et al. 2013). The use of comet assay as a standard approach for studying the genotoxic effects has been also extended to studies on different stress conditions in plants and information provided by this assay may be combined with other DNA-damage indicators, such as oxidative stress, impaired cell division or cell death (Santos et al. 2015; Lanier et al. 2015). Recent protocol improvements as described in detail by Pourrut et al. (2015) and recommendations for technical aspects and assay parameters given in (Koppen et al. 2015) open up the prospect of its increased use to study genotoxicity. Figure 4.4 illustrates images representing the classes of visual scoring comet damage levels as indicated by the amount of DNA in the comet tail in the roots of *Allium cepa* by different concentrations od the herbicdes malathione (Srivastava and Singh 2020).

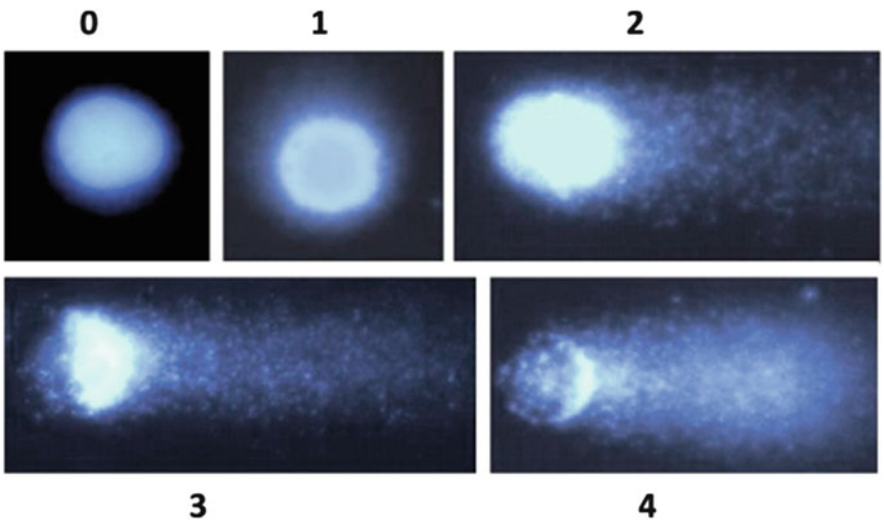

Fig. 4.4 Images representing the classes of visual scoring comet levels as indicated by the amount of DNA in the comet tail. A = undamaged cells (tail DNA% < 5); B = low damaged cells (tail DNA% 5–25); C = moderately damaged cells (tail DNA% 26–45); D = highly damaged cells (tail DNA% 46–80); E = extremely damaged cells (tail DNA% > 80). The figure is constructed by AB from photos uploaded on the web and is freely available in Google search

4.2.5 Molecular Markers

Considerable evidence indicated that genotoxicity in plants following exposure to chemical genotoxins can be evaluated by molecular markers. The early attempts were made using the random amplified polymorphic DNA (RAPD) by Atienzar and Jha (2004, 2006) who considered the gain of new bands in the RAPD fingerprinting profiles is associated with the generation of new alleles (loci) as in Fig. 4.5. The changes in the inter-simple sequence repeats (ISSRs), genotoxicity was observed as bands loss, which could be associated with unrepaired DNA damage hindering the amplification of the sampled sites as well as point mutations at the annealing site (Sukumaran and Grant 2013). ISSRs profiles of *Plantago* exposed to aluminum treatments were also considered as gain and/or loss of bands compared with the controls (Correia et al. 2014). A locus related to glutamine metabolism was linked to gain of ISSR marker in *Pisum sativum* cells treated with high concentrations of lead (Rodriguez et al. 2013). However, (Kumar et al. (2020) found that fingerprinting using ISSR markers showed no polymorphism, associated with phyto-stimulatory effect in the seedlings of *Psophocarpus tetragonolobus* after priming the seeds with fabricated silver nanoparticles (AgNPs). The simple sequence repeats (SSRs) fingerprinting revealed more gain of bands of DNA from cells treated with procymidone and iprodione (Bernardes et al. 2015). This was attributed to the nature of SSRs as tandem repeats regions likely to have a higher rate of mutation due to unrepaired double-strand DNA mismatches (Yauk 1998).

4.2.6 Chlorophyll Mutations

In addition to genotoxicity assays, some point mutations have been applied to detect genotoxicity. The most widely reported of these mutations is the chlorophyll mutations in leaves. Chlorophyll mutations have been regarded as a common indicator for evaluating the genetic effects of mutagens since it results from a

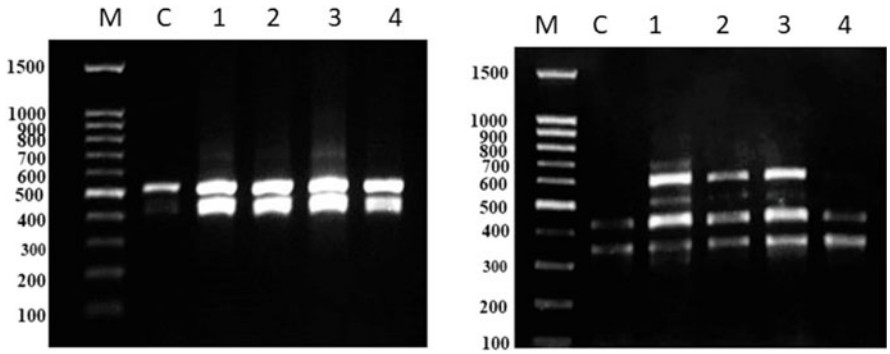

Fig. 4.5 ISSR images illustrating gain of loci in pea ISSR profile following exposure to 20, 40, 80, and 160 mg/L of AgNPs (Images donated by Dr. Mai Labib, Kafr Elsheikh University)

Fig. 4.6 Examples of chlorophyll mutations in: A = Sesame, B = Pepper, C = Barley. The figure is constructed by AB from photos uploaded on the web and freely available in Google search

point mutation indicating a loss of one nucleotide in the DNA molecule (see Sect. 4.3). Zhao et al. (2020) reviewed the molecular regulation mechanisms of plant leaf color mutations Fig. 4.6. Chlorophyll mutation is scored in M_2 generation and is widely used in mutation breeding for evaluating the mutagenic induced genetic traits by mutagens such as Gamma rays and treatments of chemical mutagens like ethyl methanesulfonate (EMS) and dimethyl sulfate (DMS). Induced mutations are useful to widen the genetic diversity of self-pollinated plants to create new starting material for breeding (Shu 2009; Kumar et al. 2009). Several authors have reported incidences of different types of chlorophyll mutations in M_2 generation following plant treatments with mutagenic agents (Kumar et al. 2009; Wani 2017). However, the chlorophyll mutation is often used in mutation breeding while its use in genotoxicity testing is not common.

4.3 Chemical Mutagens

Chemical mutagens work mostly by inducing point mutations. Point mutations occur when a single base pair of a gene is changed or deleted. Base changes are classified as transitions or transversions. Transitions occur when a purine is converted to a purine base (adenine to guanine or vice versa) or a pyrimidine base is converted to a pyrimidine (thymine to cytosine or vice versa). A transversion results when a purine is converted to a pyrimidine or a pyrimidine is converted to a purine. Three major classes of chemical mutagens are routinely used. These are base analogs and alkylating agents and intercalating reagents. Each class of chemical mutagens has specific effects that can lead to transitions, transversions or deletions on nucleotide bases (Griffiths et al. 2012).

4.3.1 Base Analogs

Base analogs are molecules which have a very similar structure to one of the four nitrogenous bases in the DNA (adenine, guanine, cytosine, or thymine). The most

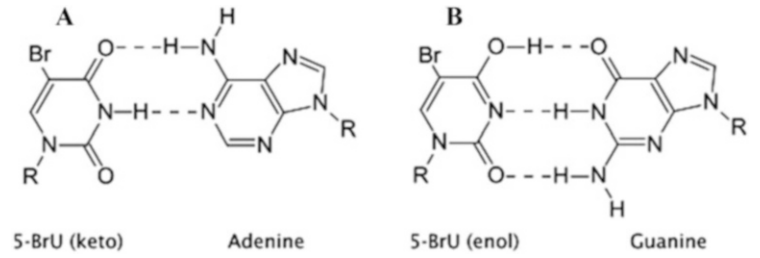

Fig. 4.7 The keto form of 5-BU pairs with adenine (**a**) but 5-BU can change to either the enol form or an ionized form that pairs in vivo with guanine (**b**). Image taken from unknown source on the web and is also found in many genetics textbooks

common base analogue is 5-bromouracil (5-BU) which is a thymine that has bromine at the C-5 position in place of the CH_3 group found in thymine. This change does not affect the atoms that take part in hydrogen bonding in base pairing in the DNA double helix, but the presence of the bromine alters the distribution of electrons in the base. The normal structure (the keto form) of 5-BU pairs with adenine but 5-BU can frequently change to either the enol form or an ionized form; the latter pairs with guanine (Fig. 4.7). Since the 1990s, a variety of compounds known as universal bases, including hypoxanthine, nitroazoles, isocarbostyril analogues, azole carboxamides, and aromatic triazole analogues have been developed and employed in degenerate PCR primers, microarray probes, ligation, and triplexes (Liang et al. 2013). Nedderman et al. (1993) denoted methoxyamine, N4-methoxycytidine and its 2′-deoxyribo analogue as transition mutagens and base analogue that can pair effectively with both adenine and guanine.

4.3.2 Alkylating Agents

Alkylating agents are reactive chemical compounds that insert themselves by binding to chemical groups (phosphate, amino, sulfhydryl, hydroxyl, and imidazole groups) found in nucleic acids bringing about changes in the DNA and RNA of cells. These agents distort the DNA double helix and chemically change DNA bases. Alkylating agents are types of chemical mutagens which exert their action by one of the following methods (Griffiths et al. 2012; Badr 2015).

4.3.2.1 Alkylation

Addition of alkyl group (methyl CH_3- or ethyl CH_3-CH_2-). This modifies guanine to 6-ethyl guanine that pairs with thymine (Fig. 4.8). Examples of alkylators include ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), diethyl sulfate (DES), and nitrosoguanidine (NG). These chemicals react directly with modified DNA bases and require DNA synthesis in order to be fixed. They are very commonly used because they are powerful mutagens in nearly every biological system.

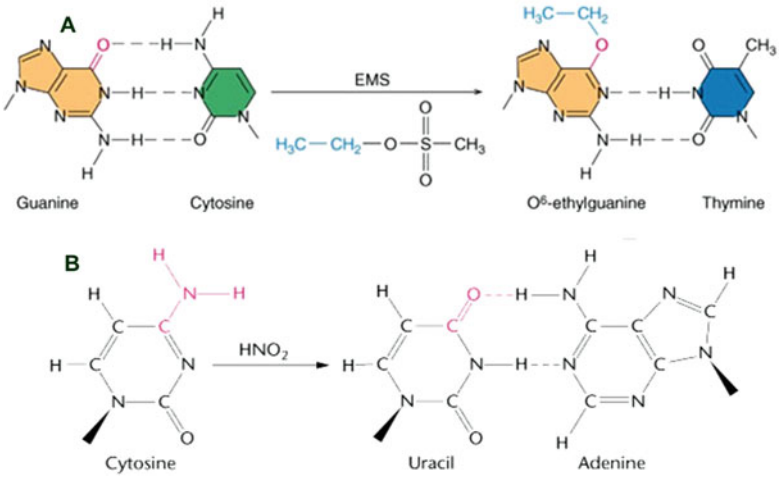

Fig. 4.8 Chemical mutagenesis by alkylation using EMS (**a**), the addition of alkyl group (methyl CH_3) by EMS modifies guanine to 6-ethyl guanine that pairs with thymine and by deamination using NHO_2 (**b**), loss of NH_2 coverts cytosine to uracil that pairs with adenine. The figure is constructed from Biology Libre Texts by Stefanie West Leacock, University of Arkansas at Little Rock

4.3.2.2 Deamination

Conversion of amino to keto group. Deamination converts adenine to hypoxanthine by loss of NH_2 that pairs with cytosine and cytosine to uracil that pairs with Adenine. This conversion is mostly done by nitrous acid (HNO_2) which alters a DNA base directly to a miscoding form and does not require subsequent DNA synthesis for its effect.

4.3.2.3 Depurination

A loss of a purine base from the 2-deoxyribose by a chemical reaction in which the β-N-glycosidic bond is hydrolytically cleaved releasing adenine or guanine from the deoxyribose. Depyrimidination of cytosine and thymine residues occur at a much slower rate than depurination. Despite the high rate of loss purines, they are generally remediated easily by base excision repair (BER) of DNA mispairing and may not lead to mutations.

4.3.3 Intercalating Reagents

These are a class of chemical mutagens, called intercalating reagents (ICRs) mutagenize by intercalating between adjacent DNA bases, perhaps making synthesis/repair systems see them as normal base (Lerman 1961). They are often regarded as base analogues and wedge into double helix of the DNA. Intercalated analogue may be read as extra base in the DNA strands. Examples include acridine dyes and

ethidium bromide. These chemicals induce frameshift mutations by adding or removing one nucleotide from a DNA strand and require DNA synthesis to cause mutations (Griffiths et al. 2012; Badr 2015).

4.3.4 Other Chemical Mutagens

Many other chemicals are known as potent mutagens, the most well-known of these are Sodium azide (NaN_3) which is a common bactericide, pesticide, and industrial nitrogen gas generator. Gruszka et al. (2012) reviewed the highly mutagenic potential of NaN_3 in several organisms including plants and animals and of course microorganisms.

4.3.5 Genotoxicity Produced by Chemical Mutagens

The most widely used chemical mutagens include EMS, MMS, DES, NG, hydroxyl amine, nitrous acid, hydrazine sulfate, maleic hydrazide, and many others. The mechanisms of these compounds to produce mutations are summarized above. These and other mutagens are widely used in plant species of interest with an objective mutation. Such mutations are used to elicit different types of genotoxicity in plants to create genetic variation. The types of genotoxicity by various mutagens have been documented in countless publications in plans by using one or more of the genotoxicity assays described in Sect. 4.2. The types of genotoxicity end points produced by these mutagens are similar to genotoxicity types produced by other classes of the chemicals described in Sect. 4.4 and only few examples are mentioned here.

The mutagenic effectiveness and efficiency of hydrazine sulfate was demonstrated by Jabee and Ansari (2005) by cytomorphological mutations in *Cicer arietinum* L. Jabee et al. (2008) reported that maleic hydrazide produced clastogenic chromosomal abnormalities including fragments, stickiness of chromosomes, and anaphase bridges at mitosis and univalents, multivalents at meiosis, and reduced pollen sterility. Various types of mitotic chromosomal aberrations, including fragments, stickiness, precocious separation, c-metaphase, ring chromosomes, unequal separation, laggards, bridges, micronuclei, disturbed anaphase (Khan et al. 2009; Bhat et al. 2007). Different treatments with the two mutagens MMS and diethyl sulphate (DES) induce meiotic aberrations such as univalent, multivalent, stickiness, bridge, laggards in the M_1 plants of *Capsicum annum* L. As the concentrations of both mutagens increased, reduction in chiasma frequency and pollen fertility was observed. MMS treatments proved to be more effective in inducing meiotic aberrations as compared to DES (Gulfishan et al. 2012). Bhat and Wani (2017) reported chromosomal aberrations at meiosis and reduction in seed germination, pollen fertility, and seedling survival of M_1 plants of *Vicia faba*.

4.4 Other Chemicals Inducing Oxidative Stress and Genotoxicity

Higher plants are exposed to chemicals of various types. The most common are the agricultural chemicals, such as pesticides and fertilizers. In addition to heavy metals, chemical mutagens, nanoparticles, natural products phytocompounds, aflatoxins produced by some fungi, in addition to industrial chemicals. Here we present major types of genotoxicity induced by selected examples of the categories of the above-mentioned chemical compounds.

4.4.1 Agrochemicals

Agrochemicals are chemicals used in agriculture and associated activities and include pesticides, fertilizers, growth regulators, soil stimulants, animal feed additives, etc. A pesticide is a substance used for repelling, destroying, or avoiding pests. Pesticides are categorized generally as herbicides, fungicides, and insecticides depending on the target killed. Increased food demand due to population human growth has forced people to use pesticides to increase crop production (Jayakumar et al. 2019). Pesticides are used to protect crops to mitigate crop damage, both on the ground before harvest and for storage after harvest. However, within hours or days of application, pesticides may be volatilized but a few studies identified pesticide residues in the air over the last few years (Raherison et al. 2018). However, agrochemical may leach to surface waters or shallow groundwater and soil leaving residues of agrochemicals as potential hazards to plants and the ecosystem for a long time.

4.4.1.1 Oxidative Stress by Agrochemicals

The normal oxygen (O_2) itself is a harmless molecule and is unlikely to participate in reactions with organic molecules unless it is activated (Apel and Hirt 2004). According to Sharma et al. (2012), activation of O_2 occurs by two different mechanisms. Stepwise monovalent reduction of O_2 leads to formation of $O_2 \bullet -$, H_2O_2, and •OH, whereas energy transfer to O_2 leads to the formation of $1O_2$. $O_2 \bullet -$ is easily dismutated to H_2O_2 either nonenzymatically or by superoxide dismutase (SOD) catalyzed reaction to H_2O_2. H_2O_2 is converted to H_2O by catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX). Free radicals can appear in plant cells resulting in ROS formation due to an inevitable leakage of electrons onto O2 molecules during electron transport in chloroplasts, mitochondria, and plasma membranes or as a by-product of different metabolic pathways (Sharma et al. 2012). ROS are a major source of DNA damage and can cause oxidative damages to the DNA by a number deoxyribose oxidation, strand breakage, removal of nucleotides and modifications in the organic bases of the nucleotides, and DNA-protein crosslinks (Imlay and Linn 1988). Further, changes in the nucleotides of one strand can result in the mismatches with the nucleotides in the other strand,

yielding subsequent mutations. Enhanced DNA degradation has been observed in plants exposed to various environmental stresses such as salinity (Liu et al. 2000).

Oxidative DNA damage caused by different types of stresses induce a number of primary actions that may lead to different types of genotoxicity including alkylation or oxidation of nucleotide bases (e.g., 8-oxo-guanine), as well as hydrolysis of the N-glycosidic bond of bases, both resulting in an apurinic/apyrimidinic site (Sharma et al. 2012). Plants exposed to pesticides often suffer from oxidative stress imposed by the production of ROS which exerts stress on many biochemical and physiological activities. This oxidative stress contributes to chlorophyll and protein degradation eventually leading to a reduction in the photosynthetic effectiveness of plants (Sharma et al. 2015). Oxidative stress via ROS generation is due to growing pesticidal levels during exposure of plants during germination, and the early seedling stage is often associated with low mitotic activity and inhibited seedling growth and chromosome aberrations (Donglin et al. 2014). More recently pesticide-mediated oxidative was found to induce genotoxicity and disrupted chromatin structure in *Trigonella foenum-graecum* seedlings (Mahapatra et al. (2019).

4.4.1.2 Genotoxicity by Agrochemicals

Genotoxicity imposed by agricultural chemicals that have been reported as mitotic abnormalities and chromosomal aberrations in plants were well documented before the association of genotoxicity with oxidative stress was understood. It was attributed to blocking of the cycle during interphase as a result of a prolonged G2 period or to the inhibition of DNA synthesis (Badr 1987; Grant 1994). Studies using *Allium cepa* and *Vicia faba* assays showed chromosomal aberrations due to chromosome breaks by to excessive pesticide use (Badr et al. 2013; Mesi and Kopliku 2013). Some pesticides that cause chromosomal abnormalities are associated with their ability to cause mutations and are considered to be consistent evidence to test the agrochemicals' mutagenic potential (Larramendy et al. 2014).

Gadeva and Dimitrov (2008) observed complete destruction of the mitotic spindle, resulting in C-mitoses as well as in numerical aberrations of chromosomes as cytotoxic and genotoxic effects of three widely used pesticides (Rubigan, Omite, and Rovral) in root meristem cells of *Crepis capillaris*. Alvarez et al. (2011) reported genotoxicity by sulcotrione pesticide on *Allium cepa* root meristem and Goujon et al. (2014) evaluated the genetic damage induced by glyphosate isopropylamine salt using *Tradescantia* bioassays. Excessive use of pesticides also leads to impairment of the important plant processes at physiological, biochemical, and cellular levels including inhibition of DNA replication, gene expression, and cell division (Shakir et al. 2016). Mitotic activity retardation, chromosomal, and nuclear aberrations have been supplemented with molecular analyses to demonstrate the toxicological effects of commercial formulations of fungicides based on procymidone and iprodione in seedlings and root tip cells of *Allium cepa* (Bernardes et al. 2019). Küçük and Liman (2018) reported on the cytogenetic and genotoxic effects of 2-Chlorophenol (2-CP), a class of chlorinated organic pollutants used as intermediate in the synthesis of chlorinated congeners, certain dyes, preservatives, herbicides, fungicides, and plastics in *Allium cepa* root meristem cells. Chromosomal abnormalities and DNA

damage indicated by comet assays were also observed. The genotoxic activity of the active compound was also demonstrated by the detection of changes in SSR and ISSR fingerprinting profile in the treated cells compared to the negative control.

4.4.2 Heavy Metals

Due to increased anthropogenic activities, rapid industrialization, heavy metal elements have increasingly released into the environment and have become a major environmental hazard. Most of the heavy metals are found in the soil. In particular inorganic and organic fertilizers, sewage sludge, pesticides, and fungicides are the most important sources of heavy metals in agricultural soil. In the industrial, heavy metals are extracted at high temperatures, such as smelling and casting, in particulate and steam form into the atmosphere (Wuana and Okieimen 2011). In addition, heavy metal elements may be released as effluents from industrial sources, such as plastics processing, textiles, microelectronics, wood storage, and paper processing. Many of these chemicals may be leached to rivers and lakes (Tchounwou et al. 2012).

4.4.2.1 Oxidative Stress by Heavy Metals

In plants exposed to heavy metals, ROS are also generated and cause oxidative harm to the macromolecules and photosynthesis of plant cells. Phytotoxicity of excessive dosages of heavy metals may result from the alteration of various cell/molecular physiological processes by inactivation of enzymes, the blockage of functional metabolic molecules, the displacement or substitution of the basic components and elements, the disruption of membrane integrity and reactive oxygen or nitrogen species, such as photosynthesis, tricarboxylic acid cycles, and Calvin cycles (Singh et al. 2020). Moreover, heavy metal toxicity can significantly affect plant health in extreme conditions, eventually leading to cell death (Sytar et al. 2013). Oxidative stress caused by chromium also includes lipid peroxidation, damaging cell membrane, chloroplast ultrastructure, pigments, and ultimately interrupting metabolism, resulting in extreme delays in plant growth and development (Wakeel et al. 2020). Elevated concentration of Cu, Hg_2C, Ni and various heavy metals induces lipid membrane peroxidation and disturbance of the mitochondrial function and cellular metabolism in plants by the generation of ROS triggered by oxidative stress (El-Beltagi et al. 2020; Singh et al. 2020; Khan et al. 2020; Natasha and Khalid 2020; Moustafa-Farag et al. 2020).

4.4.2.2 Genotoxicity by Heavy Metals

An early evaluation of genotoxicity using the SCE assay was done for heavy metal salts and compared to the mutagen maleic hydrazide, and the herbicide paraquat for comparison (Panda et al. 1996). Al-Qurainy (2010) used the Inter-Simple Sequence Repeats (ISSRs) analysis to detect the genotoxicity of the heavy metals Cd^{2+}, Pb^{2+}, Zn^{2+} in *Eruca sativa* and observed genetic variability between seedlings exposed to different heavy metal levels. Comet assay studies in Arabidopsis showed that

mechanism of Boron toxicity involves double stranded breaks (DSBs) which blocks replication and Condensin II Alleviates DNA Damage (Sakamoto et al. 2011). In the RAPD profiles of *Urtica dioica* plants, nuclear DNA damage caused by heavy metals was detected (Gjorgieva et al. 2013).

The high levels of heavy metals in soils caused inhibition in germination rates of *Cicer* plants with impaired radicle existence and increased levels of chromosomal abnormalities like bridges, stickiness, and fragmentation of chromosomes (Siddiqui 2015). Monteiro et al. (2012) also indicated that high levels of Cd could lead to the production of Cd-DNA adducts leading to DNA–DNA/DNA–protein connections or the formation of longer DNA fragments or the impairment of mechanisms of DNA repair. In addition, the comet assay used to identify the genotoxicity of 3-mercaptopropanoic (MPA) coated CdSe/ZnS quantum dots (MPA-CdSe/ZnS QD) in *Medicago sativa* cells in suspension culture. The number of DNA single and double-strand breaks increased with increasing MPA-CdSe/ZnS QD concentrations (Santos et al. 2013).

4.4.3 Nanoparticles and Nanomaterials

Particles having characteristic dimensions of between 1 and 100 nm and having properties that do not share the same chemical composition as non-nanoscale particles may be classified as a nanoparticle (Foss Hansen et al. 2007). In addition, Auffan et al. (2009) argued that nanoparticles typically <30 nm would have interfacial properties with different dissolution, oxidation, adsorption/desorption, electron transfer, redox cycles, Fenton reactions, and potential mechanisms of toxicity. Many metal nanoparticles are currently used in various economic sectors for multiple applications. Environmental nano-TiO2 levels are rising steadily and rapidly, along with increasing research on their possible risks (Grande and Tucci 2016). Meanwhile, cerium oxide nanoparticles (CeO2)-based nanomaterials have a high interest in the use of catalysts, UV radiation protective agents, and polishing agents in environmental treatment, mechanical polishing, sensing and catalysts, and biomedicine but CeO2 release to the environment is an issue of extreme importance (Andreescu et al. 2014).

