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Preface

The world population is increasing at an alarming rate. While it has already crossed the seven billion line, it is expected to continue rising in the near future. To feed the teeming humanity in the new millennium, a huge responsibility lies on the shoulders of plant scientists to discover newer ways of enhancing crop production. Along with the inputs from classical breeding, molecular breeding, and biotechnology sciences, will nanotechnology also help in this venture? Will the birth of so-called 'Nanobiotechnology' science prove a boon? Nanotechnology in a naïve sense may appear as a paradigm of the physical sciences. This is however an understatement of the potential of nanotechnology. As it turns now, the advanced and modern nanotechnology science is equally relevant to life sciences and may play a major role in improving the quality of human life in the future years. Based on nanotechnology principles, novel inventions are being made everyday in the field of medicine. Nanoparticles are receiving much attention because of their unique physicochemical properties. The nanoparticles are thus being employed as "smart" delivery systems in life sciences. No wonder, the Noble laureate in Physiology Paul Ehrlich referred these compounds as "magic bullets". In agriculture, nanoparticles are proving important as compound fertilizers and nanopesticides. Most excitingly, it is shown in recent years that nanoparticles may act as chemical delivery agents for targeting molecules such as genes/DNA to specific cellular organelles like nuclei in plants.

Considering that gaining a deeper understanding of the role of nanotechnology in relation to plant systems is of paramount importance, we felt that a dedicated book on bringing together varied aspects of plant and nanotechnology is the need of the hour. Our book *Nanotechnology and Plant Sciences: Nanoparticles and Their Impact on Plants* presents a holistic view of the use of nanoparticles in complex and dynamic aspects of plant research. The inclusion of nanoparticles in commercial products and industrial applications has significantly increased. To further extend these commercial gains, it is important to understand the interaction mechanisms between the nanoparticles and biological systems at the molecular level. The latter aspect has been emphasized in this book. As a new emerging field, nanobiotechnology unlocks new frontiers in genetic engineering science. However,

the information available on the use of nanoparticles in genetic transformation of plants is still scarce. We have tried to bring together the views of experts of these subjects under one platform of this book to address the above issues.

This book has 14 chapters written by experts with considerable experience in the area of research. The contents of each chapter are based on the research findings of active workers in nanotechnology. The book covers various important topics related to nanoparticles and plants. It provides an understanding of the mechanisms involved in the response of plants to nanoparticles. We firmly hope that the readers of this book will be exposed to new challenges and at the same time new vistas of future line of action in the area of plants and nanotechnology. We believe that students and researchers of plant molecular biology, plant physiology, agriculture, botany, biochemistry, biotechnology, environmental biology, microbiology, and forestry will be hugely benefitted by the contents of this book. We also hope that NGOs dealing with civic problems caused by rapid environmental degradation will find this book useful. The book will lead to a better understanding of the interdisciplinary field of functional biology and nanoparticles. The aim of writing this book was to bring together all possible approaches to tackle the aim of the improvement of current crops and introducing crop plants into areas not currently being used for cultivation. We have tried our best to realize these goals in bringing out this book and now we want the readers to evaluate how far we have been successful in this aim.

The editors convey their heartfelt gratitude to all the contributors for their excellent, informative, and up-to-date contributions and for their consistent support and cooperation. We are particularly grateful to Christina Eckey, Senior Editor, Plant Sciences, Springer and Anette Lindqvist, Project Coordinator for their continuous support and technical advice.

We also thank Dr. Anil Grover for his critical assistance and for encouraging me from time to time during the preparation of this important book.

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Chapter 1
Chemistry, Biochemistry of Nanoparticles, and Their Role in Antioxidant Defense System in Plants

C.M. Rico, J.R. Peralta-Videa and J.L. Gardea-Torresdey

Abstract As time passes, engineered nanoparticles (ENPs) are more frequently found in medical and consumer products, as well as in industrial and agricultural applications. The intensive production, use, and disposal of ENPs-containing wastes increase the likelihood of emission of such products to the environment. During the last two decades, a body of scientific literature has shown that ENPs interact with living components of ecosystems in different ways. The literature indicates that ENPs impact on plant growth, cell structure, and physiological and biochemical functions. In this chapter we discuss the stress induced by ENPs on higher plants. Although some references about carbon-based ENPs are included, most of the references are related to metal-based ENPs. The discussion is mainly focused on the effects of ENPs on photosystems and the mechanisms of generation/scavenging of reactive oxygen species (ROS). Effects on the enzymes catalase (CAT), guaiacol peroxidase (GPOX), ascorbate peroxidase (APOX), superoxide dismutase (SOD), glutathione reductase (GR), and dehydroascorbate reductase (DHAR) are discussed. Information about low molecular weight antioxidant thiols (GSSG or GSH) and ascorbate is also included.

Keywords Engineered nanomaterials · Vascular plants · Physiology · Biochemistry · Reactive oxygen species

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1.1 Introduction

Metabolic processes in aerobic organisms, like plants, generate reactive oxygen species (ROS) molecules as intermediate products of the reduction of ground state oxygen (O_2) to water (Apel and Hirt 2004; Karuppanapandian et al. 2011). Oxygen is reduced for energy production and in the process, the following ROS are sequentially produced: Singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydroperoxy radical ($HO_2^{\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) (Apel and Hirt 2004; Karuppanapandian et al. 2011).

Plants continually produce ROS in structures such as chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum (ER), and plasma membranes (Karuppanapandian et al. 2011). Likewise, ROS molecules do not build up because they are continually removed nonenzymatically or enzymatically by a complex defensive system. Components of the defensive system have been classified according to their catalytic activity, molecular weight, compartment where they act, and level of defense or mechanism of action (Pradedova et al. 2011).

There are several biotic and abiotic factors that alter the equilibrium between production and removal of ROS. Insect attacks are among the most studied biotic stressors. At the penetration point, there is a local hypersensitive response and subsequent production of phytoalexins and other pathogenesis related proteins in preparation for programmed cell death (PCD). This process generates the production of ROS (De Gara et al. 2003). According to De Gara et al. (2003), excess ROS produces alterations in the "levels and/or redox state of ascorbate and/or glutathione (GSH), as well as in the activity of their redox enzymes." Phosphorylated proteins have shown to be another response to biotic stress in plants. Huang et al. (2011) quantified changes on phosphoproteins in *Arabidopsis thaliana* leaves treated with compounds mimicking biotic stresses. They found and characterized 75 phosphoproteins very likely associated with biotic stressors. Tyagi et al. (2014) found that rice plants invaded by bacteria and fungi showed upregulation of OsSAP1 and OsSAP11. The functional role of OsSAP1 in plant defense responses has been explored through overexpression in transgenic plants (Tyagi et al. 2014). Additionally, the gene family GF14 of rice plants is up-regulated under pathogen attack; while in other plants, phytohormones like ethylene, salicylic acid, and jasmonic acid increased under biotic stress (Fraire-Velázquez 2011).

Abiotic stress is produced by a series of factors like extreme temperatures, chemical compounds, unbalances in water conditions, and excess of heavy metals. Mizoi et al. (2012) reviewed recent literature about plant stress responses under temperature and water conditions. According to the literature, plants have binding proteins that activate the expression of abiotic stress-response genes. Responses to water and temperature stresses are regulated by a large family of transcription factors named AP2/ERF that shares a well-conserved DNA-binding domain. Calcium-dependent protein kinases, Ca^{2+}, and ROS are also well-characterized signaling molecules upregulated under abiotic and biotic stresses (Fraire-Velázquez 2011). The genetic pathway includes several gene families upregulated

under abiotic stress. Hashimoto et al. (2004) reported that in rice, the gene RO-292 is upregulated under salt and drought stresses.