Nano zinc oxide (ZnONP) is one of the main nano-industrial applications used widely in cosmetical, textile, manufacturing, ceramics as well as in the rubber and wastewater processing industries (Ghodake et al. 2011). Copper oxide nanoparticles (CuONP) are used extensively in solar cells and in lithium-ion batteries, grained oils, polymers, inks/ceramic pigments, gas sensors and catalysts, electronics (Anjum et al. 2015). The CuNPs were more effective than the respective bulk types which allowed a lower dose of Cu to be used in crop protection (Giannousi et al. 2013). Also, silver nanomaterials (AgNM) can be used in a wide range of products like cosmetics, pharmaceuticals, food processing, and wastewater treatment. In comparison with other NMs made from metals, AgNM has a high-water solubility which could worsen their harmful effects on various biota (Wijnhoven et al. 2009).

4.4.3.1 Phytotoxicity of Nanoparticles

Data on the phytotoxicity of NMs are consensual and differ widely according to several published reports (1) in terms of their NM dimension, (2) concentrations used (3) in terms of the plant species used, (4) exposures and duration of exposure and circumstances (Conway et al. 2015). The global distribution of these NMs tends to be closely associated with their unique electrical, optical, and thermal properties. The environmental level of nanotechnology is steadily and rapidly expanding and the studies on its possible phytotoxicity are increasing (Grande and Tucci 2016). Meanwhile, the importance of CeO2 NM for plants has been moderately explored without looking into the physiological, biochemical or molecular effects of this NM, although most research has only been focused on biometric, grow-up, and productivity approaches. CeO2NM tends to be one of the more investigated in plant systems in comparison with other metallic NMs, in particular with regard to its effect on antioxidant metabolism. The regulation of CeO2NM induced plant responses depends on various bibliographic documents that rely on several factors like exposure dose and plant species (Zuverza-Mena et al. 2017). The increased use of NMs ultimately facilitates the release and later accumulation of NMs in the environment.

4.4.3.2 Oxidative Stress of Nanoparticles

The generation of ROS is one of the major harmful effects of the plant treated with nano-materials (NMs). The mechanisms for the toxicity of metal nanomaterials depend on their particulate properties (e.g., size and shape), however, Soares et al. (2018) stated that some of their toxicity and impacts are similar to their bulk counterpart and zinc salts once they are within plant cells. Zinc oxide nano-particles ZnONPs tend to be more toxic/stressful than bulk-ZnO (Amooaghaie et al. 2016) because Zn NMs can form secondary NMs (aggregates) in the cell which can be more harmful to the individual ZnO NMs (Lee et al. 2013a). However, various mechanisms and action modes can trigger the toxicity of ZnO NMs including the integration of NMs, aggregation in root surface and tissues, and Zn ion separation from MPs (Ma et al. 2013). Copper (Cu) is the critical nutrient required for growth, development, photosynthetic efficiency, mitochondrial respiration, sensing of ethylene, oxygen metabolism reactive, hormone signals, and cell wall remodeling (Burkhead et al. 2009). Plants only need trace quantities of Cu and their increases are toxic to them. Cu′s redox property is also toxic, as it can catalyze ROS overproduction through Haber–Weiss or Fenton reactions as a transitional redox factor (Halliwell and Gutteridge 1984), causing oxidative stress damage. Intracellular AgNP dissociates into highly toxic Ag + which inhibits Rubisco and causes CO_2 assimilation to be slower. Excess excitement energy, therefore, promotes ROS production in the chloroplast (Jiang et al. 2017). Besides, AgNP toxicity is well-known not only by releasing ions of the Ag + (Kaveh et al. 2013) but also by its shape and size and its ability to cause oxidative damage (Sun et al. 2016).

4.4.3.3 Genotoxicity of Nanoparticles

Cytotoxic and genotoxic effect of Ag NPs on *Vanilla planifolia* plantlets showed a small reduction in the mitotic index and suggesting a dose-dependence increase in the frequency of cells with chromosomal aberrations and micronuclei (Bello-Bello et al. 2018). In addition, Kumar et al. (2020) concluded that the DNA fingerprinting using ISSR markers confirmed the genetic uniformity of nano-priming with Ag NPs and the seedling showed normal phenotype in all characters without any symptoms of toxicity. López-Moreno et al. (2010) demonstrated that the high concentrations above 2000 mg/L of CeO2NPs had genotoxic effects on soybean plants through changing of RAPD profiles by the novel appearance of new bands. Lee et al. (2013b) reported changes in the DNA, RAPD profiles were changed indicating genotoxic effect by SiZnO NPs and CuONPs on *Fagopyrum esculentum*. Also, Moreno-Olivas et al. (2014) reported that TiO2NPs genotoxic effects on *Cucurbita pepo* plants displayed as RAPD profiles differences in bands number and their intensity compared to control. Khan et al. (2019) also observed concentration dependent mitotic anomalies induced by application of Nano TiO_2 in lentils.

Labeeb et al. (2019) reported that AgNPs reduced mitotic division and concentration dependent chromosomal abnormalities and cladistic aberrations in pea, such as chromosome bridges, rings, breaks, and micronuclei indicating a genotoxic potential at high concentrations. Rodriguez-Garraus et al. (2020) reviewed the various methods for estimating genotoxicity of nanoparticles and concluded that AgNPs cause genotoxic effects at all DNA damage levels evaluated either in vitro or in vivo assays in microorganism, animals, cell lines, or plants. Effect of Cerium oxide nanoparticles (CeO_2) which is commonly used in various applications, such as TV tubes, fuel cell, solar cell, gas-sensor and ultraviolet absorption, glass/ceramic polishing agent; was studied by Liman et al. (2019) on *Allium cepa* root mitotic cells. All concentrations significantly reduced the mitotic index (MI) and increased chromosomal abnormalities (CAs) like chromosome laggards, disturbed anaphase-telophase, stickiness, and bridges, and also DNA damage. High concentrations of silica nanoparticle (SiO2NPs) caused cytotoxic and genotoxic effects on the root meristem cells of *Allium cepa* (Liman et al. 2020). In addition, in *Allium cepa* roots, DNA damage caused by TiO2NPs is confirmed by a comet test and linked to chromosomal aberrations (Pakrashi et al. 2014). AgNPs showed a greater effect in roots than in shoots and cause DNA damage in *Allium cepa* and *Nicotiana tobacco* (Ghosh et al. 2012). DNA damage increase in *Brassica rapa* ssp. was dose-dependent and was confirmed by DNA laddering and the Terminal deoxynucleotidyl transferase-mediated dUTP nickend labeling (TUNEL) assay that detects DNA damage linked to non-apoptotic events such as necrotic cell death caused by genotoxic chemical exposure (Thiruvengadam et al. 2015).

4.4.4 Natural Products and Aflatoxins

Natural products from plants, algae, and microorganisms have been major sources of attractive compounds that are essential for human health as pharmaceutical and

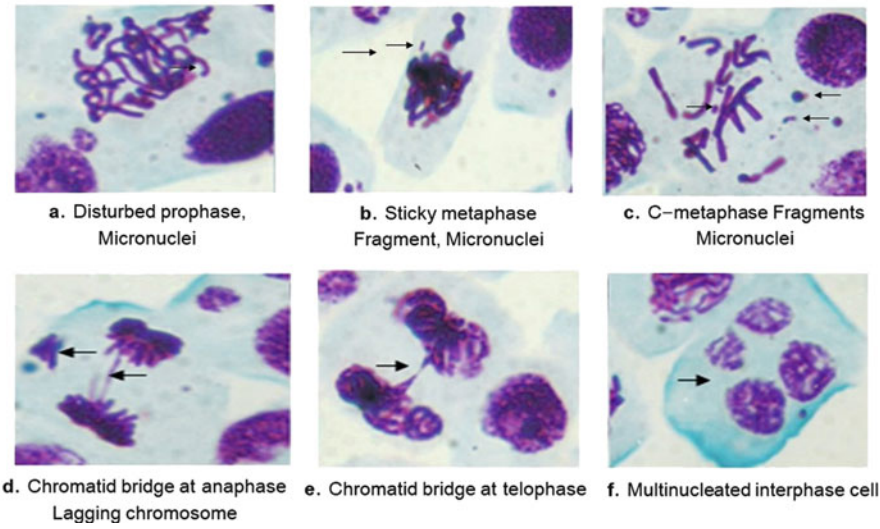

Fig. 4.9 Photograph illustrating chromosomal aberrations and micronucleus induced by the aflatoxin B1 in the root meristem cells of *Vicia faba*. (Images are taken from AB and HHE collection)

extractions for many other uses. Natural products are generally viewed as safe because of strong opinions about the absolute value of natural as opposed to anthropological compounds (Durnev and Lapitskaya 2013). However, experimental data that were obtained during genotoxicity screening of herbal compounds listed established and/or presumed genotoxic activity of many plant products, such as allyl isothiocyanates, anthraquinones, aristolochic acids, hydrazines, propenyl benzenes, pyrrolizidine alkaloids, and single flavonoids (Snyder 2010). Also, the genotoxicity and carcinogenicity of herbal products have been recently documented (Poivre et al. 2017).

During production, harvest, storage, and processing of foodstuffs aflatoxins contaminate a range of foods including animal feeds and other foods, such as nuts, maize, grains, oils, and dehydrated foods (Bennett and Klich 2003). Aflatoxins are a class of mycotoxins and are recognized as human carcinogens (class 1) by the international agency for research on cancer (IARC) and consist of four major groups, namely, B1, B2, G1, and G2. The production of these abnormalities indicates that this toxin induced partial inhibition of mitotic apparatus. Genotoxicity of aflatoxins was reported by El-Sheikh and El-Shazly (1998) and El-Shazly and El-Sheikh (2000). However, aflatoxin B1 is the most potent genotoxin that are highly mutagenic and carcinogenic metabolite known so far (Zain 2011). Chromosomal aberrations and micronucleus induced by the aflatoxin F1 in the root meristem cells of *Vicia faba* are shown in Fig. 4.9. This biotransformation of aflatoxin B1 induces DNA adducts which leads to mutation, genetic and oxidative damage, thus resulting in cancer (Bhat et al. 2010).

4.5 Plants Mitigation of Oxidative Stress

In response to the toxicity by oxidative stress, stress-related proteins and hormones, antioxidants, and so on are generated in plant cells. The ROS may act as damaging or signaling molecule depends on the delicate equilibrium between ROS production and scavenging. For the cells to control the level of ROS to avoid oxidative injury, scavenging or detoxification of excess ROS is achieved by an efficient antioxidative system comprising of the nonenzymic as well as enzymic antioxidants (Noctor and Foyer 1998). In the plant cells, two very important antioxidant protection mechanisms are used by plants to fight stress to hold the essential metals needed for plant homeostasis. Two very important antioxidant protection mechanisms are used by plants to fight stress to hold the essential plant homeostasis. The first mechanism is to decrease the uptake of toxin by cellular and root exudates which protect the plants from toxicity and prevent the toxin from entering the cell. The second strategy is to add chelates that sequester the toxic substance into the vacuole of the plant cells (Ghori et al. 2019).

The activation of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) for immediate inactivation of reactive radicals. Other mechanisms include accumulation by heavy metal mediated toxicity neutralization of non-enzymatic antioxidants, such as flavonoids, tannins, and lignin carotenoids, ascorbate (ASA), glutathione (GSH), alkaloids, and the proline for heavy metal detoxification induced ROS and subsequent elimination of free radicals by scavenging (Latif and Mohamed 2016; Sofy et al. 2020). Arsenic-induced oxidative stress has been shown to increase the level of low molecular weights (LMWTs) compounds, such as cysteine, glutathione, G-glutamyl cysteine, and phytochelatins that eventually detoxify the effects through binding to the arsenic (Mohamed et al. 2016). On the other hand, the cadmium induced oxidative stress may be mediated via membrane lipid peroxidation (El-Beltagi and Mohamed 2013). At the molecular level, plants respond to induced oxidative and genotoxic damage via the rapid change in the expression of the responsive genes at the transcriptional level. Certain transcription factors play crucial role in triggering plant defense responses. In addition to transcriptional response, epigenetic modifications have also been found to be essential for maintenance of plant genome stability under genotoxic stress (Dutta et al. 2018).

4.6 Conclusion

Chemicals are major environmental threats to the plants around the globe. In response to direct or indirect exposure to different chemicals, plant cells generate ROS which impose oxidative stress that can be involved in stimulating genotoxicity in plants by inducing DNA damage which results in a variety of cytotoxicity and genotoxicity end points. The most widely used plant-based genotoxicity assays for testing permanent DNA-damage indicators have been described, these are cell division abnormalities, chromosomal aberrations, sister chromatid exchange,

nucleus malformation, and micronucleus formation. In addition, molecular markers and chlorophyll mutations have been also applied as genotoxicity indicators. Both cytotoxic and/or genotoxic damages lead to genome instability and thus eventually severely affect plant growth and crop yield. Plants respond to induced oxidative and genotoxic damage via the rapid change in the expression of the responsive genes at the transcriptional level by activating transcription factors.

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5 Metal Induced Genotoxicity and Oxidative Stress in Plants, Assessment Methods, and Role of Various Factors in Genotoxicity Regulation

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Abstract

Genotoxicity in a broader term includes all the adverse effects on genome and can be explained as the capability of chemical agents to damage genetic material like DNA or some cellular components involved in cell cycle and cell division like spindle apparatus, DNA polymerases, and DNA repair systems. Chemicals, physical agents, metal elements, free radicals, etc. are known to have mutagenic or genotoxic effects on organisms and popularly known as genotoxins. Plants are continuously exposed to numerous biotic and abiotic stresses such as radiations (UV light, infrared, etc.), salinity, drought, flood, nutrient imbalance, soil and airborne plant pathogens, etc. These factors either directly impede plant growth or indirectly through oxidative stress and overproduction of ROS, causing DNA damage which ultimately leads to genomic instability. Heavy metal plays an important role in different stages of a plant life cycle, their presence in more than required quantity may result in cytotoxicity or genotoxicity. Heavy metals can cause oxidative damage to the macromolecules and photosynthetic apparatus present in a cell resulting in physiological and/or biochemical irregularities with lower membrane stability and photosynthesis, nutrient imbalance, suppression or inhibition of cell division, DNA replication, and gene expression. Although plants have evolved sophisticated and complex regulatory mechanisms to adapt under heavy metal stress, but under extreme conditions, it may affect plant health severely leading to cell death. Plants respond to heavy metal stress-mediated toxicity through complex interlinked mechanisms and these metals induce the changes through direct and indirect interactions with genetic material.

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Keywords

Genotoxicity · Oxidative stress · Genotoxins · Heavy metal stress · DNA damage · Comet assay · Ames test

Abbreviations

(A/N) comet assay	Alkaline unwinding-neutral comet assay
A. rhizogenes	Agrobacterium rhizogenes
AgNPs	Silver nano-particles
Al	Aluminum
AP site	a-purinic/a-pyrimidinic site
As	Arsenic
Ca2C channels	Ca-calmodulin signaling
Cd	Cadmium
Cr	Chromium
D, glaucum	Dipterygium glaucum
DNA	Deoxyribonucleic acid
DSBs	Double-strand breaks
FISH	Fluorescent in situ hybridization
H_2O_2	Hydrogen peroxide
Hg	Mercury
IONPs	Iron oxide nano-particles
IR	Infra-red
ISSR	Inter simple sequence repeats
LP	Lipid peroxidation
MAPK	Mitogen-activated protein kinase
MMS	Methyl methane sulfonate
MNU	Methyl nitrosourea
Ni	Nickel
nm	Nanometer
O_2^{*-}	Superoxide radical
OC	Organo-chlorine
OH*	Hydroxyl radical
OP	Organo-phosphorus
Pb	Lead
PCD	Programmed cell death
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
TUNEL	TdT-mediated dUTP nick end labeling
UV	Ultraviolet
μM	Micrometer

5.1 Introduction

Genotoxicity and oxidative stress are the results of adverse effects of genotoxins on the integrity and structure of genetic material. Chemicals and physical agents, metal elements, free radicals, etc. are known to have mutagenic or genotoxic effects on organisms, popularly known as genotoxins. The prime effects of these genotoxins are chromosomal aberrations like deletion of chromosomal segment or change in base pairing resulting in different kinds of genome instabilities. These genotoxins impede plant growth and development by overproduction of ROS, resulting in genotoxic and oxidative stress by forming unrepaired DNA damages. Overproduction of ROS can also cause oxidative damage to the macromolecules and photosynthetic apparatus present in cells resulting in various physiological and/or biochemical irregularities like decreased stability of plasma membrane, low photosynthesis rate and inhibition of gene expression, DNA replication, and cell division. In this chapter, we will discuss metal induced genotoxicity in plants and various assessment methods used to evaluate the effect of genotoxins on genetic material.

5.2 What are Genotoxicity and Oxidative Stress?

Many chemical agents damage the genetic material leading to alterations in the genetic information. This property of chemical agents is known as genotoxicity. This seems a similar phenomenon to mutations as it also results in heritable alterations in the genetic material of an organism but there are certain key differences between them. Genotoxicity includes all the adverse effects on genome and can be explained as the capability of chemical agents to damage the genetic material or some cellular components involved in cell cycle and division like spindle apparatus, DNA polymerases, DNA repair systems, etc. It also includes DNA damage assessments. These damages may or may not be transferred to the next generation. Mutagenicity is the capability of some chemical agents to cause heritable DNA damage or genetic alteration. We can say that all mutagenic substances are genotoxic as they cause a change in genetic material, but all genotoxic agents are not mutagenic as they may be not able to cause heritable genetics changes. Primary genotoxicity occurs as a result of direct reactivity of genotoxins with DNA, while secondary genotoxicity may be due to formation of reactive oxygen species (ROS). Somatic cell genotoxicity may result in cancer, whereas germ cell genotoxicity often led to infertile or diseased progeny.

An imbalance between free radicals and antioxidants inside an organism is known as oxidative stress. The uneven number of electrons present in free radicals facilitates their reaction with other molecules which may result in large chain chemical reaction known as oxidation, which can be either beneficial or harmful to the organisms. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in both stressed and unstressed plants. Oxidative stress is a complex chemical and physiological state resulted from overproduction and accumulation of these reactive

oxygen species (ROS) and/or reactive nitrogen species (RNS) in cells/tissues that bring biotic and abiotic stress conditions in higher plants.

There are various physical, chemical, and biological agents which act as genotoxin and cause DNA damage. Among physical agents, UV and IR radiations are known to produce reactive oxygen species which cause damage to cellular system and DNA. The UV radiations have an electromagnetic spectrum ranging from 200 to 400 nm further subdivided into UV-A, UV-B, and UV-C, out of these UV-A and UV-B are most effective in causing DNA damage. Sometimes high temperature or heat may also cause DNA damage. Chemical agents causing DNA damage include heavy metals such as lead (Pb), arsenic (As), mercury (Hg), chromium (Cr), aluminum (Al), and cadmium (Cd). These heavy metals induce DNA damage via double-strand breaks (DSBs) generation, ROS production, and inhibition of the biochemical functions of some prime proteins. Most common ROS produced are O_2^{*-} (superoxide radical), H_2O_2 (hydrogen peroxide), OH^* (hydroxyl radical), singlet oxygen, etc. The pesticides being used in farming and health practices have also been reported to induce mutation, chromosomal alterations, and DNA damages. It can induce double-strand breaks in DNA in association with pesticides such as fluoxastrobin and imazamox (Demirci et al. 2016). Apart from these pesticides, various industrial chemicals such as fluoroalkyl substances, brominated flame retardants, dichloro-ethane, vinyl chloride, hydrogen chloride, etc. are reported to cause serious DNA damages by affecting the DNA repairing machinery. Studies have shown that herbicide 2,4-D can also induce alterations in chromosomes in Chicory (Khan et al. 2009); therefore, the fact cannot be ignored that herbicides can also act as genotoxic agent. Some nitrate and nitrite chemicals used as food preservatives against bacterial growth are also reported as highly toxic and carcinogenic. There are some secondary metabolites such as free radicals (ROS, RNS), alkaloids, etc., synthesized in plant cells and are known to cause genotoxic effect and oxidative stress when present in more than the required quantity in the cellular system. For example, sanguinarine (an alkaloid) obtained from *Argemone mexicana* has been shown to act as a potential genotoxic agent and known to induce chromosomal aberrations, micronucleus formation, and DNA damage.

5.3 Mechanism of DNA Damage Under Oxidative Stress

At present, the exact mechanism of oxidative stress-mediated DNA damage has not been clearly understood but one hypothesis named as "fenton reaction of oxidative damage to DNA" has been most acceptable. Overproduction of ROS/RNS leads to a reduction in the level of cellular antioxidants ensuing an imbalance between free radicals and cellular antioxidant mediated defense system which ultimately triggers the oxidative stress-mediated DNA damage. Studies revealed that chemical hydroxylated de-oxyguanosine (8-hydroxydeoxyguanosine, 8-OHdG) production may act as one of the key biomarkers of oxidative damage to DNA by chemical xenobiotics. The effect of these chemicals includes de-amination of cytosine

nitrogenous base of DNA changing it into uracil and removal of nitrogenous base resulted in generation of AP site (a-purinic/a-pyrimidinic) in DNA.

5.4 Metal Induced Genotoxicity and Oxidative Stress in Plants

Heavy metals may induce genotoxicity and genome instability in plants. Chemical toxicity induced by heavy metal ions has become a major environmental threat with continuous use of chemical fertilizers and expeditious industrialization, etc. which disturb the structural, enzymatic, and non-enzymatic plant cell components leading to loss of cell viability. Soil with excess amount of heavy metals, viz. cadmium and lead, generates metal stress resulting in the inhibition of plant growth which is prominent at seed germination stage and at early stages of the growth and development of seedling. Initially, heavy metal ion competes with essential nutrient cations for absorption at root surface and after entering into plant cell, they disrupt the structure and function of proteins by directly attacking thiol group of proteins. A conformational change occurs in the protein's structure which ultimately impedes its function. Heavy metals cause oxidative damage to the cellular organelles resulting in physiological and/or biochemical irregularities with less stability of plasma membrane, lower pigment production and photosynthesis, hormonal/nutrient imbalance and suppression of gene expression, etc. Heavy metal uptake initiates a variety of stresses in plants depending upon metal type, their concentration, and developmental stage of the plant.

Genomic stability and integrity play a prime role in the survival of any species. Due to changing climate and environmental pollution plants are exposed to various stress. A hypothesis has been proposed named general adaptation response (Achary and Panda 2010), first described in bacteria and later in mammals, fungi, algae, and higher plants. According to this hypothesis, different kinds of stress induce similar adaptive response indicating the role of formation of reactive oxygen intermediates (ROI). Generally, ROI are involved in plant defense, signal transduction, plant growth and development but they can also damage genetic material resulting in genotoxicity and programmed cell death (PCD). General adaptation response is one of the primary strategies that plants utilize inherently to withstand the adverse climatic conditions leading to genomic protection. In general adaptation approach first, the cells are exposed to low (non-cytotoxic) dose of genotoxic agent which triggers resistance response in these cells when treated with higher doses of the genotoxic agent (Calabrese et al. 2007). The exact mechanism behind general adaption approach is still unclear but studies show possible role of cell's DNA repair network, synthesis of unique proteins and polypeptides, or involvement of epigenetic mechanisms. Various studies have shown that lower doses of metal ions, various alkylating and oxidative agents, neutrons, and ionizing radiations can trigger different mechanisms for genotoxic adaptation in prokaryotic as well as eukaryotic cells (Dimova et al. 2008).

Among environmental pollutants, aluminum metal is of prime importance as it contaminates water, soil, and food chain which can be a menace for human health,

environment, and ecosystem. Industrial mining and smelting of bauxite ore, wrapping sheets, cosmetics, medicines, food additives, etc. are the prime sources of aluminum. Studies suggested that Al excess (dose range 1–200μM) triggers oxidative burst at surface of root cells which generate an oxidative stress condition in root cells of *A. cepa* through induction/repression of definite antioxidant enzymes (Patra et al. 2000). Studies showed the expression of some specific genes in *Arabidopsis* subjected to Al stress and about 256 Al responsive genes have been identified (Goodwin and Sutter 2009). It was observed that Al genotoxicity induces cell wall disruption, plasma membrane disintegration, and most importantly changes in nuclear DNA structure (Kochian et al. 2005). Later, it was documented that Cd can induce genotoxic adaptation against methyl nitrosourea (MNU) in the root cells of *H. vulgare* (Achary and Panda 2010) when present in low concentrations. Dual role of the Al triggered ROI production was also documented, which was found to induce genotoxic adaptation at low concentrations, conferring genomic protection, and induced damages to genetic material when present in high concentrations. Therefore, the concentration range of Al (ROI production) is critical for the induction of genotoxicity adaptation in plants.

Pollutants present in wastewater released from industries particularly pesticide industry can also cause genotoxicity in plants. Major pollutants released are organo-phosphorus (OP) and organo-chlorine (OC). EI-Gawad (2016) detected high concentration of some pesticides of organo-chlorine group, viz. alpha-BHC, gamma-BHC, aldrin, heptachlor, and heptachlor epoxide in different water samples collected from industries. Pesticide residues present in soil and water even in low concentrations adversely affect vegetables and fruits and thus can raise serious health issues. Studies revealed mutagenic nature of these water pollutants as they can induce heritable changes in the DNA of an organism. Large number of mutagens have been extracted using various organic solvents (dichloro-methane, n-hexane, acetone, ethyl acetate, aceto-nitrile, etc.) and were identified as aromatic amines, polycyclic aromatic hydro-carbon, polychlorinated compounds (Zeyad et al. 2019). These pollutants are main cause of cancer, chronic kidney diseases, sterility, immune system suppression, leading to neurological or behavioral disorders (Kaur et al. 2014).