Heavy metals are well known abiotic plants' stressors. In a recent review, Ovecka and Takac (2014) highlighted the strategies used by plants to manage the stress imposed by heavy metals. These authors concluded that the response of plants to heavy metal stress is genotype-specific but "to some extent, modulated by environmental conditions." Several metal transporters have been found to be involved in maintaining heavy metal homeostasis in plant cells. Detailed information about families of transporters like ZIP, HMA, CDF, NRAMP, phytochelatins, and others, was analyzed by Ovecka and Takac (2014). Studies have also been aimed to study the effects of heavy metals on ROS production and proton pumps at vacuolar and plasma membrane levels. Kabała et al. (2008) reported that Cd, Cu, and Ni did not affect the proton pumps; however, these metals modified the structure and properties of plasma membrane fatty acids. Lipid peroxidation is another typical effect of oxidative stress imposed by heavy metals in plants. In barley (*Hordeum vulgare* L.), μM concentrations of Cu (10), Zn (4), Cr (4), Ni (4), Pb (0.1), and Cd (22) were found to induce lipid peroxidation (Juknys et al. 2012). Moreover, Zn and Cd reduced the dry biomass production at concentrations as low as 0.1 and 3 μM, respectively (Juknys et al. 2012). Other types of stress produced by heavy metals include reduction in photosynthesis (Cu U, Zn, Cd), changes in root ultrastructure and architecture (Al, Cd, Cu), and alteration in cellular ionome (Cd, Pb, U) (Viehweger 2014). Silver is another heavy metal that has shown to cause stress in plants, even at low concentration. Kaveh et al. (2013) reported that after 10 days of exposure to 5 mg Ag^{+}/L, there were 84 genes upregulated and 53 genes downregulated in *A. thaliana*. Some of the upregulated genes were linked to oxidative stress and some of the downregulated genes were linked with response to pathogens and hormonal stimuli.

1.2 Nanoparticles and Their Interaction with Plants

Nanoparticles (NPs), natural or manmade, are materials with at least two dimensions between 1 and 100 nm (ASTM 2012). Manmade NPs (engineered nanoparticles, ENPs) can be carbon-based or metal-based (Peralta-Videa et al. 2011). Carbon-based are of two main types, fullerenes and carbon nanotubes; while metal-based are grouped in metals, metal oxides, and quantum dots (Peralta-Videa et al. 2011). Among the most produced and used metal-based ENPs are zinc oxide (*n*ZnO), titanium dioxide (*n*TiO_2), gold (*n*Au), silver (*n*Ag), cerium oxide (*n*CeO_2), and copper (*n*Cu) NPs (Keller et al. 2013). Other NPs like *n*Mn, *n*Fe_3O_4, *n*CuO, and *n*$CoFe_2O_4$ are also widely used.

Investigations have shown that both carbon-based and metal-based ENPs are able to produce stress, generating excess ROS with the potential to affect proteins, lipids, carbohydrates, and DNA in plants (Fig. 1.1). Carbon nanotubes, one of the carbon-based ENPs have been reported to induce ROS accumulation enhancing lipid peroxidation in cell culture (Liu et al. 2010) and seedlings' root tips (Liu et al. 2013). On the

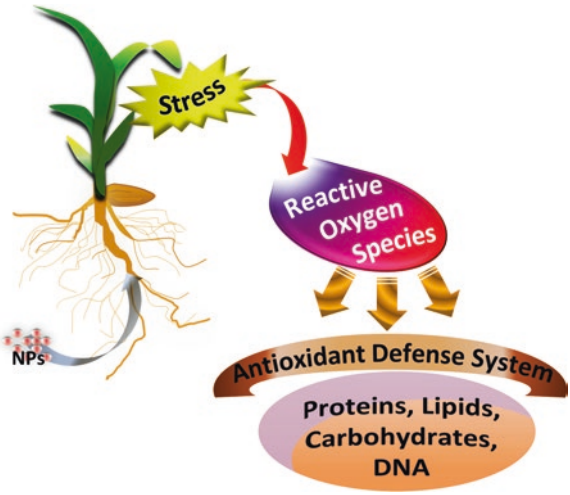


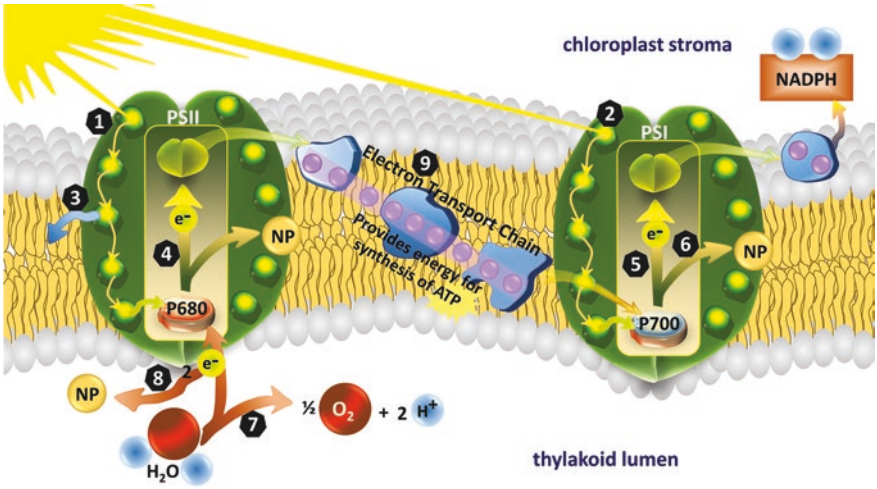
Fig. 1.1 Stress induced by engineered nanoparticles and the antioxidant defense system in plants

other hand, metal-based ENPs and/or the released ions from the NPs have been found to produce stress inducing ROS accumulation in plants. For instance, several reports indicate that *n*Au and *n*Ag affect photosynthesis in different ways (Barrazzouk et al. 2005; Bujak et al. 2011; Olejnik et al. 2013).

Other NPs like $n\text{CeO}_2$, $n\text{Fe}_3\text{O}_4$, $n\text{CoFe}_2\text{O}_4$, and $n\text{TiO}_2$ have also shown to affect chlorophyll and ROS generation (Mingyu et al. 2007; Ursache-Oprisan et al. 2011; Rico et al. 2013b). Studies have shown that both rutile and anatase crystalline phases of $n\text{TiO}_2$ were found to generate ROS in spinach (Fenoglio et al. 2009). The stress imposed by *n*ZnO and *n*CuO has been associated to the NPs and released Zn and Cu ions (Shi et al. 2011; Kumari et al. 2011; Lee et al. 2013; Nair and Chung 2014). On the other hand, contradictory results have been reported about ROS generation by $n\text{CeO}_2$ (Rico et al. 2013b; Gomez-Garay et al. 2014). In the following sections we discuss the stress imposed by ENPs/released ions on plants and their defensive mechanisms.

1.2.1 Interaction of ENPs with the Photosynthetic Machinery

Photosynthetic efficiency is a convenient parameter to detect stress induced by biotic and abiotic factors. Disturbance in the photosynthetic activity results in oxidative stress in plants. NPs alter the photosynthetic efficiency, photochemical fluorescence, and quantum yield in plants; thus, knowledge on the interactions of NPs with the photosynthetic machinery provides understanding on NP-induced oxidative stress and antioxidant defense system in plants. The current knowledge on the influence of NPs on plants photosystems is summarized in Fig. 1.2.



Legend	NPs	Chemistry/mechanism involved	Plants	References
1,4	TiO_2-A	Increased light absorption and quantum yield in PSII	*Spinacea oleracea, Lycopersicon esculentum*	Mingyu et al. 2007, Yang et al. 2007, Lei et al. 2007ab, Qi et al. 2013
1,2,4,5	TiO_2-A	Decreased light absorption	*Ulmus elongata*	Gao et al. 2013
1,2	Au	Increased light absorption due to plasmon resonance effect	*Glycine max*	Falco et al. 2011
1,2	CdSe/ZnS	Decreased light absorption	*Chlamydomonas sp.*	Lin et al. 2009
1,2	CeO_2	Decreased light absorption and photochemical efficiency	*Medicago arborea*	Gomez-Garay et al. 2014
1,2,4,5	CuO	Decreased light absorption and quantum yield in PSII	*Lemna gibba*	Perreault et al. 2010
4	TiO_2-R	No effect on quantum yield in PSII	*Vicia faba*	Foltete et al. 2011
3	CuO, TiO_2-A	Enhanced non-photochemical fluorescence	*L. gibba; Ulmus elongata*	Perreault et al. 2010, Gao et al. 2013
2,5	CdSe/ZnS	Enhanced light absorption and improved quantum yield in PSI	*C. reinhardtii*	Jung et al. 2010
4	Ag	Improved quantum yield in PSII	*Brassica juncea*	Sharma et al. 2012
6	Au	Decreased quantum yield due to chlorophyll to nanoparticles electron/energy transfer	*G. max*	Falco et al. 2011
7	Mn, TiO_2-A	Enhanced splitting of water and evolution of oxygen	*S. oleracea, Vigna radiata*	Pradhan et al. 2013, Lei et al. 2007ab
9	Ag	Enhanced the production of secondary quinone electron acceptors in the electron transport chain (ETC), but inhibited electron transport	*Chlamydomonas reinhardtii*	Matorin et al. 2013
	Mn, TiO_2-A	Improved the photophosphorylation activity in ETC	*S. oleracea, V. radiata*	Gao et al. 2013, Lei et al. 2007ab, Pradhan et al. 2013
	CuO, TiO_2-A	Decreased ETC activity	*U. elongata; C. reinhardtii*	Gao et al. 2013, Saison et al. 2009
	CeO_2	Increased ETC activity	*M. arborea*	Gomez-Garay et al. 2014