5.5 Genotoxicity and Oxidative Stress Inducing Potential of Soils Under Agriculture

High level of radiation and release of genotoxic chemicals have increased the pollution level which affects the ecosystem, health of humans, and most of the other organisms. Sometime excessive use of fertilizers containing specific element results in an increase in the level of that element to the toxic level. Excessive use of phosphate-based fertilizers may increase Cd level in the soil which dissolve in soil and have extremely toxic nature. Heavy metals toxicity (especially Cd and Pb) resulted in various physiological and clastogenic chromosomal aberrations leading to abnormalities in cell division, DNA replication, absorption and transportation of

crucial elements, disturbance in metabolism, affects growth and reproduction in plants.

A very general detrimental effect of these heavy metals is the production of high quantity of ROS and free radicals, which can lead to permanent damage of biomolecules like proteins, lipids, chromosomes, and genetic material. Plants possess complex anti-oxidative defense system comprising of enzymatic (SOD, CAT, APX, GST, DHAR, MDHAR, detoxifying lipid peroxidation (LP) products like ascorbate and glutathione) and non-enzymatic (tocopherols, carotenoids, and phenols) components that play a key role in ROS scavenging. These types of system are mainly present in plant organelles, viz. mitochondria, chloroplasts, and peroxisomes. The enzymatic components can convert the potentially dangerous hydrogen peroxide (H_2O_2) and superoxide radical into water and oxygen molecules, thus prevents plant from cellular damage (Scandalios 2005). Under heavy metal stress H_2O_2 gets accumulated in cells resulting in inactivation of catalase (Olteanu et al. 2011) which plays an important role in protecting the cell from oxidative damage. It was documented that Cd increases the binding affinity of some metal ions to the sulfydryl group of superoxide dismutase enzymes, due to this binding phytotoxicity of metals increases which results in decreased activity of superoxide dismutase (Kaur et al. 2014).

5.6 Genotoxic and Oxidative Stress Regulation in Plants Undergoing Heavy Metal Stress

Although plants have developed complex and sophisticated regulatory mechanisms to adapt and survive chemical toxicity triggered by heavy metal stress, it may have adverse effect on plant health under extreme stress conditions, leading to death of the plant cells. Several metals like Al, Cd, Hg, Cr, and Pb cause chlorosis, lower photosynthetic rate and biomass, water imbalance, and altered nutrient assimilation ultimately leading to plant growth inhibition, senescence, and ultimately yield losses. The roots are the first organ to encounter the heavy metal stress which initially results in root growth inhibition due to mitosis inhibition in root meristem. Further, heavy metal stress disrupting auxin transport in roots is another important reason for root growth inhibition. Plant cells have developed several complicated interlinked mechanisms including short-term and long-term processes to combat with toxicity induced by heavy metals. The short-term or immediate responses include fast alteration at the transcription rate of numerous responsive genes resulting in the changes at the physiological and metabolic levels. The long-term responses are an integral part of plant stress responses, associated with the permanent genetic modifications in genomes by making changes in the transcription level of stress-responsive genes to control and regulate gene expression. Therefore, plants respond to both oxidative and genotoxic stress induced by heavy metal toxicity through an integrated approach of various components of stress perception and signaling networks at various steps during stress.

5.7 Heavy Metal Stress Activates Signaling Cascades in Plants

Under heavy metal stress condition several signaling pathways are induced in different plant species. In one important signaling cascade event plants sense external stress signal and transmit it to the downstream to activate appropriate measures to overcome genotoxicity or oxidative stress, which regulates biochemical, physiological, and molecular function of a stressed cell. It has been observed that at an early stage of heavy metal stress, plants start accumulating secondary metabolites known as stress-responsive secondary metabolites. A complex signal transduction network activates immediately after plant recognizes heavy metal content in extra-cellular environment, this stimulates several signaling networks like calcium–calmodulin pathway, ROS directed signaling, phyto-hormonal response, and mitogen-activated protein kinase (MAPK) mediated phosphorylation cascade in plant (Dutta et al. 2018). Calcium-calmodulin signaling (Ca2C channels) in plants is generally associated with abiotic stresses, viz. salinity stress, high or low temperature stress, osmotic stress, and oxidative stress. Under heavy metal stress Ca2C channel shows some modification which increases the calcium flux inside the cell. This Ca acts as a messenger to regulate the expression of downstream genes associated with transport, metabolism, and tolerance of heavy metal stress (Ruta et al. 2014).

Phyto-hormonal signaling occurs with other cascades like ROS signaling and mitogen-activated protein kinase (MAPK) signaling pathways in plants. In case of phyto-hormonal response, the level and balance of phyto-hormones play crucial role to initiate the signaling. Under heavy metal stress (Cd) ethylene biosynthesis was found to increase in some different species of plants like *Arabidopsis*, pea, mustard, and soybean (Arteca and Arteca 2007; Rodriguez-Serrano et al. 2009). The increased level of ethylene promotes synthesis of other hormones (Auxin, cytokinins, GA, ABA, jasmonic and salicylic acids) and antioxidants production to increase stress tolerance level in plants. These hormones are known to involve in the activation of several transcription factors for the upregulation of the expression of stress-responsive genes. For example, in rice, studies on gene expression followed by As stress revealed increased expression of OsNCED2, OsNCED3, and four other genes, involved in the biosynthesis of ABA (Huang et al. 2012). Under heavy metal stress the MAPK signaling cascade also regulates plant response. It has been reported that under Cu and Cd stress, seedlings of alfalfa activate four different cellular MAPKs signaling cascades in roots, viz. SIMK, MMK2, MMK3, and SAMK (Jonak et al. 2014). Likewise, in soybean during early developmental stages, exposure of seedlings to Cd triggers ethylene biosynthesis with upregulated expression of those genes involved in the metabolism of polyamines, NO generation, and MAPK signaling cascade (Chmielowska-Baak et al. 2013).

5.8 Evaluation of Genotoxicity Induced by Plants

Plants species are poorly evaluated for their genotoxicity and mutagenic effects rather their products and derivatives are used. According to WHO, in Asian and African countries, traditional medicines are the main source of health care for about 80 per cent of populations. In recent times global interest towards herbal products has increased as these are natural and believed no harm for health. However, limited scientific evidences are available till now regarding the safety of use of these plant sources. For example, *Dipterygium glaucum* is a perennial shrub used for many medicinal purposes (as a trachea-dilating agency for miss-breathing problems) and is a source of volatile alkaloid, cyanide, flavonoide, and cumarin (Al-Zugut 1989) but there are no cytogenetic and molecular reports available regarding genotoxicity and biological activities of this plant (Altwaty et al. 2016). It should be kept in mind that safety and potency of the herbal products depend on the fact that safe resources should be used, and this safety check of raw materials must be carried out before their use in the development of herbal medicinal products. Although the use of medicinal plants as herbal medicine are continuously increasing (Atere and Ajao 2009) concern about the toxicity and adverse effect of these plant based remedies on health has been raised from time to time (Saad et al. 2006). As a traditional medicine consumption of these plants are assumed to be safe as these are in long time sage based on knowledge accumulated over centuries, but many plant species used as food or traditional medicine may be potentially mutagenic, toxic, or may have carcinogenic effect (Fennell et al. 2004). Therefore, there is a need to supplement health programs with traditional herbal preparation with certain genotoxicity tests to assure their non-toxic nature (Akintonwa et al. 2009). In recent years, some plants such as *Allium cepa*, *Vicia faba, Brassica sps.* Have been used as good bio-indicators of genotoxicity induced by environmental pollutants, and micronucleus, comet or chromosomal aberration assays and were used to measure the genotoxic effects (Asita and Matebesi 2010). Root tips frequently used in past from *V. faba* served as an excellent source for clastogenic studies of physical and chemical agents. Molecular and cytogenetic assessment provides evidence of genotoxic effects of *D. glaucum* extracts on mitosis in the roots of *V. faba,* which showed decreased mitotic activity and different chromosomal abnormalities including sticky chromosomes, chromosomal fragmentation, chromatin bridges, micronucleus formation, and inversions probably due to loosening of DNA/nucleic acid from histone packaging or due to de-polymerization of nucleic acid (Altwaty et al. 2016). Chromatin bridges and fragments were observed due to stickiness and chromosome breakage. The other effects of these plant toxins may be inhibition of centromeric and spindle activity, formation of lagging chromosomes, stray chromosomes, etc. These types of studies are useful to understand the genetic deformities and distortion induced by plant species when used as herbal medicine without prior scientific information and help in fixing specific dose and duration of the therapy.

Generally primary roots or adventitious roots are used to assess DNA or chromosomal damages in plants as these are actively dividing cells and any type of chromosomal aberration can be easily observed during metaphase of mitosis. With

the development of in vitro plant culture and transformation techniques, transformed hairy roots have been developed which opened a new possibility to use these transformed root cultures as an attractive source of actively dividing mitotic cells. As these cells are identical with respect to chromosomes or genetic material any kind of alteration or damage due to the genotoxin can be easily detected with the help of suitable detection method. An example is culture of transformed hairy root line obtained from *Agrobacterium rhizogenes* mediated transformation, characterized by lateral branching which easily provides many root tips cells. These can be useful in research related to plant genome for cytogenetic analysis of chromosomes to assess structural and numerical aberrations (Siroky et al. 2001). Another example is the use of *C. capillaris* hairy roots, used for karyotype and morphology stability as they show fast growth, genetic stability in in vitro culture, and have simple karyotype which is convenient for evaluating chromosome damage (Juchimiuk and Maluszynska 2005). It has been recorded that transformed root cells are more sensitive for mutagenic analysis as compared to primary roots at both chromosome and DNA levels. When hairy roots and seedling roots were exposed to same doses of X-rays different frequencies of chromosomal aberrations were observed. Two times higher frequency of DNA fragmentation was recorded in hairy roots than seedling roots when exposed to same dose of irradiation. These results suggest that all described features of *C. capillaris* hairy roots, notably their relatively higher sensitivity towards genotoxins, make them an auspicious system for plant bioassay (Juchimiuk and Maluszynska 2005).

5.9 Cytogenetic Assessment of Genotoxicity: Analysis of Genotoxicity at the Level of Chromosome and DNA

It is crucial to study and understand the changes that can be induced by a genotoxin into the structure and function of chromosome and DNA of an organism. DNA of an organism, when exposed to genotoxin may result in genomic instabilities and multiple mutation events, error in DNA replication and repair pathways. The exact molecular mechanism of the action of these genotoxins is still not very clear, possibly they induce the changes through direct and indirect interactions with genetic material. Genotoxins may induce damage to the structure and sequence of genetic material by interacting at specific locations and base sequences of DNA. This interaction results in breakage followed by deletion or fusion of chromosomal segments and mis-segregation/non-disjunction which ultimately leads to mutation. For example, in its high-valent oxidation state, transition metal Cr interacts with DNA, causing DNA lesions, potentially contributing to carcinogenesis. It has been recorded that the mechanism of interaction and DNA damage by these transition metals are similar to DNA damages in vivo, where it causes cancer in human populations when exposed to Cr (Mohamed et al. 2017). It is also reported that ROS cause oxidative lesions in DNA and when these ROS and free radicals are present in cellular system, they may alter the structure of lipids, proteins, and of the genetic material.

Table 5.1 Effect of genotoxins on genome and common testing methods (Ren et al. 2017)

Effect of genotoxins	Testing method used
1. Chromosomal damage	
a. Structural aberration	Chromosomal aberration test, micronucleus assay
b. Numerical aberration	Micronucleus assay
2. DNA damage	Comet assay
3. Gene mutation	Ames test, gene mutation assay using transgenic rodent's somatic/germ cell, mouse lymphoma assay (MLA), hypoxanthine guanine phosphoribosyl transferase test

The effect of these genotoxins has been studied in detail in plants, animals, and rodents and various tests have been developed for the assessment of their impact. They affect genome by damaging chromosomes and DNA, thereby inducing gene mutations. Chromosomal changes include both structural and numerical aberrations. It is quite possible to use a wide range of plant species for genotoxicity assessment because of the highly conserved chromosome structures. The most widely used approaches use laboratory rodents, bacterial indicator species, insects, yeast, fungi and mammalian cells in testing of genotoxicity. For the screening and monitoring of environmental mutagens, some higher plants bioassays have been developed (Maluszynska and Jjuchimiuk 2005). The common testing methods used to assess these changes are summarized in Table 5.1.

Genotoxin such as 1-4 benzoquinone (BQ), pyrrolizidine alkaloids (PAs) possessed ability to induce aberrations in chromosomal and DNA structures. Genotoxicity assessment aims to prevent adverse effects of genotoxins on an organism. There are some rapid short-term tests such as Ames test, micronucleus assays, and in vivo cytogenetic test to evaluate the genotoxic potential of hazardous chemicals. Mohamed et al. 2017 have described certain agents, viz. ROS, UV radiation, ionizing radiations, topoisomerase inhibitors, protein synthesis inhibitors can induce DNA damages directly or indirectly. They also described various tests for genotoxicity evaluation summarized in Table 5.2.

The purpose of in vitro testing is to establish whether a genetic damage is caused by a substrate, product, or an environmental factor. The clastogenic (loss of acentric chromosomal fragments) or aneugenic (breakage, exchange, or mitotic loss of chromosomes) effects of genotoxin increase the occurrence of structural or numerical aberrations in genome. The purpose of *in vivo* testing is to know the potential of a genetic damage affecting structure of chromosome or deteriorating mitosis process leading to numerical change in the genome. Exploration of genetic markers like randomly amplified polymorphic DNA (RAPD) has upgraded the detection process of DNA alterations occurred in plants when exposed to many genotoxins. RAPD-PCR has been identified as a reliable technique for the detection of DNA damage as amplification stops at the damaged site. Certain changes occur in RAPD profile of a

Table 5.2 Some of the standard tests used for genotoxicity evaluation with description

Test	In vivo/ in vitro	Description
Ames test	In vitro	The test was first developed by Ames et al. (1973), known as *bacterial reverse mutation test* or Ames test. In this the amino acid (histidine) dependent strains of *S. typhimurium*/*E. coli* are used to detect mutation (may be substitution, addition, or deletion of DNA base pairs). The identified mutation reverted so that the mutant cells (reverent) get back the ability to synthesize histidine. This is commonly used as an initial screening test for genotoxicity and it is rapid, inexpensive, and easy
Comet assay	In vivo	It is the most common in vivo test used to assess hazardous potential of genotoxic agents for mutagenicity/genotoxicity. In this method, cells are lysed by detergents and salts to release its genetic material which is subjected to electrophoresis. Cells containing DNA with more DSBs will migrate faster to anode and give a greater number of bands. It can detect a broad variety of primary DNA lesions which are difficult to identify by any other tests and require very few cells and can be applied to a wide variety of cells or tissues to detect low levels of DNA damage
Micronuclei test/ chromosome aberration test	In vivo/ in vitro	Both in vivo and in vitro micronucleus test are similar as it investigates structural and numerical chromosomal aberrations. This test detects the occurrence of chromosomal or spindle damages when the cells are exposed to mutagens. At the time of division, the cell develops a small micronucleus in addition to main nucleus. A micronucleus formed by separation from the nucleus is a small structure having nuclear DNA segments or one or more complete chromosomes which were not incorporated in progeny cells at mitosis. The frequency of cells with these micronuclei is strong measure of the cytogenetic effects of tested chemical
Ames *Salmonella*/ microsomal test	In vitro	The Ames *Salmonella*/microsomal test is a short-term bacterial reverse mutagenicity assay which is used to detect potential of a wide variety of the chemical substances to cause gene mutations. In this test *Salmonella* tester strains are used which are histidine dependent each carrying different mutations in different histidine operon genes. These mutations function as hot spot for mutagens causing damage to DNA. When *Salmonella* tester strains being grown on minimal medium with limited quantities of histidine, only those bacteria which revert themselves to histidine independent (his^+) can grow. This test is used to examine the mutagenic potential of toxic chemicals. When a mutagen is

(continued)

Table 5.2 (continued)

Test	In vivo/ in vitro	Description
		applied to the media containing plate, revertant colonies number/plate increases in a dose dependent manner (Mortelmans and Zeiger 2000)
Mung bean seed assay	In vitro	Mung bean seed assay is a short-term genotoxicity assessment assay which uses various characters like seed germination and seedling vigour index, etc. to evaluate the effect of genotoxin on plant growth
DNA nicking assay	In vitro	The DNA nicking assay is used to measure the antioxidant and prooxidant effects of a plant extract on cellular component such as DNA. In vitro DNA nicking assay allows a rapid screening of potentially capable in vivo antioxidant substances. It is based on the Fenton reaction that mimics the biological conditions in vivo by producing hydroxyl-free radicals from endogenous substances like intra-cellular iron. H_2O_2 is cleaved to •OH during reaction by electron transfer from iron, a highly reactive and strong oxidizing species. The initial supercoiled plasmid DNA structure changes from its supercoiled to open circular and nicked linear forms that present altered mobility related properties on the electrophoresis gel
Allium test/the Allium chromosome aberration test	In vitro/ in vivo	It is a classical test developed by Levan (1938) to study different effects of chemical agents on the root tip cells of *Allium.* It has 16 large chromosomes which are helpful in detecting chromosomal aberrations
TUNEL test	In vitro	TdT-mediated dUTP nick end labeling (TUNEL) test is used to detect apoptotic DNA fragmentation, identification, and quantification of apoptotic cells and detection of excessive DNA breakage in individual cells. In this test terminal deoxynucleotidyl transferase (TdT) enzyme is used that acts as catalyst in joining of deoxynucleotides which are tagged with a fluorochrome or another marker to the 3′-hydroxyl terminus of double-strand breaks of DNA
FISH	In vitro	In plants, fluorescent in-situ hybridization (FISH) is used to evaluate chromosomal aberrations occurred due to mutagenic effect of genotoxin. It facilitates the identification of chromosomal rearrangements with a more precise position of them

certain genome after genotoxin treatment. These changes include gain or loss of a band and variation in the intensity of bands. These changes are further verified by analyzing and comparing the exposed and non-exposed individuals for difference in band intensities and/or band gain/loss variation between them. The inter-simple

sequence repeat (ISSR)-PCR method is more sensitive than RAPD DNA assay due to exhibiting specificity of the sequence-tagged-site markers and high reproducibility potential ratio owing to the use of longer primers.

5.10 Role of Plant Products and Environmental Factors in Oxidative Stress Regulation in Plants

Each genotype interacts with its environment, a process known as genotype x environment interaction. So, plants are influenced by the external environment and this environment has the capacity to change the phenotypic expression and thus, characters of a plant species. Apart from genotoxins, various environmental factors such as high temperature, drought, salinity, metal induced toxicity, UV light, pathogen infection, and insect–pest infestation, etc. can induce oxidative stress in plants either directly or in an indirect way by production and accumulation of ROS. In plants production of ROS takes place in various cellular organelles as a natural bi-product of various biochemical reactions. ROS activation is an energy dependent mechanism requiring an inescapable electrons leakage from electron transport systems of many cell organelles like mitochondria, apoplasts, peroxisomes, chloroplasts, endoplasmic reticulum (ER), plasma membranes as well as cell wall, etc. (Sharma et al. 2014) but chloroplast and peroxisomes are the prime sites of their generation under the light and mitochondria under dark conditions.

Some of the effects caused by overproduction of ROS are flower and leaf abscission, growth retardation, less seed germination, cell senescence, disruption of plasma membrane, protein denaturation, and damage to the genetic material (DNA and RNA), etc. that result in different level of yield losses and decrease quality of crop produce, e.g., in sweet orange, overexpression of CitERF13 gene leads to degradation of chlorophyll pigment and ROS accumulation (Xie et al. 2019). Plants produce some proteins to withstand these oxidative stress conditions, e.g. in potato, AtCYP21-4 protein's overexpression leads to heavy tubers and this protein produces tolerance against oxidative stress. In rice transgenic plant, overexpression of OSCYP21-4 gene resulted in 10–15% higher biomass and productivity with high seed weight (Park et al. 2017). Many antioxidants and plant products are known to scavenge ROS and stop chain reactions initiated by these ROS. The aqueous extract of *Ganoderma lucidum* contains notable antioxidant property with the potential of protecting DNA from damages due to radiation or chemical, also methanolic extract of *C. carandas* leaves has been reported to inhibit DNA damage. Brinjal has been reported to have anti-genotoxic effect and has inhibitory potential against urethane-induced mutagenicity and its extract was also found very effective in the protection of oxidative DNA damage (Singh and Sharma 2019). It has been reported that these plant products chelate heavy metals and protect the cells from DNA damage. In addition, some vitamins play a role in decreasing oxidative damage. Vitamin C is an active antioxidant capable of donating an atom of hydrogen and creating a relatively stable ascorbyl-free radical. Vitamin C, vitamin E, and β-carotene also known as antioxidant vitamins have been reported to act as quenchers of free radicals and thus

saving cells from DNA damages caused by them (Wang et al. 2012). The available reports confirm that medicinal plants contain a lot of antioxidants which have potential to use as therapeutic supplements to protect DNA from oxidative stress-mediated consequences. In past several years research has been extended from animal to plant crops which increased our understanding about the role and action of oxidative stress in defense and also about the regulation of these ROS by the environment through environment-induced responses.

5.11 Conclusion

Effect of heavy metal mediated stress on productivity and growth of plants has been a major concern in various terrestrial ecosystems worldwide. The detrimental effect of environmental stress on plants health puts restrictions on the production of world food crops. These factors are closely connected with raised requirement to develop abiotic stress tolerance in plants. Plants exposure to numerous biotic and abiotic stresses has adverse effects on plant health that may give rise to instabilities at genomic level. Plants respond to these stresses by changing the expression level of the stress-responsive genes. Studies suggested that apart from the regulation at transcriptional level, there is a key role of alterations in structure of chromatin material for the regulation of expression of abiotic stress-responsive genes. DNA and histones undergo several dynamic epigenetic changes in response to these stresses inter-play between ROS and these epigenetic modifications have become a prime focus for researchers to study the potential of crop plants for stress tolerance. There is a strong connection between chromatin modification and change in the pattern of gene expression in plants in response to these stresses. Undoubtedly, these stresses and their effects are complex in nature, but genetic engineering approaches are playing an important role in understanding their full potential which may provide an important avenue in future for the development of several improved crop species. The various biotechnological tools and techniques rely upon modulating the expression of stress-responsive genes in order to develop heavy metal stress tolerance in crop plants which encode proteins and synthesize metabolites involved in stress signaling to confer tolerance. Several species of plants have been recognized as natural heavy metals accumulator with appreciable growth potential in soils under heavy metal stress. Apart from transgenic and biotechnological approaches, these metal quencher species have opened up new targets for genetic modifications and manipulation with respect to heavy metal stress tolerance in crop plants. Different genotoxicity assessment has also played an important role in understanding the cause and effect of these biotic as well as abiotic elements on genetic material of plants and also to know the potential effects on the gene expression of these crop species.

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Mechanisms of Genotoxicity and Oxidative Stress Induced by Engineered Nanoparticles in Plants

6

Yasmin M. Heikal and Nicoleta Anca Şuţan

Abstract

Engineered nanoparticles (ENPs) are commonly used in various industrial sectors, manufacturing processes and product categories, but their special properties lead to deleterious effects on all types of living beings. Up to the present, the theoretical basis for the cytotoxicity, genotoxicity and oxidative stress induced by most engineered nanomaterials (NMs) has not yet been fully understood. Intense usage of ENPs has resulted in a much greater environmental and organism's exposure. The pathways involving the most basic principles of the toxicity of ENPs have recently been intensively studied. Chronic and acute exposures to ENPs are known to cause oxidative stress, genotoxicity, cytotoxicity and carcinogenicity in the biological system. Recent studies on in vitro and in vivo genotoxicity have explored the possible mechanisms of genotoxicity caused by ENPs. There are critical factors that can impact genotoxicity and oxidative stress. These essential determinants, including abiotic factors, physico-chemical properties and experimental conditions, respectively, and biotic factors, are outlined in this chapter. The present investigation is focused on the characteristics of the ENPs, the currently available genotoxicity methodological approaches and a survey of the recent genotoxic studies of the ENPs, standardized testing protocols and their potential mechanisms. A special attention should be paid to the risk evaluation of the ENPs, to understand the distinct and precise interactions between the ENPs and plants, as an integral basic segment in any ecosystems.

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Keywords

ENPs interaction · Oxidative damage · Genotoxicity biomarkers · DNA damage · Antioxidant machinery · ROS regulation

Abbreviations

$[O_2]^{\bullet}$	Superoxide anion radicals
$[OH]^{\bullet}$	Hydroxyl radicals
$^1O_2$	Singlet oxygen
8-OHdG	8-hydroxy-2′-deoxyguanosine
ABA	Abscisic acid
AgNPs	Silver nanoparticles
Al_2O_3NPs	Aluminium oxide nanoparticles
AlNPs	Alumina nanoparticles
APX	Ascorbate peroxidase
ASA	Ascorbic acid
ATP	Adenosine triphosphate
AuNPs	Gold nanoparticles
BrdU	Bromodeoxyuridine
CA	Chromosomal aberrations
CaBPs	Ca^{2+}-binding proteins
CAT	Catalase
CBMN	Cytokinesis-block micronucleus assay
CeO_2NPs	Cerium oxide nanoparticles
CO_2	Carbon dioxide
CSD2	Cu/Zn superoxide dismutase 2
CTA	Cell transformation assay
CuNPs	Copper nanoparticles
CuONPs	Copper oxide nanoparticles
DHAR	Dehydroascorbate reductase
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSB	Double-strand breakage
ENPs	Engineered nanoparticles
Fe_2O_3NPs	Iron oxide nanoparticles
Fe_3O_4NPs	Iron oxide nanoparticles
FISH	Fluorescent in situ hybridization
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
LDH	Lactate dehydrogenase

MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase
MN	Micronuclei
MPs	Microparticles
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue
MWCNTs	Multiwalled carbon nanotubes
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide reduced
NiNPs	Nickel nanoparticles
NMs	Nanomaterials
NPs	Nanoparticles
POD	Peroxidase
PrxR	Peroxiredoxin
QDs	Quantum dots
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROOH	Lipid peroxide
ROS	Reactive oxygen species
SCE	Sister chromatid exchange test
SCGE	Single-cell electrophoresis gel
SiNPs	Silicon nanoparticles
SnO_2	Tin (IV) oxide
SOD	Superoxide dismutase
SSB	Single-strand breakages
SWCNHs	Single-wall carbon nanohorns
TBARS	Thiobarbituric acid reactive species
TF	Transcription factor
TiO_2NPs	Titanium dioxide nanoparticles
UV	Ultraviolet
ZnONPs	Zinc oxide nanoparticles

6.1 Introduction

In 1925, Nobel Laureate in Chemistry Richard Zsigmondy introduced the term "nanometer" into science to characterize nano-sized particles (Hulla et al. 2015). Nanoscience, defined as the science and the study of matter with dimensions between 1 and 100 nm (Bayda et al. 2019) opened promising paths to the world of nanotechnologies. The amount of interest given to nanotechnology has increased since 1960, when Nobel Laureate in Physics Richard Feynman launched the

hypothesis "Why can't we write the entire 24 volumes of the Encyclopedia Britannica on the head of a pin?" (Feynman 1960).