Fig. 1.2 Influence of nanoparticles on the photosystems

1.2.1.1 Metal Nanoparticles

The implications of metal nanoparticles in the chemical energy production of a photosynthetic system have been explored. The experiment designed by Govorov and Carmeli (2007) where photosynthetic reaction center of a photosystem I (PSI) was bound to *n*Au and *n*Ag, revealed two competing effects affecting the photosystem efficiency: improved light absorption by chlorophyll molecule due to plasmon resonance effect of metal nanoparticles, and decreased quantum yield by photosystem due to enhanced chlorophyll to metal nanoparticles energy transfer. Similarly, electron transfer from excited fluorophore to *n*Au or *n*Ag has been

reported (Barrazzouk et al. 2005; Nieder et al. 2010; Beyer et al. 2011; Bujak et al. 2011; Matorin et al. 2013; Olejnik et al. 2013).

Falco et al. (2011) determined the effects of *n*Au on photosystem II (PSII) chlorophyll *a* fluorescence quenching in soybean leaves. The chlorophyll was extracted and after mixing with *n*Au of different sizes (5, 10, 20 nm) and different concentrations (0, 3.6, 7.2, 10.6, 14.0, 17.3 μM), the absorbance at 538 nm (the characteristic absorption band of *n*Au surfaces) and fluorescence spectra at typical PSII region (625–800 nm) were measured. Data revealed that absorbance and fluorescence quenching increased at increased *n*Au concentration. The absorbance increased primarily due to higher amount of *n*Au that absorbs light, whereas fluorescence quenching was enhanced due to more *n*Au available for electron transfer. On the other hand, the lowest absorbance was recorded at the highest *n*Au size while the highest fluorescence quenching was registered at the lowest *n*Au size. Low *n*Au size enhanced fluorescence suppression due to its higher surface area that could adsorb large amount of chlorophyll molecule which facilitates better the chlorophyll to metal nanoparticles electron transfer. Similarly, *n*Au (8 nm) increased fluorescence quenching in a chlorophyll solution which was attributed to the enhanced electron transfer from excited chlorophyll molecules to *n*Au (Barazzouk et al. 2005).

In the same study, Falco et al. (2011) investigated the chlorophyll fluorescence in soybean leaves in vivo. Soybean seeds were inoculated with *n*Au and allowed to germinate until the cotyledon, unifoliate, and trifoliate leaves appeared. Chlorophyll fluorescence was measured directly on the surface of each of cotyledon, unifoliate, and trifoliate leaves. Results showed a shifting of fluorescence band to the higher wavelength and a *n*Au-induced quenching of chlorophyll fluorescence. The fluorescence was also measured when the *n*Au was deposited directly either on the surface or bottom surface of the leaves, and the results showed a similar *n*Au-enhanced fluorescence quenching in leaves.

In a similar study, Matorin et al. (2013) examined the influence of *n*Ag on the photosynthetic activity of green algae *Chlamydomonas reinhardtii*. They found that *n*Ag had no direct effects on PSI, but inhibited the electron transfer in PSII, and enhanced the production of secondary quinone electron acceptors (Q_B). These observations were opposite to those reported by Sharma et al. (2012a) wherein *n*Ag improved the quantum efficiency of PSII in *Brassica juncea*.

The modulations in photochemistry of *Vigna radiata* exposed to *n*Mn have been extensively investigated by Pradhan et al. (2013). The analysis of photoreduction activities in isolated chloroplasts revealed that *n*Mn modulated the activity of PSII by enhancing the splitting of water and evolution of oxygen, and improving the photophosphorylation activity of electron transport chain (ETC). Related studies also showed the effects of quantum dots on the photosynthetic activity in *Chlamydomonas* sp. Lin et al. (2009) exposed *Chlamydomonas* sp. to CdSe/ZnS quantum dots and found that QDs decreased light absorption that reduced the photosynthetic activity in the algae. In contrast, Jung et al. (2010) examined the photosynthetic process in PSI purified from *C. reinhardtii* and integrated with CdSe/ZnS QDs. They found that QDs could absorb light and transfer energy efficiently to PSI.

1.2.1.2 Metal Oxide Nanoparticles

Chlorophyll *a*, the major photosynthetic pigment in plants, is more sensitive to photodegradation than other pigments, and could be a more useful indicator of NPs toxicity compared with growth characters. For example, $n\text{CeO}_2$ did not induce apparent signs of toxicity but severely decreased the chlorophyll content in rice (*Oryza sativa*) (Rico et al. 2013a). $n\text{Fe}_3\text{O}_4$ and $n\text{CoFe}_2\text{O}_4$ also showed no toxic effects on sunflower seedlings, but the chlorophyll content decreased, relative to the control, by 50 % in $n\text{Fe}_3\text{O}_4$ and 28 % in $n\text{CoFe}_2\text{O}_4$ treatments (Ursache-Oprisan et al. 2011). A related study also showed that *Scenedesmus obliquus* exposed to 50 mg/L $n\text{SiO}_2$ exhibited a marked reduction in chlorophyll *a*, despite its normal growth (Wei et al. 2010). In contrast, superparamagnetic iron oxides nanoparticles (SPIONs) improved chlorophyll levels without trace of toxicity in soybean (Ghafariyan et al. 2013).

Chlorophyll *a*/*b* ratio is considered a global indicator of photosynthetic activity. It is an indicator of plants response to light and N availability (Hikosaka and Terashima 1996). Ursache-Oprisan et al. (2011) found that $n\text{Fe}_3\text{O}_4$ and $n\text{CoFe}_2\text{O}_4$ (20–100 µL/L) did not affect the germination rate nor caused toxic effects in sunflower; however, chlorophyll ratio in both $n\text{Fe}_3\text{O}_4$ and $n\text{CoFe}_2\text{O}_4$ decreased significantly, compared to the control. On the contrary, Rico et al. (2013a) found that $n\text{CeO}_2$ increased the chlorophyll ratio, despite decreased chlorophyll content in rice, whereas Ghafariyan et al. (2013) did not find changes in chlorophyll *a*/*b* ratio in soybean exposed to SPIONs, indicating that the photosynthetic efficiency was not affected. However, these studies did not clarify the mechanisms causing the changes in chlorophyll *a*/*b* ratio.

The chlorophyll *a* fluorescence in *Lemna gibba* exposed to 0.1–0.4 g/L *n*CuO has been investigated (Perreault et al. 2010). The study showed that *n*CuO markedly decreased the quantum yield which inhibited the photosynthetic processes causing retardation in plant growth. *n*CuO also strongly suppressed the photochemical fluorescence quenching and greatly enhanced the non-photochemical fluorescence quenching, indicating major modifications in PSII photochemistry. Overall, the findings illustrated that *n*CuO decreased conversion of absorbed light energy via PSII electron transport. Similarly, Lalau et al. (2014) reported that *n*CuO caused disruption of mitochondria, dilation of chloroplast membrane, distortion of stroma and grana of the chloroplasts, and alteration of photosynthetic pigments in *Landoltia punctata*. *n*CuO coated with polyacrylic acid also severely damaged the PSII electron transport system in the unicellular algae *C. reinhardtii* (Saison et al. 2009). Here, the toxicity of *n*CuO was attributed to its dissolution and release of copper ions.