Since then, the nanomanufacturing and industrial applications of nanomaterials (NMs) have expanded considerably. The global nanotechnology is estimated at a value of about $124 billion by 2024 (Adiguzel 2019). Today, hundreds of household and industrial products containing NMs have found their applicability and are widely used in electronics, optics, agriculture, food and beverage, medicine, cosmetics, waste-water treatment technologies or environmental remediation processes (Shafiq et al. 2020). In the meantime, several thousand NMs are under research and evaluation for various applications.

Although engineered nanoparticles (ENPs) are very small in a large world, by their unique properties they can raise big issues. Simple question about the behavior of nanoparticles (NPs) in the environment and safety of their handling call for broad, coherent, and reasoned answers. Through their extraordinary physical, chemical and biological properties, ENPs lead the ordinary laws of science to a collateral line (Handy et al. 2008; Ovissipour et al. 2014).

The physical and chemical properties, source, fate and dose are crucial in assessing the potential harmful effects of the ENPs on living systems, including crops. Exposure route, co-exposure with various pollutants, prooxidant effect, along with important biotic factors such as species, tissue and cell type direct nano-biointeractions in a favorable or unfavorable way, even from one generation to the next (Winnik and Maysinger 2013; Husen and Siddiqi 2014).

In this chapter, we reviewed current literature on basic understanding of the NPs–plant interaction pathways and cellular responses, including oxidative stress, genotoxicity, cytotoxicity and apoptosis. The knowledge presented here could improve the understanding of nano–bio interactions and the current methods for assessing the in vivo or in vitro genotoxicity of NPs in plants.

6.1.1 Engineered Nanoparticles: Definition, Types, and Its Impact on Plant Cell

Nanotechnologies represent the deliberate manipulation of NMs in practical applications (Bayda et al. 2019). Materials with internal or external structures of nanometric dimensions are called nanomaterials. Based on the number of their nanoscale dimensions, NMs are classified into (1) zero-dimensional nanostructures, including various types of quantum dots, noble metal and magnetic nanoparticles (Wang et al. 2020); (2) one-dimensional nanostructures with one dimension exceeding nanoscale, just as nanorods, nanobelts, nanofibers, nanotubes and nanowires; (3) two-dimensional which has two nanoscale dimensions, and one micrometric dimension or larger, such as sheet-like graphene and graphene oxide; (4) three-dimensional NMs, also known as bulk NMs, consisting of equiaxed nano-sized structures which can form three-dimensional structures that exceed nano-sizes (Saleh and Gupta 2016; Zhang et al. 2018a, b; Sudha et al. 2018).

NPs have been defined as objects with a variable size between 1 and 10 nm (Abdullaeva 2017) or objects with three external dimensions in the nanoscale (Jeevanandam et al. 2018). Beyond the lower limit of NPs, there are clusters—small groups of atoms, molecules or ions closely related to each other, and above the upper limit are nanocrystals made up of separate crystallites or phases, with dimensions not exceeding 100 nm (Abdullaeva 2017).

NMs are not only the by-products from combustion engines, furnaces and welding, but also the result of natural hazards such as dust storms, volcanic eruptions and forest fires (Roco 2005; Nowack and Bucheli 2007; Cupaioli et al. 2014), or can be produced either using chemical, physical, biological synthesis or through evaporation-condensation and laser ablation (Iravani et al. 2014; Saleh and Gupta 2016).

Two manufacturing methods are used to produce nanoparticles: top-down and bottom-up, which compete in terms of the quality and speed of NP synthesis, cost-efficiency and effectiveness. The top-down approach consists in etching away bulk material until the nanostructure architecture is reached. A top-down nanofabrication approach is nanolithographic processes. The bottom-up approach involves the principle of self-assemblance and self-organization through supramolecular interactions, under the action of external stimuli, to form functional materials (Iqbal et al. 2012).

The bottom-up approach provides the possibility to create NMs by chemical reduction of specific organic or inorganic agents. For assembling nanostructures, the bottom-up approach uses physical and chemical processes, such as UV irradiation photoreduction (Zhou et al. 1999), photoinduced reduction (Shchukin et al. 2003), radiation-assisted method (Abid et al. 2002; Fierascu et al. 2019), microwave heating (Nadagouda et al. 2011) or bio-based protocols. Through biological method, NPs were synthesized intracellularly and extracellularly. Intracellular synthesis was reported using bacteria, such as *Lactobacillus casei* subsp. case (Korbekandi et al. 2012) and *Pseudomonas stutzeri* AG259 (Klaus et al. 1999). Extracellular synthesis of NPs by fungi from the species *Fusarium oxysporum* (Ahmad et al. 2003) or by alga *Spirulina platensis* (Govindaraju et al. 2008) was also revealed. The use of plant extracts is a simple, cost-effective and eco-friendly alternative for synthesis of NPs. Pteridophyte and spermatophyte species have been successfully used in the synthesis of metal nanoparticles. From pteridophyte, such as *Asplenium scolopendrium* (Şuțan et al. 2016) and *Polystichum setiferum* (Şuțan et al. 2019a), from gymnosperms, such as *Cycas* (Jha and Prasad 2010), from angiosperms, such as *Aconitum toxicum,* were obtained alcoholic or hydro-alcoholic extracts, which acted as the biological reducing agents in the formation of NPs from metal salts (Şuțan et al. 2019b).

ENPs can have different sizes and shapes, such as spherical, tubular (Nowack and Bucheli 2007), cubic, nanowires (Sun and Xia 2002), nanorods (Gu et al. 2006), nanohorns, nanostars (Lee et al. 2019) or irregularly shaped (Wu et al. 2020) and can be available as fused, aggregated or agglomerated forms (Nowack and Bucheli 2007).

Depending on the components used for their synthesis, ENPs are divided into organic (polymeric), inorganic and composites (Sekhon 2010; Teleanu et al. 2018).

The group of organic ENPs includes dendrimers, nanogels, micelles and liposomes. Being characterized by a high biocompatibility and biodegradability, polymeric nanoparticles are widely used as a drug delivery system (Kumar et al. 2012) and carriers of proteins and deoxyribonucleic acid (DNA) (Soppimath et al. 2001). Inorganic ENPs are deliberately synthesized by humans using various materials, including metals (Ag, Au, Ti, Cu, Zn, Au, Ni, Fe, Co, Zn, In), metal oxides and binary oxides (TiO_2, SiO_2, CeO_2, Fe_3O_4, ZnO, Co_3O_4, Al_2O_3, In_2O_3, SnO_2, Mn3O4, $LiCoO_2$) (Xu et al. 2012; Faisal et al. 2016; Faisal and Kumar 2017; Bozon-Verduraz et al. 2009; Gui et al. 2020), nonmetals, such as silica and quantum dots (Khademolhosseini et al. 2020; Antolini and Orazi 2019), carbon, such as carbon nanotubes, carbon dots, graphene and fullerenes (Georgakilas et al. 2015) or green materials, like chitosan (Kumar et al. 2020).

Although a large number of studies concerning the effects of ENPs on the environment and living things have been reported, there is a lack of a complete understanding of their toxicity to plants (Zuverza-Mena et al. 2017). The phytotoxicity can be evaluated in terms of morphophysiological, cellular and molecular changes induced by ENPs (Večeřová et al. 2016; Yan and Chen 2019).

The most studied morphological parameters for investigating the effect of NPs in plants include root/stem growth potential, germination ratio, biomass, leaf number and leaf area (Ruttkay-Nedecky et al. 2017; Yan and Chen 2019). Several studies have shown that ENPs didn't have an adverse effect on seed germination. This could probably attribute to a selective permeability of the seed coats (Mahmoodzadeh and Aghili 2014). For example, copper oxide nanoparticles (CuONPs) did not inhibit the germination of *Arabidopsis thaliana* (L.) Heynh seeds (Wang et al. 2016) and *Zea mays* (Wang et al. 2012), single-wall carbon nanohorns (SWCNHs) at a concentration of 100μg/mL increased seed germination of tomato, barley, corn, rice and switchgrass seeds (Lahiani et al. 2015) and titanium dioxide nanoparticles (TiO_2NPs) increase the germination of spinach seeds (Zheng et al. 2005). Wang et al. (2016) noticed a significantly reduced germination of pollen grains and seeds harvested from *A. thaliana* plants, previously exposed to CuONPs, suggesting a transgenerational inhibitory effect of CuONPs and a potential negative impact on plant productivity/yield and food quality. Transgenerational transmission of carbon and fullerene nanomaterial and their likely impact on the food chain have also been reported in an extensive review (Husen and Siddiqi 2014).

The physiological assessment of NP-induced phytotoxicity focuses on the changes in chlorophyll content, water and nutrient absorption capacity, transpiration rate, and interaction with pathways of synthesis or signaling of endogenous hormones. Zuverza-Mena et al. (2016) found that direct exposure of radish (*Raphanus sativus*) seeds to 125, 250 and 500 mg/L silver nanoparticles (AgNPs) suspension diminishes the water content and impairs the uptake of nutrients in seedlings. Immersion of Oriental hybrid lily (*Lilium* cv. Mona Lisa) bulbs in the AgNPs solution of various concentrations (25, 50, 100 and 150 ppm) enhanced plant growth, flowering, leaf and bulb biomass (Salachna et al. 2019). Thakur et al. (2018) reported that gold nanoparticles (AuNPs) increased chlorophyll content in *Solanum lycopersicum* L.

The multitude of methods for preparing samples and evaluating the nanophytotoxicity predisposes to an inconsistency between various estimates of potential effects of ENPs, which can be amplified or diminished (Jośko and Oleszczuk 2014).

It is notorious that NPs exert species-specific phytotoxicity, which varies in the same species depending on the mode of application, exposure time (Wang et al. 2016) and the type of organ (Hossain et al. 2020). Stampoulis et al. (2009) found that multiwalled carbon nanotubes (MWCNTs), AgNPs, copper nanoparticles (CuNPs), zinc oxide nanoparticles (ZnONPs) and silicon nanoparticles (SiNPs) did not induce negative effects on seed germination, but the root length was affected by CuNPs. The same authors considered that seed germination rate and root elongation toxicity tests are not the most suitable to assess NP-induced phytotoxicity in terrestrial plants. Wang et al. (2016) stated that compared to shoots, the roots were much more sensitive to CuONPs. At the same time, phytotoxicity depends on the NPs properties, such as size (Larue et al. 2012; Hawthorne et al. 2014), charge (Santana et al. 2020), shape, chemical composition, zeta-potential, stability and hydrophobicity (Avellan et al. 2019).

6.1.2 Mechanism of Cytotoxicity and Genotoxicity Induced by Engineered Nanoparticles

The data presented in the scientific publications highlight the contrasting effects of ENPs on plant development and the food chain, emphasizing the need for thorough investigation of NPs–plant interactions. However, NPs–cell interactions are highly vulnerable to many biotic or abiotic factors. Alteration in cellular structure and disruption in the cell division process are indicators of nanophytotoxicity at the cellular and molecular level. Disproportionate increase in reactive oxygen species (ROS) can be the possible mechanism of assessing NPs-induced phytotoxicity.

6.1.2.1 Exposure Routes of Engineered Nanoparticles: Entry Mechanisms into the Plant Cells

Exposure of living organisms to ENPs could be the result of their uncontrolled release into the environment, from everyday objects, landfills, industrial leaks, etc. (Długosz et al. 2020). This unconstrained exposure could be amplified in time and space by uncontrolled discharge and delivery of ENPs. Industrial processes, such as water treatment technology, bioremediation, removal of pollutants (Remédios et al. 2012), deliberate use of products to improve crop yields are important sources of ENPs released into the environment. In recent years, ENPs have been designed as nano-delivery systems of biomolecules (DNA, proteins) in plants (Martin-Ortigosa et al. 2014; Vega-Vásquez et al. 2020), nanopesticides and nanofertilizers (Fraceto et al. 2016; Rehmanullah et al. 2020), nanozeolites and hydrogels for soil quality improvement, as well as nanosensors for real-time monitoring of crop health and soil quality (Fraceto et al. 2016). In order to anticipate the impact of ENPs on organisms and ecosystems, the shape, mass and route of entry into cells, mechanisms of

absorption, translocation and accumulation, bioavailability, cytotoxicity, potential adverse effects on growth, development and biogeochemical processes must be evaluated (Klaine et al. 2012). Moreover, the effects of coexistence of ENMs with various organic or inorganic pollutants can change the absorption rate, translocation, and interaction of plants with each material (Deng et al. 2017). In a co-exposure experiment performed by Li et al. (2018), graphene oxide significantly enhanced polycyclic aromatic hydrocarbons accumulation in rice (*Oryza sativa* L.). In another study, CuONPs reduced the arsenic absorption by rice grain (Liu et al. 2018), and co-exposure to AgNPs associated with magnetic field improved quantitative yields in *Zea mays* L. (Berahmand et al. 2012).

Regardless of the exposure route, NPs adhere to the surface of roots or leaves via electrostatic, hydrophobic and van der Waals forces (Schwab et al. 2011; Zhang 2015). After foliar exposure, ENPs are retained on the cuticular wax from the outermost barrier and internalized through trichomes (Khan and Rizvi 2014) or stomata and transported to the roots via phloem together with photosynthesis products (Kranjc et al. 2017).

The absorption of NPs over the underground parts takes place mainly through physiologically active lateral roots and root hairs (Khan and Rizvi 2014). NPs are captured in border-like cells-associated mucilage and then internalized in the root cap (Avellan et al. 2017). NPs pass through the root apex to the stars and are transported via xylem to the shoots (Wang et al. 2012, 2016).

Depending on their size, NPs internalization occurs through cell wall pores (Davison et al. 2013), endocytosis (Kurepa et al. 2010) and plasmodesmata (Zhai et al. 2014). Permeation of lipophilic compounds across the plant cuticles and the pectin's pores size variable between 0.6 and 4.8 nm (Wild et al. 2005) favor the accumulation of small neutrally charged NPs (Schwab et al. 2015). Cell uptake of positively charged NPs is achieved by clathrin-mediated endocytosis, caveolin-mediated endocytosis (Manzanares and Ceña 2020), and neutrally charged NPs endocytosis is clathrin-independent (Onelli et al. 2008). Binding of ultrasmall NPs to carrier proteins (Rico et al. 2011) and ion transporters could be another likely way of NPs uptake (Fig. 6.1). Although a relatively recent study showed that carbon nanotubes resulted in a significant up-regulation of NtPIP1 gene aquaporins (Khodakovskaya et al. 2012), there is a lack of consistent studies regarding the NPs penetration via aquaporins and ion channels. The wounds were also gatewaying for fluorescent quantum dots (QDs) (Al-Salim et al. 2011) or AgNPs (Lu et al. 2010), but the translocation of NPs to the apoplast was limited. Symplastic transport of NPs is possible by plasmodesmata (Zhai et al. 2014), but studies to support and demonstrate this type of transport are very rare.

The absorption capacity of NPs by plant roots varies depending on the species, NPs charge and the anatomical and physiological properties of the plants. Lahiani et al. (2015) observed a different NPs absorption capacity depending on the species, SWCNHs being absorbed in a higher proportion of corn roots compared to soybean roots. NPs charge significantly influence the uptake, translocation and spatial distribution within the plant tissues. The polarity of phospholipid molecules inside the cell membrane allows a higher absorption rate of positively charged NPs compared to

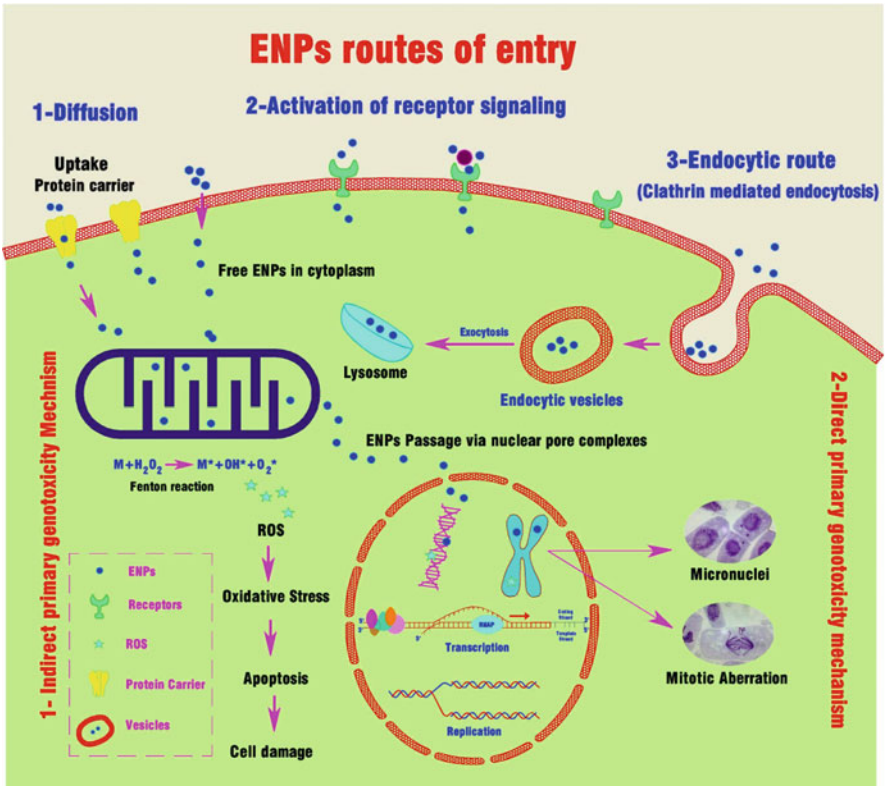

Fig. 6.1 Engineered nanoparticles (ENPs) routes of entry through plant cell

negatively or neutral charged NPs (Mu et al. 2014; Verma and Stellacci 2010). However, the interaction of NPs with the cell surface is not limited to simple statements that generalize their behavior. Thus, compared to positively charged cerium oxide nanoparticles (CeO_2NPs), those negatively charged adhered significantly less to the root surface, but were more efficiently translocated to shoots. At the same time, translocation of CeO_2NPs to shoots was more efficient in dicotyledons than in monocotyledons, suggesting that the anatomical and physiological diversity of plants are determinants of NPs transport and distribution (Spielman-Sun et al. 2019).

6.1.2.2 Nano-Bio-Interactions and Toxicity of Engineered Nanoparticles

The increased application of NMs in various fields, constant risk of exposure, uptake and uncontrolled translocation have led to the initiation of comprehensive researches on their safe use and safe applications. The physicochemical interactions between ENPs and biomolecules depend on several factors, such as the elemental and protein composition (type, amount and conformation) of the corona of NPs (Behzadi et al. 2017; Jackson et al. 2017), porosity, size, shape and surface area, surface crystallinity, ligands, solid–liquid interface, contact surface with an organic molecule

(Bhaumik et al. 2014), microenvironmental factors (Yuan et al. 2012; Pulido-Reyes et al. 2017). Depending on this multitude of factors, bio-nano-interactions can induce a wide variety of cellular responses (Juárez-Maldonado et al. 2019).

NPs are characterized by a high surface/volume ratio and implicitly by a high level of superficial free energy, due to which they have a high reactivity (Powers et al. 2007) to themselves, forming aggregates, and also to other molecules/biomolecules (Loosli et al. 2015). Several studies regarding the size of NPs have shown that the smaller NPs, the more reactive they become and the more toxic they are to cells (Krug and Wick 2011; Liu et al. 2020). The size of NPs matters (notably, in cell absorption) even is not mandatory for the induction of cytotoxicity (Karlsson et al. 2015). The mechanisms and ability of the body to assimilate, translocate, eliminate toxicity potential, i.e., cytotoxicity, genotoxicity, mutagenicity, apoptosis (Powers et al. 2007) depends on the size of NPs. For example, 25 nm TiO_2NPs did not affect seed germination rate or root growth, while 12 nm TiO_2NPs were absorbed in the roots and translocated in the parenchyma cells and vascular cylinder (Larue et al. 2011). Inhibition of seed germination and toxicity were dependent on the size of ZnO particles, ZnONPs inducing greater toxicity than micro-sized ZnO particles (Lee et al. 2010). In *Allium cepa* L. ZnONPs <10 nm passed easily through the cell membrane and formed agglomerates with other molecules within the cell (Ahmed et al. 2017). In a study on the effect of AuNPs of 10, 14 and 18 nm diameter, Siegel et al. (2018) noticed that the smallest NPs had a negative effect on primary root growth of *A. thaliana*, inducing root hair growth, while Thakur et al. (2018) found that smaller sized AuNPs were more efficient for inducing growth of *Solanum lycopersicum* L. plants.

Not only size matters, but also shape of the NPs. During the germination period, silver nanosphere decreased the roots and shoots growth and diminished root hair abundance of the annual ryegrass (*Lolium multiflorum*), whereas nanocubes and nanowires did not exhibit toxicity (Gorka et al. 2015). Syu et al. (2014) showed that although the decahedral and triangular AgNPs are similar in size, the decahedral AgNPs promoted root growth associated with the lowest levels of Cu/Zn superoxide dismutase2 (CSD2) in *A. thaliana*.

The level of toxicity depends on the direct contact between NPs and cell membranes, by aggregation, interaction of NPs-cells being remarkably diminished (Zeyons et al. 2009). Following in vitro studies, it has been reported that NPs suspended in culture media form agglomerations whose bioavailability is altered by changing their contact surface, charge and solubility (Jiang et al. 2009; Oukarroum et al. 2015). Dobrucka et al. (2019) noticed that biosynthesized, spherical shaped and locally agglomerated AgNPs do not exhibit any toxic effects against *Linum flavum* and *Lepidium sativum* seeds.

The toxicity of NPs varies with exposure time, suggesting that standard experiments may underestimate the effects of chronic exposure. Accordingly, the severity of the phytotoxic effects of ZnONPs on the *Lemna minor* model species gradually increased after 4–6 weeks, with the increase of Zn content in plants (Chen et al. 2018).

Bioavailability of metallic NPs is highly dependent on their colloidal stability. Dissolution of metallic NPs increases the bioavailability of metal ions so that the toxicity of NPs in aqueous solutions is highly dependent on the concentration of metal ions released. Depending on the interaction between the redox potential of NPs and of cell microenvironment, internalized metallic NPs and metal oxides NPs release metal ions, whose toxicity is lower compared to NPs in equivalent concentrations (Wu et al. 2012). More specifically, other publications have revealed that the toxicity of AgNPs is due to the generation of Ag^{+} by oxidative dissolution (Liu and Hurt 2010) associated with the production of ROS (Simeone and Costa 2019). The toxicity of ferric oxide nanoparticles (Fe_2O_3 NP) is determined by the release of Fe^{2+} (Auffan et al. 2008). Adenosine triphosphate (ATP) forms chelated complexes with Zn^{2+}, in the binding reactions being involved phosphate and adenine (Bhaumik et al. 2014). Additionally, the materials solubility and the concentration gradient at the particle/solution interface, in the milligram per liter concentration range, affect dissolution (Borm et al. 2006). Baalousha (2009) and Baalousha et al. (2015) noticed that iron oxide nanoparticles (Fe_2O_3NPs) form smaller aggregates at concentrations between 10 and 200 mgL^{-1}. It is also important to notice that NPs with a diameter <25 nm have a higher rate of dissolution and release of toxic ions (Bottero et al. 2011). At the same time, dissolution and aggregate size of NPs vary in a concentration-environment dependent manner. For instance, coatings of NPs may represent a solution for mediating the bioavailability of NPs and the dissolution of ions within the soil, rhizosphere and microenvironment of cells, with limitation of toxicity to plants (Cartwright et al. 2020). The cellular microenvironment can induce changes in the coating and NPs itself. Using shoots and leaves of mesquite plants (*Prosopis* sp.), Parsons et al. (2010) reported for the first time the biotransformation of nickel nanoparticles (NiNPs) into Ni (II)—organic acid complex. Soybean (*Glycine max*) plants induced a reduced dissolution of CeO_2NPs and biotransformation from Ce (IV) to Ce (III) (Hernandez-Viezcas et al. 2013).

NPs can influence the production of phytohormones (Yang et al. 2017), amino acids, fatty acids, sugars and phenols (Rico et al. 2014). Notably, AgNPs toxicity is generally manifested from the seedling growth stage to the fully developed plant (Yin et al. 2012a), through morphological, physiological (Ma et al. 2010; Tripathi et al. 2017) and genotoxic changes (Şuțan et al. 2016; Şuțan et al. 2019a, b; Heikal et al. 2020).