Nano-anatase ($n\text{TiO}_2$-A) generally improved the photosynthetic activity in plants due to its large specific surface area, high thermal conductivity, and high photocatalytic ability (Mingyu et al. 2007; Lei et al. 2007a, b; Yang et al. 2007). Studies have shown that $n\text{TiO}_2$-A (5 µM) treatment of spinach (*Spinacia oleracea*) chloroplast resulted in enhanced light absorption in chlorophyll *a*, fluorescence quantum yield in PSII, electron transfer activities, and oxygen evolution rate (Mingyu et al. 2007;

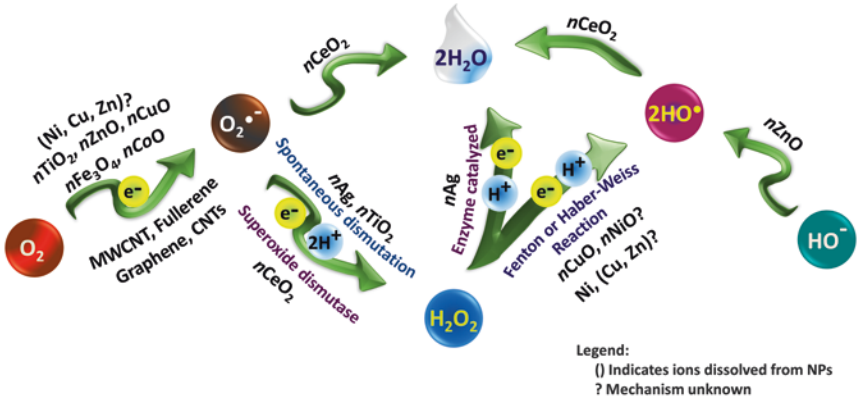
Yang et al. 2007). Spinach and tomato exposed to $n\text{TiO}_2$-A exhibited superior efficiency in the absorption, transfer, and conversion of light in PSII (Lei et al. 2007a, b; Qi et al. 2013). Lei et al. (2007b) hypothesized that the photogenerated electron holes in $n\text{TiO}_2$, h^+, capture electrons from water which accelerated water photolysis and oxygen evolution in PSII. On the contrary, foliar application of 0.1–0.4 % $n\text{TiO}_2$-A in *Ulmus elongata* exposed to light intensity of 800 and 1,600 μmol m^{-2} s^{-1} resulted in lower PSII quantum yield, chlorophyll fluorescence, photochemical quenching, and electron transfer rate, but higher non-photochemical quenching and water loss, relative to the control (Gao et al. 2013). The marked reduction in photosynthetic activity was due to increased water loss caused by decreased mesophyll activity. These researchers also believed that $n\text{TiO}_2$-A reduced electron transfer rate by blocking the electron transfer from Q_A to Q_B. In a related study, altered $n\text{TiO}_2$ (nano-rutile coated with aluminum hydroxide and dimethicone films) at 5–50 ppm concentrations did not change the PSII maximum quantum yield in *Vicia faba*, even after 48-h exposure (Foltete et al. 2011).

Fluorescence emission analysis of 8-month old *Medicago arborea* exposed to $n\text{CeO}_2$ (100–400 mg/L) has been performed (Gomez-Garay et al. 2014). The study revealed that, relative to control, the 100 and 200 mg/L $n\text{CeO}_2$ treatments reduced the photochemical efficiency. At 200 mg/L, $n\text{CeO}_2$ increased the fluorescence levels of fully oxidized and completely reduced plastoquinone electron acceptor pool (Q_A), indicating that the PSII was damaged and the electron transport system was impaired. These results are in stark contrast to those observed when isolated chloroplasts were incubated with $n\text{CeO}_2$ wherein the ROS scavenging ability of $n\text{CeO}_2$ protected the chloroplasts from ROS damages and improved its photosynthetic activity (Boghossian et al. 2013; Giraldo et al. 2014).

1.2.2 Reactive Oxygen Species (ROS) and Oxidative Damage

Reactive oxygen species generation is a toxicological mechanisms of heavy metals (Sharma et al. 2012b) and NPs (Begum et al. 2011; Rico et al. 2013a) in plants. Under various biotic and abiotic stresses, the amount of ROS could increase and result in oxidative damage and cell death in plants. The effects of NPs on the oxidative stress in plants have been widely investigated using techniques that measure either just H_2O_2 or ROS in general. Oxidative damage, also referred to as cell death, is commonly measured by lipid peroxidation (thiobarbituric acid reactive species, TBARS), electrolyte leakage (conductivity test, K^+ leakage), and propidium iodide fluorescence assay.