Using beans and wheat, Lee et al. (2008) found a dose-dependent accumulation and phytotoxicity of CuNPs. Currently, most of the studies on ENPs toxicity to plants revealed that high concentration of ENPs has often been identified as a responsible factor for toxicity (Table 6.1). It should not be overlooked that a consistent number of studies have indicated the positive effects of metal NPs and metal oxide NPs on plant growth. Zhu et al. (2008) observed that iron oxide nanoparticles (Fe_3O_4NPs) were absorbed, translocated and accumulated in the tissues of pumpkin plants, showing no toxic effects. Other NPs, such as ZnONPs, are non-toxic, biosafe and biocompatible (Zhou et al. 2006a).

Table 6.1 Summary of literature related toxic effects induced in plants by various concentrations of NPs

Nanoparticles	NPs concentration	Plant species	Assessments	References
Metal nanoparticles				
Ag	12.5, 25, 50, 100 mg L^{-1}	*Vicia faba*	Genotoxic effects	Patlolla et al. (2012)
Ag	0,12.5, 25, 50,100 and 200 mg L^{-1}	*Stevia rebaudiana* B. in vitro culture	Inhibits development increase in chlorophyll a, b and total contents	Castro-González et al. (2019)
Au	1, 10, 100 mgL^{-1}	*Arabidopsis thaliana*	Significantly decreased the number and length of lateral roots	Siegel et al. (2018)
Mn	0.05 mg L^{-1}	*Vigna radiata*	Enhanced the net flux of nitrogen assimilation	Pradhan et al. (2014)
Si	25, 50, 75, 100, 200μg/mL	*Lens culinaris*	Chromosomal aberrations, decrease in the mitotic index, decrease in the germination percent	Khan and Ansari (2018)
Zero-valent iron (nZVI)	1000–2000 mg L^{-1}	*Linum usitatissimum*, cv. Electra, *Lolium perenne*, cv. Tove, *Hordeum vulgare*, cv. Annabell	Complete inhibition of germination	El-Temsah and Joner (2012)
Bimetallic nanoparticles				
Cu-Ag	100 ppm	*Prosopis glandulosa*	Significantly reduced the chlorophyll, epidermal polyphenol content and photochemical efficiency	Gonzales-Mendoza et al. (2019)
Au-Ag	10% and 20% ethanolic extract of *M. officinalis* with ag - au NPs	*Allium cepa*	Clastogenic aberrations	Fierascu et al. (2017)
Metal oxide nanoparticles				
ZnO	85/200, 400, 800 mgL^{-1}	*Allium cepa*	Increased cytotoxicity in	Ghosh et al. (2016)

(continued)

Table 6.1 (continued)

Nanoparticles	NPs concentration	Plant species	Assessments	References
			meristematic root cells	
ZnO	50, 100, 200, 500, 1000μg/mL	*Allium cepa*	Decreased mitotic index, metaphase and anaphase chromosomal aberrations	Ahmed et al. (2017)
TiO2	200μg/mL	*Lens culinaris*	Decrease in total chlorophyll content, excessive increased production of lipid peroxidation, decreased mitotic index, augmented DNA damage and aberrant mitotic cell division	Khan et al. (2019)
CuO	30 ± 10/10, 200, 1000 mg L^{-1}	Transgenic cotton (Bt-29,317)	Elevated expression of Bt-toxin protein in leaves and roots	Van et al. (2016)
CuO	1000 mg (CuO NP) L^{-1}	*Oryza sativa*	Complete loss of PSII photochemical quenching, enhanced malondialdehyde and proline contents, increase in the expression of ascorbate peroxidase and superoxide dismutase	Da Costa and Sharma (2016)
CuO	150 and 200 mg L^{-1}	*Zea mays*	Complete germination retardation	El-Shazoly and Amro (2019)
$Cu(OH)_2$	25, 75 mg/L	*Medicago sativa*	Reduced root elongation, significantly reduced the concentration of K, P in alfalfa seedlings, significant up-regulation of cu/Zn SOD	Cota-Ruiz et al. (2018)

(continued)

Table 6.1 (continued)

Nanoparticles	NPs concentration	Plant species	Assessments	References
NiO	30 nm/nearly spherical/ 1000 mg L^{-1}	*Lemna gibba*	Strong increase in ROS formation, strong inhibitory effect on the PSII quantum yield	Oukarroum et al. (2015)
Co_3O_4	1.0 mg/mL	*Solanum melongena cv. Violetta lunga 2*	Decreased seed germination, increased level of DNA damage, alterations in mitochondrial cristae, peroxisomes abundance and inordinate vacuolization, and cell death	Faisal et al. (2016)
CeO_2	2000 mg L^{-1}	*Glycine max*	DNA damage, mutations	López-Moreno et al. (2010)
BO	50 mg L^{-1}	*Allium cepa*	DNA damage	Liman (2013)

6.2 Nanoparticle-Induced Oxidative Stress in Plants

One of the most frequently reported toxic effects associated with NPs is the production of ROS that generate oxidative stress (Azam et al. 2020). ROS are by-products of aerobic metabolism and play a dual role. When maintained at basal levels, ROS constitute signal molecules that have an important role in growth, differentiation, cell signaling and in improving stress tolerance in plants, on the other hand, excess ROS causes irreversible DNA damage and cell death (Huang et al. 2019).

Stress caused by biotic and abiotic factors, such as drought, salinity (Molassiotis et al. 2016; Shah et al. 2017), chilling (Zhou et al. 2006b; Hu et al. 2015), metal toxicity (Shah et al. 2001; Juknys et al. 2012), UV-B radiation (Barta et al. 2004; Han et al. 2009), pathogen attacks, (Grant et al. 2000; Li et al. 2019) may induce increased ROS production in plants. NP-induced oxidative stress is due to both acellular factors, such as particle surface, size, elemental composition, and cellular factors, such as mitochondrial respiration, NPs–cell membrane interaction (Manke et al. 2013).

6.2.1 Roles and Mechanisms of ROS, Oxidative Stress, and Oxidative Damage

In physiological context, the main sources of ROS production in plant cells are organelles with high oxidative metabolic activity or an intense rate of electron flow, cytochrome-catalyzed detoxification reactions in the cytoplasm and endoplasmic reticulum. In the mitochondrial matrix and intermembrane space, during the process of ATP synthesis, a small percentage of oxygen is not completely reduced, leading to the generation of superoxide anion and other radicals (Møller 2001; Yin et al. 2012b). The superoxide anion radicals are produced by complex I (NADH ubiquinone oxidoreductase) and complex III (co-enzyme Q, bc1 complex, and uniquinone/cytochrome c reductase) activity (Tahara et al. 2009). In chloroplasts, ROS production through Mehler reaction (Asada and Takahashi 1987) is stimulated by unfavorable environmental factors that limit CO_2 fixation. In microbodies, ROS are produced as a side-product of fatty acid oxidation in lipid catabolism (Møller 2001).

Biologically relevant ROS are superoxide anion radicals ($[O_2]^{\bullet}$), hydroxyl radicals ($[OH]^{\bullet}$), singlet oxygen ($^1O_2$), lipid peroxide (ROOH), hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) (Dickinson and Chang 2011). Of all types of ROS, $[OH]^{\bullet}$ has the highest reduction potential and is very reactive with other types of biomolecules (Halliwell and Gutteridge 1989). Production of ROS, such as H_2O_2 is initiated by the plasma membrane NAD(P)H oxidase for cell wall polysaccharides cleavage during cell growth (Schopfer and Liszkay 2006) or in response to abiotic stimuli, such as chilling temperatures (Piotrovskii et al. 2011). Small increases in H_2O_2 allow for an overall improvement in stress tolerance, but a considerable accumulation of H_2O_2 triggers local responses that inevitably lead to programmed cell death (Almagro et al. 2009).

ROS cause oxidative changes in cellular components, leading to the disruption of cellular functions and cell death. ROS bring severe damage to different targets, including protein, lipids, nucleic acids and pigments. According to Anjum et al. (2015) products of lipids and protein oxidation are significant biomarkers of abiotic stress in plants. Lipid oxidation leads to the formation of lipid radicals, which indirectly induce damaged proteins and DNA (Smirnoff 2000, cited by Das and Roychoudhury 2014). Lipid peroxidation by ROS affects cellular integrity, which allows ion leakage and causes disruption of cellular metabolism.

Direct damage of protein consists of various chemical changes, including nitrosylation, carboxylation, carbonylation, disulfide bond formation, and glutathionylation, the protein radicals generated during the process serve as a marker of protein oxidation (Møller et al. 2007). Carbonylated proteins aggregate irreversibly, losing their function permanently (Amici et al. 1989).

ROS can act indirectly on the proteins with which DNA is associated or directly on the DNA in the absence of protective histones. Oxidative damage of DNA includes oxidation of the deoxyribose sugar residue by extracting the C-4 H-atom, modification of the nucleotide bases (Bjelland and Seeberg 2003), breaks in either DNA strand, DNA-protein cross-links (Das and Roychoudhury 2014) and chromosomal instability (Limoli and Giedzinski 2003). Due to their low redox potential,

nucleotide bases are vulnerable to the direct action of ROS (Bjelland and Seeberg 2003). Oxidative DNA damage, including that induced by NPs, results in a mismatch of DNA bases (Bridge et al. 2014), inhibition of amino acid synthesis and replication (Huh and Kwon 2011), genomic instability and mutation (Poetsch 2020).

DNA damage affects the ability of cells to grow and proliferate and can be associated with cell death. Plant stem cells are extremely sensitive to DNA damage and are prepared to enter cell death to protect meristems against the accumulation of mutations (Fulcher and Sablowski 2009).

6.2.1.1 Overproduction of ROS and Cell Damage

Overproduction and mismanagement of ROS can lead to oxidative stress, in which cells can no longer maintain the redox-regulated functions in normal parameters (Halliwell and Gutteridge 1989). It has been stated that a first probable step in the interaction between NPs-cell surface is due to the electrostatic attraction between the integral and peripheral membrane proteins and the corona of NPs. The direct contact between NPs and cell membrane components is the result of decreased free energy surface and hydrophobicity manifested against the water molecules in the apoplast (Nel et al. 2009). This interaction determines the adjustment of cell surface charges and modification/inhibition of the activity of integral membrane proteins like receptors and transport proteins that disturb metabolic pathways and gene expression (Zuverza-Mena et al. 2017). It is assumed that all types of NPs exert this type of interaction with the cell surface, regardless of their chemical composition (Juárez-Maldonado et al. 2019). Adaptations of the cell membrane to the interaction with NPs trigger significant changes, which can range from biostimulation to toxicity (Jackson et al. 2017), from positive to negative (Bell et al. 2014). NPs-induced oxidative stress alters mitochondrial respiration and cellular homeostasis (i.e., calcium homeostasis), antioxidant enzymes activity, activates the NAD(P)H oxidase system, causes mitochondrial apoptosis and ultimately tissue damage (Sharma et al. 2019).

6.2.1.2 Dependence of ROS Production on the Properties of Engineered Nanoparticles

Due to their chemical instability, NPs can generate a sharp increase in ROS. Key factors that induce the prooxidant effects of ENPs include a decrease/depletion of antioxidants or an increase in ROS production (Manke et al. 2013). NPs cause depolarization of mitochondrial membrane potential and interfere with/block the electron-transport chain by activating NAD(P)H-related enzymes, thus increasing the intracellular level of ROS via the Fenton reaction (Fig. 6.1) (Xia et al. 2006; Soenen et al. 2011). Dissociated metal ions (i.e., Ca^{2+}, Ag^{+}) can cause reduction of mitochondrial membrane potentials (Kang et al. 2012), can interfere with the expression of oxidative stress-related genes and antioxidant genes (Lee et al. 2002; Zhang et al. 2018a, b) and thus accelerates the intracellular accumulation of ROS.

ROS formation in cell is dependent on the physicochemical properties of NPs (Table 6.2), test systems, interaction with environmental factors, and cell types. The physical and chemical characteristics of NPs that lead to the formation of ROS

Table 6.2 Summary of literature related with NP-induced oxidative stress in plants

Nanoparticles	ENPs characteristics (size/shape/ concentration)	Plant species	Oxidative damage	References
Ag	20 nm/spherical/ 0, 2, 10, 20 mg L^{-1}	*Solanum tuberosum*	Altered activity of SOD, CAT, APX and GR depletion of non-enzymatic antioxidants (GSH and ASA)	Homaee and Ehsanpour (2016)
Cu^{o}	30–40 nm/NA/ 69.4μM	*Zea mays*	Increased anthocyanin content, enhanced activities of SOD and APX	Nguyen et al. (2020)
CuO	50 nm/ aggregated/10, 50, 100, 500, and 1000 mg L^{-1}	*Cucumis sativus*	Increased antioxidant enzyme activities	Kim et al. (2012)
	39 ± 3 nm/ spherical, truncated, and uneven nature/ 1500 mg/L	*Brassica juncea*	Excessive presence of H_2O_2, increased proline and malondialdehyde content	Rao and Shekhawat (2016)
ZnO	50 nm/nearly spherical/10, 50, 100, 500, and 1000 mg L^{-1}	*Cucumis sativus*	Increased antioxidant enzyme activities	Kim et al. (2012)
	18 nm/NA/100, 250, 500 and 1000 mg L^{-1}	*Solanum melongena*	Prominent ROS formation, MDA production at higher concentrations (500 and 1000 mg/L)	Baskar et al. (2018)
TiO2	44 ± 4 nm/ spherical shape/ 1500 mg L^{-1}	*Brassica juncea*	Excessive generation of H_2O_2, higher proline and malondialdehyde content	Rao and Shekhawat (2016)
Al_2O_3	100μg mL^{-1}, spherical shape, 20 ± 5 nm	*Trigonella foenum-graceum*	Increase in the malondialdehyde content and CAT activity	Owji et al. (2019)
CdO	7–60 nm/ 2.03 ± 0.45105 per cm^3 of air	*Hordeum vulgare* L.	Markedly increased phenylalanine, tryptophan, valine, leucine, asparagine and tyrosine content	Večeřová et al. (2016)
MWCNT	10–30 nm diameter/5–15μm length/NA/20 mg/ L	*Oryza sativa* L.	Significant increase of intracellular ROS	Tan et al. (2009)
NiO	30 nm/nearly spherical/1, 10, 100, 1000 mg L^{-1}	*Lemna gibba*	Strong increase in ROSproduction	Oukarroum et al. (2015)

(continued)

Table 6.2 (continued)

Nanoparticles	ENPs characteristics (size/shape/ concentration)	Plant species	Oxidative damage	References
NiO	23.34 nm/ crystallites spheres/0.025 to 2.0 mg/mL	Tomato seedling roots	Enhanced the activities of CAT, SOD and GSH	Faisal et al. (2013)
CeO_2	10 ± 1 nm/ aggregates 2124 ± 59 nm/ 400 and 800 mg NPs/kg soil	*Zea mays*	Increased CAT and APX activities, increased H_2O_2 levels	Zhao et al. (2012)
	8 ± 1 nm, particle size 231 ± 16 nm/ rod/ 0–500 mg L^{-1}	Rice	Ion leakage at higher concentrations	Rico et al. (2013b)

include elemental composition, size, shape, oxidation status, surface area, surface coating, surface positive charge, solubility, aggregation and agglomeration degree (Fu et al. 2014). In soybean, 30–60 nm aluminium oxide nanoparticles (Al_2O_3NPs) positively regulate energy metabolism, while 5 nm and 135 nm Al_2O_3NPs had a negative effect (Mustafa and Komatsu 2016). It is worth mentioning here that the size is not always a factor in amplifying or decreasing the toxicity of NPs. In a comparative study of *Trigonella foenum*, Owji et al. (2019) showed that alumina NPs did not involve higher toxicity compared to bulk (macrometer-sized particles) alumina.

Nigella arvensis treated with 50 to 1000 mg/L of Al_2O_3NPs exhibited significantly enhanced activities of antioxidant enzymes, such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and superoxide dismutase (SOD), and antioxidant compounds (total iridoids, total saponin and total phenolic) along with DPPH scavenging activity (Chahardoli et al. 2020). Similarly, in the range of 200–500 mg/L Al_2O_3NPs induced an elevated activity of SOD and CAT in *Triticum aestivum* seedlings (Riahi-Madvar et al. 2012). Although most NPs are considered redox-active, Al_2O_3 and SnO_2 are considered redox-inactive (Chemicals 2018, cited by Sousa et al. 2019).

The multitude of factors involved in the evaluation of the oxidative activity of NPs provides the basis for contradictory statements. For example, it is claimed that CeO_2NPs exhibited unique antioxidant activity by eliminating free radicals (Thakur et al. 2019). Therefore, 125 mg L^- treatment with CeO_2NPs (rod with primary size of 8 ± 1 nm, particle size of 231 ± 16 nm in solution, surface area of 93.8 m^2g^{-1} and 95.14% purity) decreased the H_2O_2 content in both *Oryza sativa* shoots and roots (Rico et al. 2013a). However, 400 and 800 mg/kg treatment with CeO_2NPs (primary size of 10 ± 1 nm, particle size of 2124 ± 59 nm in solution, with a zeta-potential of 22.8 ± 4.5 mV) increased the H_2O_2 content in *Zea mays* shoots (Zhao et al. 2012).

These results suggest that biotic and abiotic factors are of significant importance in defining the response of plants to oxidative stress.

Disruption of redox homeostasis by ZnONPs has been highlighted in several plant species, such as *Lolium perenne* L., *Cucurbita mixta* L. (Wang et al. 2011), *Spirodela polyrhiza* L. (Upadhyay and Panda 2010), *A. cepa* L. (Kumari et al. 2011). Toxicity was not attributed to ZnONPs per se, but to their solubility (Franklin et al. 2007). Using seedlings of *A. thaliana* exposed to the action of CuONPs, an increase in the concentration of anthocyanins (Gill and Tuteja 2010), superoxide and hydrogen peroxide (Nair and Chung 2014) was observed in the roots and leaves. It has been suggested that transition metal ions such as Cu^{2+} ions resulting from the solubilization of CuONPs catalyze the excess production of OH (Halliwell and Gutteridge 2007). In a virtual simulation, Wang et al. (2017) highlighted four main descriptors in the induction of oxidative stress by AuNPs, namely the number of surface ligands, the preference for hydrophobic contact, the potential for interaction with water molecules and electrostatic positivity.

6.2.2 Consequences of Oxidative Stress and ROS-Mediated Pathways: Cellular Signaling Stress Response

In response to oxidative stress, plants have developed antioxidant systems and complex signaling networks to preserve metabolic homeostasis and to integrate various cellular signals (Sipari et al. 2020). Enzymatic ROS scavenging systems include CAT (Owji et al. 2019), APX, SOD (Rao and Shekhawat 2016), glutathione peroxidase (GPX), peroxiredoxin (PrxR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Foyer and Noctor 2005). Other non-enzymatic systems by which plants protect themselves from oxidative stress include defense chemical compounds, namely primary (Sipari et al. 2020) and secondary (Tavares et al. 2019; Zhao et al. 2005) metabolites. The most important non-enzymatic antioxidants are ASA, GSH, carotenoids and phenolics (Kasote et al. 2015).

The initial response to abiotic stress in plants includes a transient increase in cytoplasmic Ca^{2+}, ROS production and intracellular activation of secondary messengers, such as inositol polyphosphate, abscisic acid (ABA) and mitogen-activated protein kinase (MAPK) pathways (Kohan-Baghkheirati and Geisler-Lee 2015; Sewelam et al. 2016). The next level of stress response involves up- and down-regulation of stress-specific genes (Kohan-Baghkheirati and Geisler-Lee 2015).

Cytoplasmic Ca^{2+} elevation is possible by mobilizing it from its accumulation sites to cytosol through Ca^{2+} channels, where it is sensed by Ca^{2+}-binding proteins (CaBPs), which in turn initiate downstream processes leading to changes in gene expression (Khan et al. 2017).

ABA also known as "stress hormone" (Zhang 2014) is a sesquiterpene synthesized de novo in plant roots and terminal buds during drying up process. The endogenous level of this hormone is determined by several stress signals, which

can stimulate the synthesis of enzymes that form ABA from β-carotene (Roychoudhury and Basu 2012; Vishwakarma et al. 2017).

Changing ROS levels modulates defense pathways. There is a close interaction between ROS production and elimination pathways determined by the intensity, duration and location of ROS signals, which are responsible for protection against oxidative damage (Mittler et al. 2004). ROS operates as intercellular signaling molecules. Each ROS has a number of chemical characteristics that ensure the specificity of the signaling pathways. $O_2^{\bullet-}$ is unstable, does not diffuse across the cell membrane due to its negative charge and oxidizes Fe-S clusters. H_2O_2 is relatively stable, can diffuse through cell membranes and can oxidize Cys residues. $OH^{\cdot}$ has a very high reactivity and is very toxic. $^1O_2$ interacts quickly with amino acids, unsaturated lipids and other chemicals in the immediate vicinity of the site where it was produced (Sewelam et al. 2016).

In addition to this signaling specificity, ROS can activate or deactivate transcription factors (TFs), which play a key role in up- and down-regulation of gene expression. The transcription factor UPBEAT1 modulates the balance between root cell proliferation and differentiation in *A. thaliana* (UniProt 2020). Tsukagoshi et al. (2010) showed that the transcription factor UPBEAT1 represses the expression of peroxidases, altering the ROS gradient ($O_2^{\bullet-}$ and H_2O_2) that controls the transition between cell proliferation and differentiation. Overexpression of UPBEAT1 is induced by H_2O_2 and is associated with shorter roots and reduced meristem areas.

TFs, protein kinases, and phosphatases are the main regulatory components in almost all signaling pathways. Protein kinases and phosphatases act by phosphorylation and de-phosphorylation of proteins (Taj et al. 2010). MAPKs comprise the family of serine/threonine protein kinases (OXI1) (Rentel et al. 2004). The MAPK cascade comprises three kinases, i.e., MAPKK kinases (MAPKKK or MEKK) that phosphorylate and activate MAPK kinases (MKK), which in turn phosphorylate and activate MAPK (Rodriguez et al. 2010). MAPK cascades play an essential role in the transduction of extracellular signals and in the activation of the corresponding cellular response, in ROS signaling and responses (Liu and He 2017). ROS, like H_2O_2, activate MAPKs (Kovtun et al. 2000) or initiate MAPK cascades (Waszczak et al. 2015). MAPK cascades regulate ROS-related genes, such as CATs and APXs (Liu and He 2017), H_2O_2 accumulation and cell death (Yue and López 2020). Although there is no direct evidence for the involvement of MAPK pathways in plant response to NP-induced stress, analogous pathways involved in AgNP-induced signaling have been proposed and it has been claimed that plants also use MAPK cascades upon exposure to AgNPs (Kohan-Baghkheirati and Geisler-Lee 2015; Marslin et al. 2017).

Oxidative burst is the plant's early response to biotic and abiotic stress. Plant respiratory burst oxidase homologues (*rboh*) have been identified as the main source of ROS during the apoplastic oxidative burst. In another system, four mechanisms have been advanced to explain the production of ROS in plant cells. One of these takes place on the inner part of the cell membrane and is mediated by NAD(P)H oxidases, the other three take place in the cell wall matrix and involve the action of

peroxidases (*Prxs*) which catalyzes the initial formation of $O_2^{\bullet-}$, poly(di)amine oxidases and oxalate oxidases, which generate H_2O_2 (Almagro et al. 2009).

NPs induce oxidative stress through mechanical damage to the cell membrane/cell wall (Dietz and Herth 2011). Tan et al. (2009) reported that the interaction between MWCNTs and *O. sativa* L. cells cultured in vitro on Murashige and Skoog basal medium (MS) determined the structural modification of signal molecules (including proteins or polysaccharides) in the cell wall constitution and led to a hypersensitive response. In another study, microscopic analysis of *A. cepa* L. root cells exposed to the action of ZnO-NPs revealed significant morphological changes in cell membrane, such as fissures, fractures and spikes. Destabilization of cell membranes may be the consequence of the formation of lipid peroxides, following the interaction of ROS with the fatty acids present in lipid membranes. Significant lipid peroxidation has been suggested by elevated levels of thiobarbituric acid reactive species (TBARS). After internalization in root cells of *A. cepa*, ZnONPs induced degeneration of nuclear constituents and dose-dependent swelling of mitochondria with alteration of mitochondrial membrane potential ($\Delta\Psi m$) (Ahmed et al. 2017). The interaction between mitochondria and ZnONPs may cause the uncoupling of respiration, thereby, increasing oxidative stress in the cells. Similar results were obtained in *Solanum lycopersicon* L. (Ahmed et al. 2018). NPs mimic Ca^{2+} and bind to CaBPs, altering the expression of stress-sensitive genes (Khan et al. 2017).

ROS-related genes were up-regulated by AgNPs (Jiang et al. 2014). Kohan-Baghkheirati and Geisler-Lee (2015) defined AgNPs as a new stressor and showed that in *A. thaliana* there are 60 AgNPs-specific genes that are affected/regulated due to physical or mechanical damage induced by AgNPs. These genes have been enriched in the anion transport process, suggesting that *A. thaliana* plants used anion transporters to maintain ionic homeostasis. Similarly, Linh et al. (2020) observed that Fe, Cu, Co and ZnONP induced up-regulation of drought-related gene expression (*GmRD20A*, *GmDREB2*, *GmERD1*, *GmFDL19*, *GmNAC11*, *GmWRKY27*, *GmMYB118* and *GmMYB17*) in roots and/or shoots of NP-treated soybean plants, under induced drought conditions. These results suggest that AgNPs may stimulate plant tolerance to drought.

Autophagy is a catabolic process by which unnecessary cytoplasmic content is eliminated through the lysosomal degradation pathways (Yun et al. 2020). In the context of the oxidative stress response, autophagy has the role of protecting cells from apoptosis (Mizushima et al. 2008) by degrading irreversibly oxidized biomolecules and damaged cell organs. When the detrimental conditions cannot be overcome autophagy is one of the processes involved in programmed cell death (Scherz-Shouval et al. 2007; Filomeni et al. 2015). The first targets of autophagy are cellular sites of ROS production and signaling (Minibayeva et al. 2012). A relatively recent study showed that cerium oxide nanoparticles (CeNP) induced autophagy in tobacco BY-2 cells. Although CeNPs induced the accumulation of Ca^{2+} and ROS in a concentration-dependent manner, significant DNA damage and alteration in the antioxidant defense system were observed mainly at higher concentrations, respectively, at 50 and 250μg mL^{-1}. At the lowest tested concentration of 10μg mL^{-1},

CeNPs did not induce genotoxicity in tobacco BY-2 cells and provided better protection against H_2O_2 exposure, while observing the formation of autophagolysosomes (Sadhu et al. 2018).

Our point of view is therefore that genotoxicity can be caused by ENPs exposure, and the mechanisms behind molecular responses need to be thoroughly investigated for adequate risk assessment, prediction and management. In updating this fast-changing field of study, we focus on the methods of investigation, the mechanisms of genotoxicity and the variables that impact the experimental results.

6.3 Genotoxicity and Cytotoxicity of ENPs in Plants

Long-term hazard to sensitive receptors, including plants, animals and humans, is a strong concern regarding ENPs genotoxicity. The ability to inflict damage to genetic material is a significant issue attributed to the toxicity of ENPs in biological media, especially given by the capacity of ENPs to cross cell membrane (Fig. 6.1). As such, there has been a rising interest in the analysis of the possible genotoxicity of NPs for crops. A further issue relates to the possible transgenerational nature of genotoxicity (Winnik and Maysinger 2013).

There are at least five major information gaps with regard to ENPs-induced genotoxicity: (1) the lack of standardized metrics and experimental conditions for the evaluation of ENPs genotoxicity (2) the effective dosage at the site of toxicity mediates the biological reaction and this would be different from the nominal exposure dose (3) genotoxic effects under specific exposure conditions such as occupational circumstances and chronic low-dose exposure are not considered (4) investigations based on extensive transcriptional activity are required to discriminate the genotoxicity caused by ENPs from that of other co-exposed agents (5) the relationship between genetic disorders, carcinogenesis and genotoxic effects as a feature of trophic level is unclear (Wang et al. 2013).

6.3.1 Mechanisms of ENPs-Induced Genotoxicity

The pathways of ENPs genotoxicity are not quite well known and therefore it is not obvious if there is a nanoscale impact on DNA. Recent studies have shown that ENPs genotoxicity can arise from two key mechanisms: primary (indirect or direct) or secondary genotoxicity. Either of these mechanisms may relate to some of the ENPs; however, both mechanisms may occur simultaneously following exposure to some of the ENPs (Kohl et al. 2020). ENPs-induced genotoxicity can be interrelated to a variety of facets, such as direct contact of ENPs with DNA, indirect injury due to ROS generation and emission of hazardous ions through soluble ENPs (Kisin et al. 2007; Barnes et al. 2008).

Direct genotoxicity results from actual contact to DNA, for example, by affecting the stacking forces between DNA bases, via affecting phosphorylation, by triggering adduct development, or through changing gene regulation/expression (Wang et al.

2013). Many considerations such as ENPs intervention with nuclear/cytoplasmic proteins, connection to mitotic spindle or its constituents, cell cycle checkpoints interruption, induced oxidative stress, suppression of antioxidant defense, increased generation of ROS or interaction with cellular components (e.g., cell membrane, mitochondria) are also known to stimulate indirect primary genes (Dhawan and Sharma 2010).

Secondary genotoxicity ENPs may be the consequence of an oxidative DNA damage (Stone et al. 2009). Based on the ENPs size, the possibility of their integration into the cells and interacting with organelles and macromolecules (RNA, DNA and protein) is so significant. These interactions can destroy DNA and organelles by traumatic harm in addition to up-regulating the biochemical mechanisms (An et al. 2010).

6.3.1.1 Direct Primary Genotoxicity Mechanisms: Direct Interaction of Engineered Nanoparticles with DNA or Chromosomes

To understand whether primary genotoxicity is direct or indirect, it is crucial to know the uptake pathway and if ENPs can enter the nucleus (Fig. 6.1). The smallest ENPs (with just a few nm in size) could enter the nucleus across nuclear pore complexes. Even so, some findings imply the presence of larger ENPs in the nucleus, indicating that there could be other nuclear absorption pathways, such as intracellular routes involving endocytosis (Kazimirova et al. 2020). Nucleus existing ENPs that (penetrate either via nuclear pores or throughout mitotic division) can associate directly with chromosome-organized DNA or chromatin, liable on the cell cycle phase (Magdolenova et al. 2014). This intervention can lead to chromosomal aberrations or DNA disorder, for instance, severe DNA defects and DNA lesions (Kohl et al. 2020).

ENPs may also reach the nucleus during mitosis, once the nuclear envelope is discarded. Formerly, ENPs in the nucleus can associate directly with DNA or DNA in chromosome structures depending on the cell cycle stage (direct genotoxicity). ENPs can attach or link to DNA molecules during the interphase and may interact with DNA transcription and replication (Fig. 6.2). ENPs can be mechanically destructive or chemically bound to DNA strands (Kisin et al. 2011). Direct clastogenic mechanisms encompass some lesions on DNA, such as oxidation of bases, generating a basic site, creating 8-OHdG (8-hydroxy-2′-deoxyguanosine), methylation, base nitration by reactive nitrogen species (RNS), oxidative depurination or deamination, ring opening, double-strand breakages (DSB) and single-strand breakages (SSB) (Benameur et al. 2012). Via in silico strategies, it has been demonstrated that the ENPs carbon binds to one strand and is integrated into double helix throughout DNA replication. Furthermore, the strong correlation of diverse ENPs with DNA bases or DNA structure in various species has been recorded (An et al. 2010; Jin et al. 2011). For instance, after in vitro exposure of human cells, AgNPs (Hackenberg et al. 2011b), TiO_2NPs (Shukla et al. 2011) and ZnONPs (Hackenberg et al. 2011a) have been originated in the nucleus of the cell. TiO_2NPs with larger aggregates (of mean size was 285 ± 52 nm and, in general, the

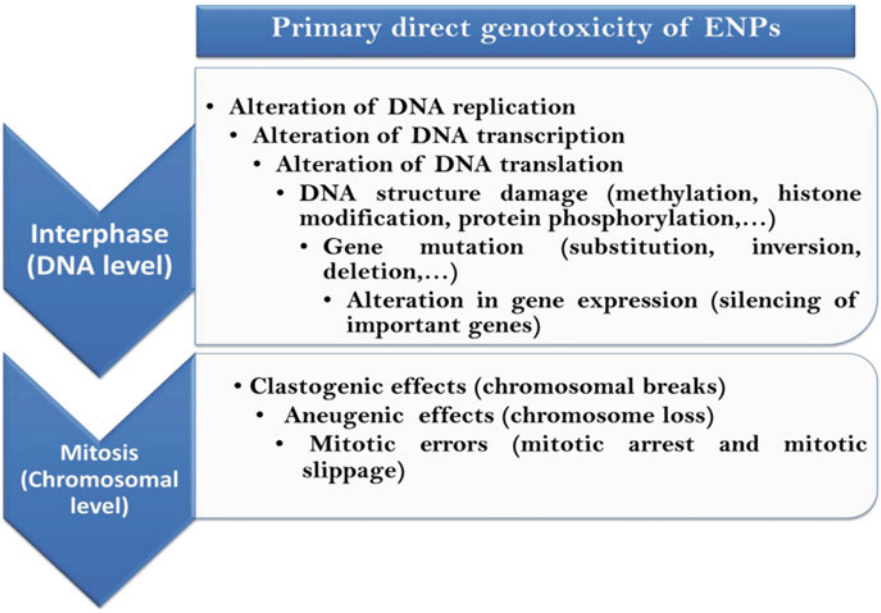

Fig. 6.2 Potential consequences of ENPs-induced direct primary genotoxicity

aggregates may be up to 2000 nm in diameter) were found in the nucleus (Hackenberg et al. 2010).

In addition to experimental studies, some computational ones have been conducted to explore interactions between ENPs and DNA. Computational approaches concluded that significant correlations between Al12X (X = C, N, Al, P) NPs and DNA nucleotides are predicted (Jin et al. 2011). They suggested that AlNPs may alter DNA stability and induced DNA structural damage. An et al. (2010) showed that carbon NPs are linked to single-stranded DNA and integrated in vivo double-stranded DNA in *Escherichia coli*, possibly during DNA replication. This indicates that DNA replication may be disrupted by carbon NPs. During mitosis, ENPs may also interact with the centrioles, spindle fibers related proteins which can interrupt mitosis; this contributes to the creation of micronuclei that might be perceived as aneugenicity or clastogenicity (Kisin et al. 2011). ENPs can cause chromosomes to split or disrupt the mitosis process, either mechanically or by chemical binding (Magdolenova et al. 2014).

6.3.1.2 Indirect Primary Genotoxicity

ENPs do not require direct interaction with DNA to convince genotoxicity in which ENPs can indirectly induce primary genotoxicity in different ways as follows: (1) interference with nuclear proteins (tangled in transcription, replication, repair) (2) interaction of ENPs with mitotic spindles or their components—aneugenic effect (3) disruption of cell cycle control features (4) transition metals from ENP surface

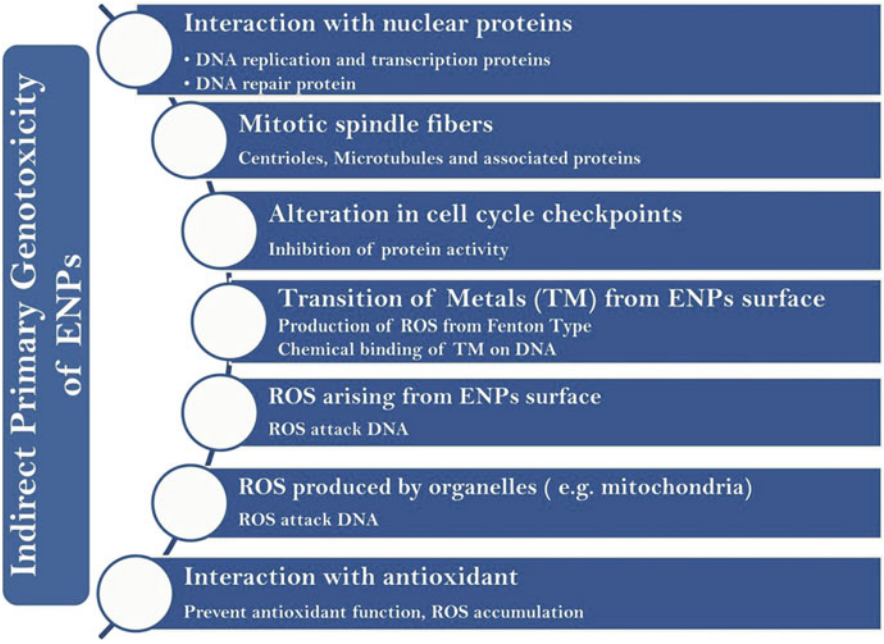

Fig. 6.3 Potential consequences of ENPs-induced indirect primary genotoxicity

(5) ROS generation (6) antioxidant defense inhibition (Fig. 6.3) (Magdolenova et al. 2014).

The interaction of ENPs with nuclear proteins may have undesirable effects on the mechanisms of DNA repair, transcription, replication and mitotic spindle function. Some experiments in silico and in vitro have examined the binding efficiency of ENPs with different essential proteins. Jugan et al. (2012) have shown that TiO_2NPs reflect the impaired activity of DNA repair in A549 cells and genotoxicity. Production of ROS was the cause of the inactivation of DNA protein repair activity. Baweja et al. (2011) exposed in silico study that C60 fullerene associates with the ATP binding domain of human DNA topoisomerase II alpha and could suppress the enzyme activity.

One of the fundamental mechanisms of primary indirect genotoxicity is oxidative stress. ROS can damage DNA that causes strand breaks and pyrimidine or purine oxidation lesions. The disruption can be remedied, but it can also lead to chromosomal abnormalities and gene mutations. ENPs may also induce DNA damage by other molecules that either interfere with DNA or interact with cellular division and DNA replication. ENPs can interfere with protein kinases which involved in controlling cell cycle activities, including cellular division and DNA replication (Magdolenova et al. 2015).

It has been reported different pathways for indirect genotoxicity caused by oxidative stress. First, the release of harmful substances through the interaction

ENPs-exposure media might be responsible for DNA damage and ROS generation. For example, free Ag^+ ions and other toxic ions produced from AgNPs or soluble ENPs might induce DNA destruction. ROS generation through Fenton-type reactions caused by some transition metal ions as Ag^+, Cr^{5+}, Mn^{2+}, Cu^+, Ni^{2+} and Fe^{2+} (Kruszewski et al. 2011). These ions can also link to DNA strand bases (Robertazzi and Platts 2005). Second, ROS can interact with NPs and increase their solubility. The interaction of AgNPs with H_2O_2 was assessed to cause in vivo Ag^+ development (Asharani et al. 2009). Third, in vitro suppression of antioxidants and ROS overproduction can eventually cause DNA damage. Silicon carbide ENPs have been linked with the degradation of glutathione (a major cell molecular antioxidant) and certain antioxidant enzymes inactivation, for example superoxide dismutase and glutathione reductase (Barillet et al. 2010).

6.3.2 Secondary Genotoxicity Mechanism

Secondary genotoxicity cannot be studied using traditional in vitro methods and has only lately been examined in vivo regarding chronic inflammation due to motivation of immune cells as neutrophils or macrophages (Evans et al. 2017). The in vitro methods for secondary genotoxicity have several difficulties, as standard systems cannot be used to decide the ENPs capacity to induce genotoxicity through inflammation. Recent advancements also rely on the co-culture of immune system cells with target cells. TiO_2NPs are known to induce apoptosis in cell lines resulting from different organs and stimulate inflammatory cytokines (Petkovic et al. 2011).

6.3.3 Cytotoxicity Assays

The cytotoxicity of the tested ENPs must be carried out before genotoxicity assessment where it is important to determine the lethal concentration (LC50 at which 50% of the cells will die) so as to choose the required range of concentrations prior to genotoxicity evaluation. The dosage range is defined by the genotoxicity test kind assigning to the ENPs evaluation. The non-cytotoxic concentration range must be (up to $50 \pm 5\%$ of cell death) (Kohl et al. 2020). If cytotoxicity is not an incorporated part of the genotoxicity assessment, false-positive outcomes of the cytotoxic compounds diagnosis as genotoxic can easily happen. There is indeed no consensus on the threshold value for cytotoxicity. Technically accurate cytotoxicity is shown to be as low as 80% viable (20% cytotoxicity opposed to untreated control), some presume that the scope of cytotoxicity throughout the genotoxicity study could be as low as about 50% viability (Reisinger et al. 2018).

Alternative cytotoxicity tests (instead of viability test) can be dependent on the membrane integrity and the use of different dyes to the membrane, allowing cell membrane to be stained, for example, the Trypan blue exclusion test. Cell death offers indirect proof of dye penetration into cells (Baker and Mock 1994). Heikal et al. (2020) concluded that cell death was observed in all concentrations and that the

number of dead cells steadily exceeded to 20 mg L^{-1} of AgNPs, when *A. cepa* roots were treated. Higher concentrations of AgNPs such as 40 mg L^{-1} and 80 mg L^{-1} were related with a reduction in Evans blue dye accumulation following damage to the plasma membrane and may use as a cytotoxicity marker. Ng et al. (2017) also observed that ZnONPs at 50 mg mL^{-1} reduced cytotoxicity at higher concentrations and caused complete cell death.

Dual staining with fluorescein diacetate for live cells and fluorescent dyes propidium iodide for dying cells through microscopy or flow cytometer testing is much more common. Dead cells may be detected by colorimetric or fluorometric approaches such as dye staining requiring metabolic activation through leakage of intracellular constituents as lactate dehydrogenase (LDH). AlamarBlue™ test is a popular and realistic test, reducing the total amount of Resazurin to fluorescent Resorufin. The AlamarBlue™ assay is a simple and reliable cytotoxicity assay to be used in combination with a comet assay that has been tested and shown to be applicable to a number of different ENPs (Efeoglu et al. 2018; Ventura et al. 2018). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay is used to monitor cell cytotoxicity, viability and proliferation which are commonly used to regulate cell response and culture health following exposure to different stimuli. It is important that the same treatment circumstances should be applied when considering a cytotoxicity assay for use in genotoxicity tests and that the tests would be carried out in tandem.

6.3.4 Biomarkers for Genotoxicity: Methods Used for In Vitro and In Vivo Genotoxicity Testing of ENPs

Genotoxicity biomarkers include a series of tests that include DNA damage, chromosomal damage and gene mutations as critical endpoints for genotoxicity. While some studies have been conducted to determine the hazards and risks of ENPs, there are no definitive data on their safety. This may be due to a variety of factors, such as (1) absence of credible and approved assays protocols; (2) improper characterization of ENPs; (3) indirect/direct interaction of ENPs with test reagents/protocols; (4) ENPs synthesis procedures (Stone et al. 2009). The absence of accuracy in the studies has contributed to a global effort to create a survey technique that can accommodate for the above-mentioned possible confounding variables and to determine accurately the mechanism of behavior, the serious health threats of the ENPs.

For genotoxicity assessment, short-term experiments were carried out to identify and evaluate genotoxicity, which is categorized in three major groups based on the abnormality type found in plants: (1) primary DNA defects, (2) gene mutations and (3) chromosome mutations. Genotoxic assessments of various ENPs have been primarily documented in vivo and in vitro studies in plants. Reported ENP studies include chromosomal fragmentation, oxidative DNA adducts, point mutations, DNA strand breakages and variations in profiles of gene expression and so may promote carcinogenesis and mutagenesis. Despite insufficient details and lack of expertise of NM protection, the amount of NMs generated is steadily increasing thus, in a

number of past and ongoing European projects, just like NanoReg, NanoGENOTOX, HISENTS, NanoTEST, RiskGONE, etc., attempts have been devoted to clarify the mechanism of action of NMs and to establish or modify validated OECD testing procedures for chemicals used in NMs. Genotoxicity biomarkers have been commonly used for the risk management of nanotoxicology (Kohl et al. 2020).

6.3.4.1 Comet Assay or Single Cell Gel Electrophoresis (SCGE) Assay

The comet assay or single-cell electrophoresis gel (SCGE) assay is a quick, responsive and relatively easy approach for quantifying DNA damage in single cells. The DNA that is subjected to the electric current relocates out of the cell, throughout the direction of the anode, emerging as a "comet" with a distinctive head, composed of intact DNA and a tail of degraded or fractured DNA fragments. The structure, size and DNA density inside the comet relate to the DNA damage level (Kisin et al. 2007). It enables the identification of single-stranded DNA breaks (SSBs, strand breaks, alkali-labile sites and imperfect excision repair sites), DNA cross-linking and oxidative DNA damage (Kumar et al. 2013). Comet assay has been frequently used to measure the ENPs genotoxic potential (Stone et al. 2009; Shukla et al. 2011, 2013). The dose-dependent reaction of AgNPs to *Allium cepa* root tips with low levels of DNA damage observed by the comet assay has been documented. The examination of the comet assay image data showed a change of DNA repair kinetic models (Heikal et al. 2020).

6.3.4.2 Gamma-H2AX Assay

The γ-H2AX analysis is one of the components of the nucleosome core of the H2AX histone family and is considered as more accurate tool for identifying double-strand fractures. The phosphorylation of this protein to serine-139 is controlled by either ataxia telangiectasia, ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK) or Rad3-related protein (ATR) resulting in the formation of γ-H2AX. γ-H2AX is present in a complicated shape in the cell and the DNA double-strand break (DSB) triggers phosphorylation. This converts the complexes into monomers that are usually needed as stimuli for the induction and maintenance of DNA repair proteins at the DSB site. Different methods, such as Western blot, flow cytometry and immunohistochemistry, have been documented to modify the expression pattern of γ-H2AX mediated by ENPs (Ismail et al. 2007; Lewis et al. 2010).

6.3.4.3 Chromosomal Aberration (CA)

Chromosome abnormalities involve thousands of DNA bases or whole chromosomes owing to two major mechanisms: clastogenic mechanisms contribute to (qualitative or direct structural) chromosome anomalies, just like dsDNA breaks, whereas aneugenic mechanisms lead to (quantitative or numerical) anomalies. The latter abnormalities comprise variation in chromosomes number by creating lesions in mitotic system proteins (Benameur et al. 2012). Chromosomal damage (chromosome breakage) may contribute to chromosome abnormalities: (a) modifications in

chromosome structure (duplications, deletions, inversions and translocations of chromosome segments), gene-based consequences that have been modified; and (b) chromosome number variations (polyploidy-multiplying of entire sets of chromosomes or aneuploidy—gain or loss of one or more chromosomes) (Ma et al. 1995).

The sister chromatid exchange test (SCE) is interpreted using methods for integrating bromodeoxyuridine (BrdU) into chromosomal DNA and separate staining of chromatids having BrdU-free chromatids and BrdU DNA. For plants, somatic recombination and sister chromatid exchange strategies have been designed for experiments with *Vicia faba* and *Crepis capillaris* root cells, in addition to with transgenic *Arabidopsis* and tobacco plants (Geras'kin et al. 2011).

Irregular chromosomal alignment, multipolar formation and separation throughout telophase and anaphase were recorded for long-term exposure to TiO_2NPs according to Huang et al. (2009). Furthermore, the genotoxic effects of engineered AgNPs on *V. faba* root tip meristems have been reported by Patllola et al. (2012). The authors noted that various concentrations massively increased the frequency of mitotic anomalies and reduced mitotic index compared to control. Also, Limana et al. (2019) demonstrated that CeO_2NPs (CNPs, <25 nm) and CeO_2 microparticles (CMPs, <5μm) have been shown to have genotoxic and cytotoxic impacts in *A. cepa* meristematic root tips. In addition, Kumari et al. (2009) and Pulate et al. (2011) explored that AgNPs had genotoxic and cytotoxic impacts on A. *cepa* root tips. The authors have shown that various treatments of AgNPs caused various forms of chromosomal abnormalities, such as bridge, chromosome splits, chromosome stickiness, micronuclei and disrupted metaphase. They also observed an increase in the frequency of abnormal cells with elevated concentrations of AgNPs and duration of exposure. Heikal et al. (2020) concluded that the *A. cepa* root tips treated with 40 mg L^{-1} of AgNPs for 4 h showed the maximum CA percentage (15.36%). A concentration-dependent increase in the CAs and micronuclei frequencies were detected in AgNPs treated root tips.

6.3.4.4 Micronucleus (MN) Assay

Micronuclei (MN) are distinct nuclear bodies which differ from the basic nucleus, one to six in number per cell, with a diameter between 1/3 and 1/16 of the nucleus. These MNs are developed during cellular division and can include entire chromosomes that have been lost throughout anaphase due to spindle protein lesions (aneugenic effect) or acentric chromosome fragments that cannot be positioned on the chromatic spindle (clastogenic effect). In order to determine whether the genotoxic material induces aneugenic and/or clastogenic impacts, the MN test may be coupled with fluorescent in situ hybridization (FISH) using pancentromeric DNA probes to give precise fluorescent visualization of the incidence (aneugenic) or absence (clastogenic) of centromeres in the MN (Benameur et al. 2012).

The mitotic errors frequency (mitotic arrest, chromosome loss and mitotic slippage) as a consequence of spindle alternations was calculated. Measurements of micronucleus in mononucleated and binucleated cells in the cytokinesis block micronucleus. CBMN assay was recommended as an additional marker to

differentiate between ENPs causing aneugenicity and clastogenicity (Kazimirova et al. 2012). Kumari et al. (2009) recorded various forms of nuclear distortions and chromosome abnormalities in NPs treated *A. cepa*. Consequently, DNA damage that is not remedied or incorrectly remedied can initiate mutation. This condition will occur if the DNA damage triggered by ENPs is severe and the DNA repair mechanism is not sufficiently effective to repair all damage (Huang et al. 2009). Also, Younis et al. (2019) recorded lower percentages of MN and CA below control in both interphasic and mitotic cells of chemically synthesized AgNPs treated root tips of *V. faba*.

6.3.5 Effect of Physicochemical Properties on NPs-Induced Genotoxicity

Genotoxicity mechanisms can also be more complex, for example, it is not understood why different ENPs physiochemical properties cause particular genetic effects. In order to determine lethal effects, several NP characteristics (surface properties, shape, solubility, composition, size, agglomeration/aggregation, NP absorption, mutagens existence and ENPs-affiliated transition metals, etc.) must be taken into account. Physicochemical characteristics of ENPs are closely related to their biological activity and many of them can contribute to negative health impacts (Vega-Villa et al. 2008).

Numerous reports have shown that the genotoxicity of ENPs depends not only on dosage and exposure duration but also on their chemical composition, surface properties, size and shape (Magdolenova et al. 2014). Furthermore, Huk et al. (2014) explored some silver ENPs with identical surface properties, but dissimilar sizes and observed that the genotoxicity of AgNPs hangs on their scale. Another research studied the genotoxicity resulted from the impacts of AuNPs surface functionalization and size (Vales et al. 2020). Studies showed that silica oxide (SiO_2)-coated TiO_2 nanoscale caused a lesser amount of DNA damage than uncoated (Falck et al. 2009). This result could be related to capabilities that minimize the formation of free radicals mediated by TiO_2NPs (Vales et al. 2015), alter the agglomeration process of the ENPs (Osman et al. 2010) and affect interaction with biological components (Charles et al. 2018). In vitro shape-dependent genotoxicity was also investigated (Gea et al. 2019). Diverse shapes (rods, bipyramids and platelets) of TiO_2NPs have been related to commercial TiO_2NPs (Gea et al. 2019).

In addition to shape, scale, surface properties, the chemical configuration also plays a crucial role in genotoxicity assessment of ENPs. Investigations on the ENPs metals genotoxicity (TiO_2, Ag, Zn, iron oxide, etc.) were distinctively studied (Rodriguez-Garraus et al. 2020). The correlation between ENPs particle size, shape, chemical conformation and toxicological impacts has been explored by Yang et al. (2009). ZnONPs and carbon nanotubes were moderately cytotoxic but lead to extra DNA damage estimated by the comet assay. This comparative research reveals that chemical composition plays a crucial role in genotoxicity and cytotoxicity. Variability in the findings of genotoxicity investigations can be due to the NPS

synthesis, source of NPs, the dispersion procedure and physicochemical parameters such as cell type used, dosage, pH, impurities existence, temperature, treatment regime, exposure time, etc. (Shukla et al. 2011).

6.3.6 Molecular Mechanisms of ENPs-Mediated Genotoxicity and Plant Interactions

ENPs are considered to have an ability to cause changes in gene expression in plants (Kaveh et al. 2013). In tobacco, carbon nanotubes can interfere in plant growth and development processes via a substantial up-regulation of the aquaporin gene (NtPIP1) and a corresponding increase in NtPIP1 protein synthesis by growing the expression levels of the important NtLRx1 (extensin) gene for cell wall assembly/cell growth and the CycB gene role in the cell cycle progression regulation (Khodakovskaya et al. 2012). By applying tomato seeds and seedlings to carbon nanohorn genes associated with cell response, stress response and metabolic processes have been up- or down-regulated. Studies conducted by Lahiani et al. (2015) have shown that nanohorns are absorbed in very small quantities by plant roots, but sufficient to alter the response of plants at transcriptome and proteome levels. Moreover, in perspective of the close association between the various "omics" technologies (genomics, transcriptomics, proteomics and metabolomics), convergence is helpful in solving natural biological difficulties at the system level (Joyce et al. 2006). There is extensive awareness that the integration of omics data provides unique insights into fields of toxicology, pathology and physiology (Heijne et al. 2005). Consequently, "omics" technologies are increasingly used in nanotoxicity investigations, there would be a trend to systematically explore the molecular mechanism of NPs interaction by incorporation of omics.

6.4 Engineered Nanoparticles and Their Carcinogenic Potential

Oxidative stress, ROS generation and ENPs inflammation eventually raise the issue of genotoxicity and/or carcinogenicity. If nanoparticles may interfere directly with DNA and cause mutagenesis (strand breaks, lesions, adducts, oxidatives, etc.), genotoxicity can activate and stimulate carcinogenesis mechanisms (Vlachogianni et al. 2013). In vivo and in vitro experiments have shown that ENPs cause mutations and DNA damage. The association between cancer and genotoxicity is also well known. Accordingly, these experiments provide valuable information to estimate the carcinogenicity of the ENPs. For instance, the carcinogenic impacts of UV radiation, ionizing radiation and other chemical carcinogens are dependent on its ability to induce DNA damage and gene mutations. Connections between metal oxides, metals, oxidative stress and cancer have been studied extensively (Pulido and Parrish 2003; Valko et al. 2005; Lee et al. 2012; Chalbot et al. 2017). It is well understood that the excessive production of ROS overwhelms the antioxidant defense system of

the cells through the oxidation of biomolecules. The role of oxygen-derived organisms in causing cell damage or death is increasingly recognized: ROS is implicated in a wide number of degenerative modifications, carcinogenesis, contributing to tissue loss, ageing and other diseases (Luo et al. 2011).

The carcinogenic potential of chemicals and ENPs is important for the safety evaluation. Any substance is genotoxic means that it could be possibly carcinogenic. A further feature of carcinogenicity is the presence of non-genotoxic carcinogens that cause their impacts by secondary mechanisms, just like oxidative stress or other inflammatory responses (Toyooka et al. 2012). Cell transformation assay (CTA) is a technique that uses the phenotypic transformation of cells as a predictor of carcinogenicity throughout in vitro technique (Kerckaert et al. 1996). In vitro transformed cells have been revealed to cause tumors when inserted into immune-suppressed animals. CTA cells are also produced from rodent embryos such as the Syrian hamster embryo (SHE), Bhas42, BALBc 3 T3 mouse and C3H/10 T cells. In general, CTAs can be useful for the identification of non-genotoxic carcinogens and should be used as an essential component of a package of in vitro tests to forecast the carcinogenic potential of ENPs (Fontana et al. 2017).

Earlier studies from Takagi et al. (2008) and Sakamoto et al. (2009) showed that the MWCNTs had potential carcinogenic effects, while Muller et al. (2009) performed similar MWCNTs tests and reported no carcinogenicity after 2 years of exposure. ENPs such as CuONP, ZnONP and TiO_2NP may also have similar effects as any other possible carcinogens such as asbestos (Abigail and Jacobs 2012). It also suppresses the immune system, resulting in elevated microbial loads resulting in cell and tissue destruction. It has been well established that free radicals cause different types of genetic damage that could cause cancer. 8-OHdG is the most investigated oxidative DNA damage agent due to its relative ease of measuring and premutagenic ability. Elevated 8-OHdG has also been found in a number of tumors, greatly impacting the etiology of cancer (Oberley 2002).

In addition, a carcinogenic bioassay has been used to assess the carcinogenic capacity of CuO instilled nanoparticles in male F344 rats. Neoplastic lesions were shown to be caused by oxidative stress caused by CuO NP at exposure (Masanao et al. 2009). As a result, in vivo tests are consistent with in vitro evidence that suggests that the toxicity to the living organism caused by ENPs is due to oxidative stress.

6.5 Conclusions and Perspectives

This study shows that the toxicity of nanomaterials on plants is species-specific and dependent on a multitude of factors and complex interactions. The challenge for even more research is therefore the uptake kinetics and interaction process inside cells, as well as the maximum amount of these ENPs that plants can take without showing any signs of stress. Extensive studies on the toxic effects of ENPs may have a substantial impact on mitigating the harmful effects in both environmental and agricultural systems. Multidisciplinary approaches using various models (from in

silico, in vitro and in vivo) and research approaches should also be used to determine the risk associated with the ENPs. In the current situation, the cytotoxic, genotoxic and carcinogenic hazards through transgenerational transmission and possible health threats of ENPs should also be distinguished on the basis of biotic and abiotic factors.

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Oxidative Stress and Genotoxicity Induced by Industrial Wastes and Effluents in Plants

7

Akansha Khare, Susheel Kumar Singh, and Shafia Siddiqui

Abstract

Intensive increase in industrialization and urbanization has created avenue for plethora of additional wastewater worldwide. The effluent from these sources is being discharged into the water bodies and makes them contain high level of heavy metals, hormones, antibiotics, pesticides, acids, and alkali. Majority of the compounds are non-biodegradable and affects the aquatic as well as terrestrial life by contaminating them. Plants irrigated with the water containing industrial effluents exhibit stunted growth, altered photosynthetic function, induction of genotoxicity and oxidative stress which eventually leads to loss of productivity. Therefore, it is essential to assess the toxicity potential of such effluents and establish the appropriate treatment methods prior to their discharge. To detect the potential of these pollutants, various bacterial and plant-based assays are available. In this chapter, types of effluents from different industries, their impact on plants, the assays used to detect them are discussed. The chapter also discusses the measures which are being taken to mitigate the level of pollution in the water and the demanding advancement in them.

Keywords

Effluents · Genotoxicity · Industrial wastes · Oxidative stress

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Abbreviations

ROS	Reactive oxygen species
GSH	Glutathione
AsA	Ascorbic acid
SOD	Superoxide dismutase
CAT	Catalase
POX	Peroxidase
APX	Ascorbate peroxidase
GR	Glutathione reductase
GPX	Glutathione peroxidase
GST	Glutathione-s-transferase
MDA	Malondialdehyde
H_2O_2	Hydrogen peroxide

7.1 Introduction

Industrialization indeed has resulted in innumerable benefits to humankind. But it has simultaneously presented a massive problem, i.e., the effluent which requires proper treatment before discharging it into the water bodies and thus gravely contaminating them (Gemeda et al. 2020). The problem is more severe in developing countries like India due to the lack of proper waste management program, viz. equipment, initiative, public awareness, and training. This increased pollutant level has an intense effect on the flora and fauna (Ferronato and Torretta 2019). Researchers around the world are exploring and coming up with technologies to treat the effluents effectively. However, it is quite an uphill task because effluent is agglomeration from various industries like food and beverages, leather, textiles, pesticides, paper and pulp, mining, livestock feedlots and sewage treatment plants. The incomplete sewerage system of several megapolis further adds sewage wastewater into ponds, streams, and ducts. The effluent consists of herbicides, fertilizers, plastics, polycyclic aromatic hydrocarbons (PAHs), synthetic dyes, antibiotics, plasticizers, hormones, and heavy metals like cadmium (Cd), mercury (Hg), arsenic (As), thallium, chromium (Cr), and lead (Pb). One of the severely impact living organisms from industrial effluents is plants. The contaminated water generates stress in the plants which induce genotoxicity and other associated damages (Arregui et al. 2019). In terms of genetics, genotoxicity is a characteristic of chemical agents that damages the DNA and ultimately causes mutations. The continuous contact of flora and fauna to hazardous chemicals may give rise to chromosomal anomalies which cause diseases to living organisms and their offspring (Mazzeo et al. 2018). To minimize the risks to living organisms the effluent should be monitored for its genotoxic potential. Many assays are available to specifically analyze the genotoxic potential of different chemical effluents. Various genetic models have been used to

report toxicity of industrial effluents such as *Allium cepa, Vicia faba, Pisum sativum, Tradescantia, Crepis capillaries, Hordeum vulgare, Zea mays,* and *Nicotiana tabacum*, of these, *Allium cepa* and *Vicia faba* are commonly used test models (Rank and Nielsen 1997; Leme and Marin-Morales 2009; Bhat et al. 2017; Mazzeo et al. 2018; Iqbal et al. 2019).

Stress is a multifaceted phenomenon that occurs due to any change in the number of abiotic factors such as light intensity, nutrients, temperature, and relative humidity, which give rise bountiful production and accumulation of reactive oxygen species (ROS) (Pandey et al. 2017). Stress ultimately causes alterations in the normal plant physiology which gives rise to early aging and decreased photosynthesis in plants (Sharma et al. 2012).

The present chapter addresses the various effluents being discharged by different industries, their effects on plant growth and development, methods to detect the level of pollutants and available methods to mitigate the level of pollutants (Table 7.1).

7.2 Characteristics of Industrial Effluents

The discharge from numerous industries, i.e. metal, textile (Alves de Lima et al. 2007; Holkar et al. 2016; Zengınbal et al. 2018), dyeing chemicals, pesticides (Köck-Schulmeyer et al. 2013; Bachmann Pinto et al. 2018; Arregui et al. 2019; Zeyad et al. 2019), fertilizers, cement, petrochemical, leather, sugar, construction, engineering, mining, carried by pipes and ducts to rivers ultimately worsen and widens water pollution. High levels of pollutants in river water causes an increase in chemical oxygen demand, biological oxygen demand, total suspended solids, total dissolved solids, and toxic metals such as Cu, As, Fe, Mn, Ni, Zn, Cd, Cr, and Pb, making such water inappropriate for aquatic life, irrigation, and drinking. These effluents are not biodegradable. Industrial effluents contaminated the soil, hamper crop production throughout the world, and became a great environmental threat. These effluents enter the food chain by accumulating in soils and plants in excess (Tariq et al. 2006).

7.3 Mechanism of Phytotoxicity Induced by Industrial Waste and Effluents in Plants

Industrial effluents contaminate the agricultural soil with industrial wastes and has become a critical environmental concern due to their ability to induce oxidation-reduction reactions, and hence oxidative stress in plants (Otokunefor 2005; Hossain et al. 2012). ROS are responsible for numerous stress-induced destruction to macromolecules and eventually to cellular structure (Mostofa et al. 2015). Oxidative stress generated as a result of imbalance between ROS generation and detoxification. It is due to the disturbance of normal cell functioning because of ROS biosynthesis due to stress and the immunity response needed for defense and adaptation. The stress response induced by oxidative stress comprises severe morphological, physiological changes in plants for, e.g. protein defragmentation, breakage of DNA

Table 7.1 Source of industrial pollutant, its composition and effects on plants

Source of pollutant	Examples of wastes and effluents composition	Impact of effluent content on plants	Reference
Food and beverages industry	Fats, oils, grease, ammonia, and phosphorous	Plant growth, biomass partitioning, photosynthetic ability, and fruit yield	Ayyasamy et al. (2008), Jiménez-Tototzintle et al. (2015), Beneduce et al. (2017)
Leather industry	Colored compounds, sodium chloride, sulfate, and toxic metallic compounds	Morphological, photosynthetic, and oxidative alterations	Karunyal et al. (1994), Singh et al. (2004), Roy et al. (2015), Chowdhury et al. (2015)
Textile industry	Acid, alkalis, dyes, hydrogen peroxide, starch, surfactant dispersing agent, soaps of metal	Impact on plant root–shoot ratio	Alves de Lima et al. (2007), Holkar et al. (2016), Zengınbal et al. (2018)
Pesticide industry	Organophosphate and organochlorine pesticides	Induction of genotoxicity	Köck-Schulmeyer et al. (2013), Bachmann Pinto et al. (2018), Arregui et al. (2019), Zeyad et al. (2019)
Electroplating, cadmium batteries, nickel and iron alloys industries	Nickel and cadmium	Impacts soil health and fertility induces oxidative damage in plants and heavy metal accumulation in the plant parts	Orisakwe et al. (2004), Kumar et al. (2015)
Mining, smelting, combustion of fossil fuels, and the sewage sludge	Zinc, arsenic, copper, iron, free cyanide, chromite, ash, and slag	Delayed germination, chlorosis, stunted growth, reduced crop yield, premature leaf fall, senescence, biochemical lesions, enzymatic changes, and decreased biosynthesis	Acheampong et al. (2013)
Pharmaceutical industry	Hormones, antibiotics, analgesics and anti-inflammatory drugs, endocrine-disrupting compounds, and chemical compounds used for cleaning and disinfection	Impact plant growth and antioxidant activity either by posing damage directly or by disrupting the soil communities	Carvalho et al. (2014), Adeel et al. (2017)

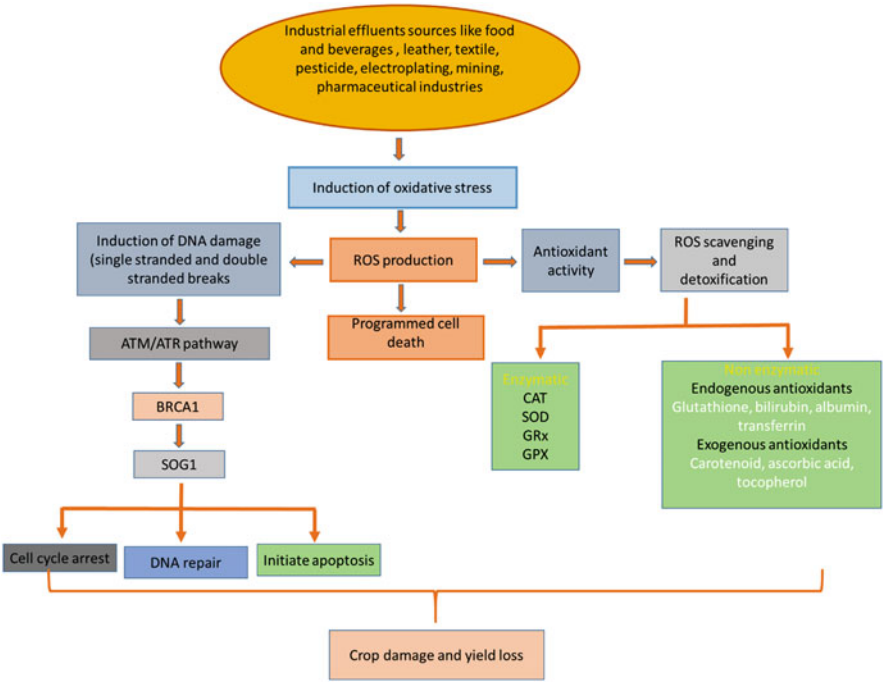

Fig. 7.1 Schematic representation of industrial effluent toxicity in plants and its downstream processing (modified from Singh et al. (2015), Dutta et al. (2018), and Jing et al. (2018))

strands, and damage of photosynthetic pigments, ultimately responsible for cell death (Gill and Tuteja 2010). Plants counteract oxidative stress by increasing both enzymatic and non-enzymatic antioxidants (Fig. 7.1).

7.4 Overview of Plant Enzymatic and Non-enzymatic Antioxidants

In plants, antioxidants play a very important role in scavenging ROS either directly or indirectly (Carocho and Ferreira 2013). The antioxidant defense system is classified into enzymatic and non-enzymatic antioxidants. The non-enzymatic antioxidants includes GSH, AsA, phenolic compounds, α-tocopherol, flavonoids, and alkaloids and they work in coordination with antioxidant enzymes, i.e. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), glutathione peroxidase (GPX), and glutathione-s-transferase (GST) in order to check overproduction of ROS (Nath et al. 2018; Laxa et al. 2019). In plants, SOD converts O2•−into H_2O_2 (Biczak 2016; del Río et al. 2018), this H_2O_2 is further converted into H_2O by the enzymes CAT, APX, GPX. In addition, GPX and GST are also vital enzymes for the detoxification of

H_2O_2 and xenobiotics (Hasanuzzaman et al. 2018). In higher plants, among non-enzymatic antioxidants, ascorbic acid (AsA) and GSH (glutathione) are the most abundant soluble antioxidants (Foyer and Noctor 2011). They play a very important role as electron donors and scavenge ROS directly through Ascorbic acid-glutathione cycle (Hasanuzzaman et al. 2019). β-carotene also reacts with ROS radicals and ultimately decrease ROS concentrations of cell (Kapoor et al. 2019).

Ascorbic Acid regulates many phytohormone biosynthetic pathways. Also, ascorbic acid regenerates α-tocopherol (vitamin E) from tocopheroxyl radical or by scavenging of •OH and O_2•−(Seminario et al. 2017). Glutathione is another important component of the antioxidant defense system which is significantly involved in the regulation of ascorbic acid-glutathione cycle for scavenging cellular ROS (Hasanuzzaman et al. 2019). Tocopherol by scavenging ROS protects the chloroplast, hence plays a significant role in photosynthesis (Kumar et al. 2013). Carotenoids are another class of antioxidant molecules, they scavenge harmful free radicals and protect thylakoid membrane stability and light-harvesting complex proteins (Hussain et al. 2019; Hasanuzzaman et al. 2020). Flavonoids also have great capability to scavenge free radicals and decrease cell damage from lipid peroxidation (Agati et al. 2012; Brunetti et al. 2013).

Phenolic acids antioxidants are mainly constituting hydroxycinnamic and hydroxybenzoic acids, they show antioxidant activity as scavengers and chelators of free radicals, mainly •OH, O_2•−, ROO•, and ONOO– (Carocho and Ferreira 2013). Alkaloids also have antioxidant ability as free radical scavengers ultimately inhibit H_2O_2-induced oxidation (Tiong et al. 2013). In addition to these, nonprotein amino acids, e.g. gamma-aminobutyric acid, citrulline, and ornithine are also considered effective as non-enzymatic antioxidant (Vranova et al. 2011).

7.5 Oxidative Stress and Genotoxicity Induced by Different Industrial Effluents in Plants

Tannery industry effluents are very harmful to the environment or ecosystem. The industry is involved in releasing of toxic substances into water bodies and responsible for contamination of the water ecosystem and ultimately the death of organisms (Kumari et al. 2016). Tannery wastewater induced oxidative stress and thus increased contents of MDA, H_2O_2 and electrolyte leakage (%) in leaves and roots of *B. napus* plants in a concentration-dependent manner. Activities of various antioxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) responded against tannery wastewater toxicity and showed a decrease in antioxidants (SOD, POD, APX, and CAT) with the addition of different levels of tannery wastewater in the soil (Ajitha et al. 2019).

Tannery effluents are potentially genotoxic on root tip cells of *Vicia faba* and inhibited mitotic index and induced chromosomal and mitotic abnormalities. The major constituent of tannery solid waste is chromium and nickel, which may involve in chromosomal and mitotic abnormalities (Gowrishanker and Vivekanandan 1994;

Chandra et al. 2004). It also showed genotoxic and phytotoxic effects on *Allium cepa* and decrease in root length, mitotic index, and induction in chromosomal abnormalities and micronuclei (MNC) was observed. Tannery effluents exhibited genotoxic effects to *Allium cepa* consist of high chemical oxygen demand, total dissolved solids, biological oxygen demand, electrical conductivity, and total chromium (Gupta et al. 2012; Kumari et al. 2016).

The textile and dyeing industry wastes contain numerous hazardous materials such as dyes, metals, polyvinyl alcohol, cellulose, surfactants, etc. (Saeed et al. 2016). It is reported that textile effluents when present in excess increases ROS or MDA content and also showed a significant increase of CAT activity in leaves of two cultivars wheat and chilli. The activity of cellular GR and APX increased with an increase in textile effluent concentration. Higher concentration of MDA and H_2O_2 indicate that stimulated antioxidant enzymes are not sufficient for excess ROS removal to decrease oxidative stress (Singh and Rathore 2018). Textile dye red HE3B also produce oxidative stress in *Allium cepa* by inducing activity of SOD, APX and GPX and suppressing the activity of the CAT. This elevates protein and lipid oxidation in a dose dependent manner (Phugare et al. 2011). Remazol red (RR), a monochloro sulphonated azo dye induced oxidative stress in *Allium cepa* and increased enzyme activities of SOD and APX and inhibited CAT activity (Jadhav et al. 2011).

Textile wastewater samples produced anaphasic aberrations and micronuclei in *Allium cepa* (Grover and Kaur 1999), and increase chromosomal abnormalities such as sticky chromosomes, binucleus, c-tumors, vagrant, etc. Genotoxic effects of raw and ozonized textile industrial effluents were investigated using *Vicia faba* micronucleus assay, and it was found that raw effluents are relatively more toxic in *V. faba* roots than ozonized effluents (Rosa et al. 2001). The silk dyeing industry also evaluated for genotoxicity on the *Allium cepa* root system with various concentrations of effluents ranged between 25 and 100% for different durations. The researchers observed that effluents hamper cell division and also found a decrease in mitotic index with an increase in effluent concentrations and treatment duration. Effluents are also involved in a variety of mitotic abnormalities, i.e. vacuolated nuclei, fragments, bridges, stickiness of chromosomes, and laggards (Sudhakar et al. 2001; Rahman et al. 2017). Textile industry effluents exhibited genotoxicity, cytotoxicity, and mutagenicity in *Allium sativum*, *Lactuca sativa,* and *Vicia faba* and authors observed a reduction in mitotic index in *V. faba* roots (Giorgetti et al. 2011). The results showed that the textile industry effluent has genotoxic potential and if released into water bodies is capable of creating an ecological disturbance in the environment.

Papermill effluents are found to be genotoxic to *A. cepa* root tip cells that showed decreased mitotic index and induced chromosomal anomalies such as c-mitosis, chromosome loss, stickiness, break, multipolar anaphase, bridge, vagrant chromosomes, micronucleated and binucleated cells (Chaparro et al. 2010; Haq et al. 2016). Sugar mill wastewater decreases the mitotic index and induces various chromosomal anomaly in root cells of *Hordeum vulgare* i.e. c-mitosis, lagging chromosomes, chromosomal bridges and multipolar anaphases (Özkara et al.

2011). Sugar mill pressmud showed the genotoxic effect on *A. cepa* and decreased root development together with the mitotic index. Sugar beet pulp waste and sugarcane vinasse have genotoxic potential on *A. cepa* that causes numerous anomalies, i.e. anaphasic bridges, micronucleus, chromosomal break, and chromosomal loss (Garcia et al. 2017; Bhat et al. 2018).

Numerous industries are very toxic for the environment as well as plants, such as pesticide industry (Mercado-Borrayo et al. 2015), electroplating industry (Ajitha et al. 2019), sewage sludge (Sommaggio et al. 2018), electronic waste leachate (Bakare et al. 2012), coal fly ash (Jana et al. 2017), semi-coking wastewater (Liu et al. 2017), petroleum refinery effluent (Bagatini et al. 2009), hospital effluents, olive mill effluent (Liu et al. 2017), and coking wastewater.

Pesticide industry wastewater induced oxidative stress in roots of *Vigna radiata* which ultimately decreased percent seed germination, radicle length, seedling vigor index, plumule length (Mercado-Borrayo et al. 2015), dry biomass of plumule, and dry biomass of radicle as compared to untreated condition. Electroplating industry effluent increased ROS level in *Chlorella vulgaris* cells and decreased the total protein and chlorophyll contents gradually in a concentration-dependent manner. SOD and CAT enzymes activities increased with increase in effluent concentration (Ajitha et al. 2019).

Sewage sludge waste exhibited genotoxic and cytotoxic effects on *A. cepa* root cells (Sommaggio et al. 2018). Olive mill effluents (10%) induced micronuclei formation in *V. faba* root cells that may be because of phenolic materials present in the olive mill waste. Semi-coking effluents are also found to be genotoxic and mutagenic in *V. faba* bioassay (Liu et al. 2017). *V. faba* and *H. vulgare* exhibited micronuclei, sister chromatid exchange, and mitotic index reductions when treated with coking wastewater. The studies exhibited that the germination index and mitotic index were found to be reduced in petroleum refinery effluent treated roots (Cavusoglu et al. 2010) (Fig. 7.2).

7.6 Conclusion

It is understood that industrial effluents are a major problem for plant growth and development, globally. Industrial effluents lead to the increase of ROS and it can cause oxidative injury in plants. The ROS metabolism had a vital function in crop growth, adaptation, development, and survival under stressful conditions. The accumulation and scavenging of ROS are important factors in the defense processes of plants. The downstream processes involve overexpression and modulation of genes that are involved in ROS detoxification and they are widely used to enhance tolerance against numerous stresses. Both enzymatic and non-enzymatic antioxidant systems maintain balance among the detoxification and generation of ROS under stressful environments. Based on the literature, ROS are known to exert a signaling role at low concentrations, hence very important for various biological mechanisms, such as cellular proliferation and differentiation. Although, ROS overproduction is responsible for genotoxicity and ultimately cell death.

Fig. 7.2 Schematic representation of industrial effluent uptake by plants (concept taken from Wu et al. (2015), and Rai et al. (2019))

So, effluents from different industries are potential challenges, mainly for plant growth and development. This raises the need to install as well as develop robust cost-effective treatment plants so that only the minutest amount will be discharged in the water bodies. There is a need to know the genotoxic potential of the pollutants for their treatment and determining discharge standards by various known plant and bacterial based assays. There is also a need to identify the novel pollutants from the industries and find their impact on plants, soil texture, and animals. Along with updating the identification, treatment and discharge limitation of the pollutants much important is compliance. Thus, along with the government in establishing the treatment plants, it is also the responsibility of the industrial authorities to be compliant with the discharge standards.

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Mechanism and Molecular Response of Induced Genotoxicity and Oxidative Stress in Plants

8

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Abstract

Exposure of plants to various environmental factors like drought, soil salinity, heavy metal toxicity, pesticides, industrial waste products, infection by pathogens, extreme temperatures, UV radiations, and air pollutants induces oxidative stress in plants, which eventually alters various important physiological processes through the generation of reactive oxygen species (ROS). ROS, which includes superoxide anions (O_2^-), hydroxyl radicals (·OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($1O_2$), activates signaling pathways in plant cells, which induces changes in physiological, biochemical, and molecular mechanisms in cellular metabolism. Higher level of ROS in cells causes "oxidative stress," a state of imbalance between generation of ROS and their detoxification by antioxidants and subsequent genotoxic effect, which results in destruction of various cellular components including proteins, lipids, nucleic acids, and metabolites, ultimately amalgamate in cell death. Hence, for aerobic organisms, maintaining the ROS homeostasis is critical, which relies on the combined effect of enzymatic and non-enzymatic antioxidants. However, at lower concentrations, ROS plays a significant role in overcoming environmental stress and maintaining normal plant growth. Generally, plants combine ROS with genetic, epigenetic, hormonal, and external signals to assist development and counteract the effects of environmental stress, suggesting, despite having potential toxicity, ROS usually plays a dual role depending on their concentration, cellular production sites and

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duration of action, different levels of reactivity, potential to cross biological membranes, previous exposure to stress, etc.

Keywords

Reactive oxygen species · Oxidative stress · Antioxidant · Genotoxicity · Programmed cell death (PCD)

Abbreviations

$1O_2$	Singlet oxygen
ABA	Abscisic acid
APX	Ascorbate peroxidase
AsA	Ascorbic acid
BRs	Brassinosteroids
CAT	Catalase
DNMTs	DNA methyltransferases
DSC	Distal stem cell
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HDACs	Histone deacetylases
IAA	Indole 3-acetic acid
O_2	Superoxide anions
OH	Hydroxyl radicals
PCD	Programmed cell death
QC	Quiescent center
RAM	Root apical meristem
ROS	Reactive oxygen species
SA	Salicylic acid
SOD	Superoxide dismutase
UPB1	UPBEAT1

8.1 Introduction

Since plants are immobile in nature, they are manifested to various environmental stresses, like ultraviolet radiations, extreme temperatures, salinity, drought, desiccation, rehydration and a large number of soil and airborne chemicals, industrial waste materials, metals and metalloids, ozone, etc. (Dutta et al. 2018, Fig. 8.1). These stress factors, actively or passively through introduction of oxidative stress and hyper-accumulation of reactive oxygen species (ROS), often damage the physical and chemical structures of DNA causing cytotoxicity or genotoxic effects (Zhu

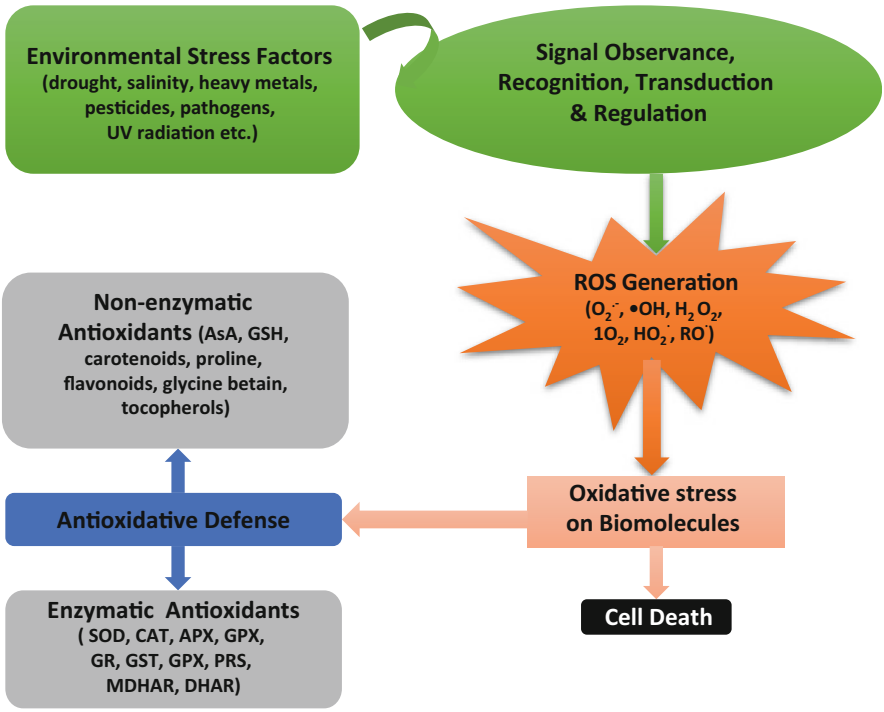

Fig. 8.1 Abiotic stress-induced ROS generation, antioxidative defense, and cell death in plant

2002). Therefore, genomic stability is affected which in turn affects the plant health and influences the crop quality and yield.

ROS are known as the natural by-products of aerobic organisms and are generated as by-products during mitochondrial electron transport. They are produced in several cellular organelles like mitochondria, peroxisomes, chloroplasts, and plasma membrane. They are also known as an unavoidable chemical entity of aerobic organism, causing irreparable DNA damage and cell death, ROS also function as signaling molecules that maintain physiological functions and regulate responses to various stresses (Xie et al. 2019). Generally, molecular oxygen (O_2) is inert in nature due to its electron configuration (Elstner 1987) but during plant metabolism, it can be converted into highly reactive ROS in various cellular compartments. ROS being reactive molecules affect various physiological and biochemical responses such as plasma membrane disruption via carbohydrate deoxidation, denaturation of proteins, lipid peroxidation, and destruction of enzymes and nucleic acids, hence avert them from carrying out their usual functions.

During stress, plants generate large amount of ROS (Table 8.1), which involved in regulation of various processes such as cell senescence, abscission, polar cell growth, stomatal behavior, biosynthesis of cell wall lignin, programmed cell death (PCD), defense against pathogen, etc. Higher concentrations of ROS in the cell is a

Table 8.1 ROS involved in plant stress responses

Stress response	Relative ROS	Gene or phytohormone	Source	References
Water logging	$O_2^{\cdot-}$, H_2O_2	Ethylene, *OsRBOHH*	Rice	Yamauchi et al. (2017)
Water stress	H_2O_2	ABA, *OsCATB*	Rice	Ye et al. (2011)
High temperature	$O_2^{\cdot-}$, H_2O_2	*OsCATB*	Rice	Zhao et al. (2018)
Disease resistance	H_2O_2	Ethylene, *OsEIN2*	Rice	Yang et al. (2017)
Cold temperature	$O_2^{\cdot-}$, H_2O_2	*AtSRC*	*Arabidopsis*	Kawarazaki et al. (2013)
Plant immune	–	*AtRBOHD*	*Arabidopsis*	Kadota et al. (2014)
Drought	O_2^{-}, H_2O_2	ABA, *AtNTL4*	*Arabidopsis*	Lee et al. (2012)
Al stress	H_2O_2	*AtPRX64*	Tobacco	Wu et al. (2017)
Organic pollutants treatment	$O_2^{\cdot-}$, NO	24-Epibrassinolide	Cucumber	Ahammed et al. (2017)
Microbial pathogens	H_2O_2	SA, *CaPAL1*	Pepper	Kim and Hwang et al. (2014)

significant threat which ultimately leads to DNA and cellular damage, resulting in either cell survival or apoptosis depending on the severity of stress. Moreover, ROS may interact with hormones and epigenetic modifiers to manage plant developmental processes and stress responses (Huang et al. 2019). Usually, lower levels of ROS are necessary for continuation of various morphological functions, such as cellular differentiation and proliferation. Plants generally cope with excessive ROS and maintain cellular redox homeostasis by utilizing antioxidative defense mechanism.

8.2 Generation and Homeostasis of ROS in Plants

Plant cells usually come into a state of "oxidative stress" when the level of ROS is higher than the internal defense mechanisms. Generally, oxidative stress is either caused directly by environmental stress or indirectly by generated ROS, which accumulates and causes cell damage before elimination. It was hypothesized that ROS production is the primary symptom of toxicity in plant cells and the mechanism has been studied widely under abiotic stress (Choudhury and Panda 2004).

8.2.1 Types of ROS

ROS can be present in either ionic or molecular states or both in plant cells, where ionic states/free radicals include superoxide anions (O_2^-), hydroxyl radicals (·OH), perhydroxyl radical (HO_2), and alkoxy radicals (RO) and molecular states/

nonradicals include hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Mittler et al. 2004; Hossain et al. 2015; Kalia et al. 2017). ROS vary with their oxidative potential and each ROS affects different physiological functions governed by different genes.

8.2.1.1 Superoxide Anions (O_2^-)

Superoxide anion ($O_2^{\cdot-}$) is generated by the one-electron reduction of molecular oxygen and is considered as the precursor of various ROS due to its instability and higher redox potential. It can be generated by both photosynthetic and mitochondrial electron transfer systems and membrane-dependent NADPH oxidase systems. NADPH oxidase transfers electrons from NADPH on the cytoplasmic side to O_2 producing O_2^- (Sharma et al. 2012), moreover, endoplasmic reticulum also mediates generation of O_2^- by Cyt P450 (Mittler 2002). Generally, O_2^- produced at lower levels could maintain the stability of cells (Zeng et al. 2017). But at higher concentrations, it causes increased production of ROS, which ultimately results in cell death (Gill and Tuteja 2010). Being the precursor for most ROS in plant cell, O_2^- could be catalyzed into other important ROS, such as hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutases (SODs) and subsequently reduced to hydroxyl radical (·OH) or water (H_2O) by peroxidases (Bose et al. 2014; Mhamdi and Van Breusegem 2018). In case of rice, roots and stems are the primary organ of oxygen production, which might be considered as an adaptive mechanism in aquatic environment.

8.2.1.2 Hydroxyl Radicals (·OH)

Hydroxyl radicals (·OH) are generated when the O−O double bond in H_2O_2 breaks. Since·OH is an active compound and has a high reaction rate constant, it generally acts very close to its production site and is most reactive ROS which can interact with any biomolecule. ·OH can oxidize polysaccharides in cell wall, which results in loosening of the cell wall (Karkonen and Kuchitsu 2015) and may also cause single-strand DNA breakage (Hiramoto et al. 1996).

8.2.1.3 Hydrogen Peroxide (H_2O_2)

Although various types of ROS are known to be produced inside the cell through metabolism of oxygen, endogenous ROS hydrogen peroxide has received the most attention due to its redox potential, specific physical and chemical properties, rapid and reversible oxidation of target proteins in cells, and significant stability within cells (half-life of 1 ms) (Mittler 2017; Mhamdi and Van Breusegem 2018). Due to its remarkably significant longer half-life of 10^{-3} s, it can cover longer distances spanning cell membranes via aquaporins causing oxidative damage (Bienert et al. 2007). During stress, the majority of endogenous hydrogen peroxide is produced from molecular oxygen via superoxide anion intermediate which undergoes enzymatic reduction to produce H_2O_2 in a stepwise manner (Černý et al. 2018). It plays a vital role in the regulation of senescence and programmed cell death (Jajic et al. 2015), stomatal behavior (Rodrigues et al. 2017), cell wall formation (Li et al. 2017a), regulation of the cell cycle and cell differentiation (Pokora et al. 2017),

photosynthesis (Exposito-Rodriguez et al. 2017), adaptation to stress (Lv et al. 2018), and antioxidative defense (Liu et al. 2016). Additionally, H_2O_2 interplays with other hormones such as abscisic acid (ABA), ethylene, brassinosteroid (BR), and auxin that have been found vital for plant development and senescence. Both ABA and BR can induce heat and paraquat (PQ) oxidative stress tolerance in tomato plants via H_2O_2 produced by NADPH oxidase (Zhou et al. 2014). In faba bean, ethylene can mediate UV-B-induced stomatal closure through peroxidase-dependent H_2O_2 generation (He et al. 2011). H_2O_2, which is transported by cell membrane-localized aquaporins, not only causes long-distance oxidative damage, but also participates in the regulation of cell signaling (Miller et al. 2010).

8.2.1.4 Singlet Oxygen ($1O_2$)

Singlet oxygen ($1O_2$) is excited oxygen, which is the product of phytochemical processes having strong oxidizability. It has a very short half-life and is highly unstable. It greatly impacts the process of photosynthesis in plants. Photosystem-I (PSI) and Photosystem-II (PSII), which are known as core of the light-harvesting complex in the thylakoids of chloroplasts are the primary sources of ROS generation. At the PSII reaction center, $1O_2$ may be generated under stress from O_2 during over-excitation of chlorophyll molecule by sunlight (Tripathy and Oelmuller 2012).

8.2.2 Production Sites of ROS

ROS can be produced in both stressed and unstressed conditions in a plant cell. When oxygen is gradually reduced under exposure to high-energy or electron-transport system, various high-energy ROS are generated. ROS activation in plants is an energy-dependent mechanism, which requires an inescapable leakage of electrons from the electron-transport systems of different cell organelles such as mitochondria, peroxisomes, chloroplasts, endoplasmic reticulum (ER), apoplasts, plasma membranes, and cell wall or produces as a by-product of various metabolic pathways in various cellular organelles (Sharma et al. 2012; Xia et al. 2015; Corpas et al. 2015).

Chloroplasts and peroxisomes function as the main site of ROS generation in presence of light while mitochondria act as the major source of ROS production in the dark (Choudhury et al. 2013). Thylakoids in chloroplast possess the core of highly efficient light-harvesting complex, viz., PSI and PSII, the main sources of ROS generation (Tripathy and Oelmuller 2012; Dar et al. 2017). At the PSII reaction center, $1O_2$ may be generated under stress from O_2 during over-excitation of chlorophyll by sunlight (Tripathy and Oelmuller 2012). Besides, $O_2^{\cdot -}$ may also be formed at PSI via Mehler reaction (Karuppanapandian et al. 2011) or at PSII during electron transfer to O_2 through QA and QB (Das and Roychoudhury 2014). Additionally, due to the activities of flavin oxidases, peroxisomes are the main sites of H_2O_2 generation. Generation of mitochondrial ROS takes place at ETC located on the inner mitochondrial membrane. During oxidative phosphorylation, leakage of

electrons from complex I and complex III of ETC leads to a partial reduction of oxygen to form superoxide.

Apart from these organelles, there is membrane-mediated generation of ROS. Plasma membrane serves as a platform for redox signal transmission and plays a significant role in sensing environmental conditions. Localized NADPH-dependent oxidase transfers a cytoplasmic enzyme, which produces ROS by transferring electrons from NADPH on the cytoplasmic side to molecular oxygen electrons (Sharma et al. 2012). ROS in apoplasts is mainly produced by plasma membrane-localized NADPH oxidases, cell wall peroxidases, and amine oxidases. Under harsh environmental conditions, the apoplast rendered H_2O_2 production by stress signals combined with ABA. ER also mediates the generation of O_2^- by Cyt P450 (Mittler 2002).

8.2.3 Oxidative Damage by ROS

ROS are generally produced as a product of normal cellular metabolism. However, at lower or moderate concentration, they act as a secondary messenger in various signaling pathways and mediate a series of reactions in plant cells, including PCD, gravitropism, stomatal behavior, and tolerance to stress. Whether ROS acts as a secondary messenger or could cause cellular damage depends on the dynamic equilibrium between their production and scavenging. However, it is evident that at higher concentrations all types of ROS are significantly harmful and cause progressive oxidative damage. When plants come under constant environmental stresses, they generate huge amount of ROS, which cannot be completely scavenged and causes various physiological changes in tissues like lipid peroxidation, oxidation of nucleic acids, protein denaturation, enzyme inhibition, activation of PCD pathway, etc. (Sharma et al. 2012; Das and Roychoudhury 2014).

8.2.4 Homeostasis/Removal of ROS

Generally, plants scavenge an excessive amount of ROS using various antioxidant defense mechanisms in the cells. However, amount of ROS can be increased dramatically during the stress conditions and the equilibrium between ROS production and scavenging can be disturbed, which cause a sudden increase in intracellular ROS levels, damaging structures of biomolecules in the cells. As plants have developed mechanisms to survive with higher ROS generation for maintaining cellular redox homeostasis, increased levels of ROS are sensed and restrictively controlled through a battery of ROS-scavenging systems. ROS-scavenging mechanisms have been categorized into two types, viz., enzymatic system and non-enzymatic antioxidant defense mechanism, which can work in a collaborative and interactive manner for protecting plants against oxidative damage and proper regulation of low ROS levels for signal transduction mechanism. Enzymatic ROS-scavenging systems in plants are superoxide dismutase (SOD), catalase

Table 8.2 Antioxidant enzymatic defense mechanism in response to oxidative stress

Stress factors	Antioxidative enzymes	Source	Recipient	References
Salinity	Cu/ZnSOD, CAT SOD	*Kandelia candel* *Arachis hypogaea*	*Tobacco*	Jing et al. (2015) Negi et al. (2015)
	PaSOD RaAPX	*Potentilla atrosanguinea* *Rheum austral*	*Arabidopsis*	Shafi et al. (2015)
	PutAPX	*Puccinellia tenuiflora*	*Arabidopsis*	Guan et al. (2015)
	OsAPX	*Oryza sativa*	*Knockout*	Cunha et al. (2016)
Drought	APX	*Solanum melongena*	*Oryza sativa*	Chiang et al. (2015) Xu et al. (2016)
Chilling	SOD, APX Glutaredoxins	*Manihot esculenta* *Arabidopsis thaliana*	*Solanum lycopersicum*	Xu et al. (2014) Hu et al. (2015)
Heavy metal	GR GSH	*Cannabis sativa* Synthetic *Pisum sativum*	*Cannabis sativa* *Oryza sativa*	Fryzova et al. (2018) Mostofa et al. (2014)
UV-B radiations	APX, SOD, POD, CAT	*Cassia auriculata*		Agrawal et al. (2009)
Pathogens	Peroxidase expression	*Oryza sativa*	Mutation	Li et al. (2017c)

(CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and peroxiredoxin (PRX) (Apel and Hirt 2004; Noctor et al. 2014, Table 8.2). Most of the enzymes are mainly located in different cellular compartments and function synergistically in ROS homeostasis. In case of rice plants, most of the genes exhibit organ/tissue-specific expression profiles. However, functions of most of the genes in ROS homeostasis and regulation of their expression remain to be understood. Among enzymatic systems, SOD is the first line of defense, which rapidly converts OH to H_2O_2, which is ultimately converted to H_2O and dioxygen by CAT, APX, and GPX. Catalase (CAT) shows dissimilarity with APX and GPX, which require ascorbic acid (AsA) and/or glutathione (GSH) regenerating cycle involving glutathione reductase, monodehydroascorbate reductase, and dehydroascorbate reductase. Glutathione peroxidase, glutathione S-transferase, and peroxiredoxin can reduce H_2O_2 and organic hydroperoxides by thiol-mediated ascorbate-independent pathways using glutathione, thioredoxin, or glutaredoxin as the nucleophiles (Dietz et al. 2006; Meyer et al. 2012; Noctor et al. 2014). Non-enzymatic antioxidant defense systems included ascorbic acid (AsA), glutathione (GSH), carotenoids, proline,

flavonoids, glycine betaine, tocopherols, which are generally low molecular weight antioxidants in cells and usually help to remove hydroxyl radicals and singlet oxygen (Gechev et al. 2006).

8.3 Roles of ROS in Plant Growth and Development

Presence of aerobic environment has given organisms an opportunity of using oxygen as an electron acceptor and traps its reactive nature for cellular metabolism and signaling (Schippers 2012; Foyer and Noctor 2016). Therefore, it was unavoidable that evolutionary mechanisms in aerobic environment would require incorporation of oxidative processes including sensing and signaling of ROS into developmental programs of plant cells. Starting from germination of seeds to senescence of plants, ROS can be produced or scavenged dynamically, making plants regulate their developmental mechanisms for adopting in different environmental conditions. Therefore, the consequences of ROS on growth and developmental processes of plants are very complex due to the temporal and spatial variability of ROS regeneration and interplay between different ROS species in cells.

8.3.1 ROS Participate in the Maintenance of Plant Vegetative Apical Meristems

Morphogenesis in plants is regulated by both internal genetic constitution and external environmental conditions. Recent evidence indicates that homeostasis of ROS can shape vegetative apex development in plants. In case of Arabidopsis, $O_2^{\cdot-}$ generally assembles in the root-tip meristem and helps in the cell division, while the accumulation of H_2O_2 mainly occurs in the zone of elongation, which confers differentiation of cells (Tsukagoshi et al. 2010). Distribution of these two ROS micro-environments is critical for the determination of the transition zone. Due to the presence of ROS species gradients, plants cells entering into the transition zone keep on multiplying (Dunand et al. 2007). When the ratio of O_2^- to H_2O_2 has been reached a certain level, cell division ceases and cells start to elongate. Hence, the ROS homeostasis in the transition zone is very important.

8.3.2 ROS Trigger Plant Organ Morphogenesis

ROS as signaling molecules are dispersed in almost every plant tissue, particularly in metabolically active tissues like meristematic tissue. Continuation in homeostasis and generation of ROS regulate germination of seeds through gibberellic acid and/or abscisic acid metabolism in Arabidopsis and signaling mechanisms in barley (Baek et al. 2015; Ishibashi et al. 2015). ROS may also play important roles in development of leaf, senescence, and organ dormancy.

8.4 Interplay Between ROS and Epigenetic Modification

Epigenetic modifications include DNA methylation, chromatin remodeling, microRNA (miRNA) expression, and modification of histone proteins to regulate the expression of various genes. If these changes are either meiotically or mitotically hereditable, then only they can be correctly defined as epigenetic markers (Chinnusamy and Zhu 2009). These alterations either individually or in consensus work to modify gene expression throughout the growth and development of plants and protect them from stresses. RNAi-dependent silencing mechanisms are also involved in transcriptional or post-transcriptional gene expression regulation after plant exposure to stress (Li et al. 2017b). It has been shown that DNA methylation regulates various molecular processes, such as chromosome stability, remodeling and transcription and plays a key role in gene expression by enhancing RNA-directed DNA methylation (RdDM) of genes and histone modifications (Yaish 2013). Studies have shown that variation in ROS levels induces obvious epigenetic modifications such as acylation, which modulates the activity of ROS-related proteins in rice leaves (Zhao et al. 2018). This interaction between ROS and acylation might have played important roles in PTMs (post-translational modifications) of leaf proteins that have key metabolic functions. Studies have also revealed a close connection between various epigenetic marks and specific redox pathways through intermediates, such as 2-oxoglutarate, FAD, NAD, and acetyl-Co A, which act as linkers between ROS and epigenetic processes (Locato et al. 2018). Alterations in the concentration of these intermediates may affect epigenetic signaling, resulting in modification of phenotypic characters. Transcription factors induced by stress may directly recruit histone-modifying complexes, e.g. COMPASS H3K4 methyltransferase complex by stress-activated bZIP transcription factors (Song 2013). Another stress-related chromatin modification is remodeling of nucleosomes that plays a crucial role in abscisic acid (ABA)-mediated stress responses (Lamke and Baurle 2017). Plants cope stress and oxidative damage by forming heat shock proteins and molecular chaperon that stabilize protein by folding and unfolding, assembling multi-protein complexes and control cell cycle (Khan and Shahwar 2020) In mammals epigenetic regulation in response to oxidative stress is generally carried out by histone deacetylases (HDACs) (Shimazu et al. 2013) which can change their conformation, consequently altering the catalytic activity or cellular localization. Conversely, when ROS level is high, they increase various histone modifications such as H3K4me2/3, H3K79me3, H3k27me3, and H3K9me2, due to inhibition of histone demethylases (Chen et al. 2006; Niu et al. 2015). Four distinct bifunctional DNA glycosylases, like DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1), (DML2), and DML3 catalyzed the active removal of methylated bases and cleaving the DNA backbone at abasic sites (Li et al. 2018). Recent studies have shown that DNA demethylase, ROS1, and DME interact directly with Fe–S cluster assembly machinery, required for active DNA demethylation. These results reveal a close relation between ROS metabolism and DNA methylation. Glutathione (GSH), a tripeptide ubiquitously present in nearly all compartments of plant cell including the nucleus, functions as a redox buffer against

ROS (Zechmann et al. 2008). Under stress condition, it prevents the oxidative denaturation of proteins by protecting their thiol groups. GSH acts as a precursor of phytochelatins and helps in chelating toxic metals which are transported and sequestered in the vacuole (Hasanuzzaman et al. 2017). Studies have shown that nuclear GSH serves not only as a vital non-enzymatic antioxidant but also as an essential regulator of chromatin structure.

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