Literature review would show that the effects of NPs on ROS generation and oxidative damage in plants have been widely investigated. ROS generation and oxidative damage are believed to cause toxicity in NP-treated plants; however, there is still a great lack of understanding on how the chemical properties of NPs induce ROS production and membrane damage in plants. The available reports on the mechanism of NPs on ROS generation or scavenging are summarized in Fig. 1.3.



NPs	Chemistry/mechanism involved	References
CeO_2	Alternates between Ce^{4+} and Ce^{3+} to scavenge $O_2^{\bullet-}$ and •OH, and mimics the superoxide dismutase activity	Bhogossian et al. 2013, Heckert et al. 2008, Horie et al. 2011, Xia et al. 2008
TiO_2	Produces free radicals ($O_2^{\bullet-}$, HO• and $CO_2^{\bullet-}$) in light or dark conditions; Ti^{4+}/Ti^{3+} oxidize/reduce $O_2^{-}/O_2^{\bullet-}$ to O_2/H_2O_2	Fenoglio et al. 2009, Lei et al. 2008
ZnO	Traps electron from ^{-}OH and produce HO•	Li et al. 2008, Mclaren et al. 2009
NiO	Probably produces HO• via Haber-Weiss reaction similar to Ni ions. However, the reaction is not confirmed.	Faisal et al. 2013
CuO	Produces HO• via Fenton reaction.	Fubini et al. 2007
Ag	Improves redox reactions by acting as electron relay center	Mallick et al. 2010
Fe_3O_4, CoO	Block aquaporins and disturb respiration	Wang et al. 2011, Ghodake et al. 2011
Fullerene, CNTs, Graphene	Not clear, probably due to aggregating on root surface and blocking aquaporins	Begum et al. 2011, Tan et al. 2009, Liu et al. 2010, Liu et al. 2013

Fig. 1.3 Proposed mechanisms on how nanoparticles generate/scavenge reactive oxygen species

1.2.2.1 Metal Oxide Nanoparticles

The ROS scavenging ability of nCeO$_2$ has been widely investigated. nCeO$_2$ possess vacant oxygen sites on the surface lattice giving them the ability to alternate between the Ce^{4+} and Ce^{3+} oxidation states and scavenge $O_2^{\bullet-}$ and HO$^{\bullet}$ in the process (Boghossian et al. 2013). The ROS generation and oxidative damage in rice seedlings germinated in nCeO$_2$ (62.5, 125, 250, and 500 mg/L) for 10 days were studied (Rico et al. 2013b). Results revealed that, relative to the control, nCeO$_2$ decreased the H_2O_2 concentration at 62.5 mg/L probably due to the radical scavenging ability of nCeO$_2$ (Heckert et al. 2008; Horie et al. 2011; Xia et al. 2008). The H_2O_2 content increased steadily from 125 to 500 mg/L treatments that was attributed to increased nCeO$_2$ SOD mimetic activity at increased

nCeO_2 concentration (Rico et al. 2013b). Gomez-Garay et al. (2014) also found that low *n*CeO_2 concentrations (100 and 200 mg/L) suppressed ROS production and enhanced cellular resistance to oxidative stress in *M. arborea*. Related studies on *A. thaliana* germinated and grown in *n*CeO_2 and *n*In_2O_3 (0–1,000 ppm) for 25 days revealed that only 1,000 ppm *n*CeO_2 induced lipid peroxidation by 2.5-fold increase relative to the control, while *n*In_2O_3 did not cause lipid peroxidation at all (Ma et al. 2010). It is possible that the increase in ROS generation, as indicated by elevated anthocyanin content, caused the lipid peroxidation in *A. thaliana* at 1,000 ppm *n*CeO_2.

Fenoglio et al. (2009) evaluated the ability of rutile or anatase *n*TiO_2 to produce free radicals ($O_2^{\bullet -}$, $HO^{\bullet}$, $CO_2^{\bullet -}$) and found that both polymorphs generated radicals in light and dark conditions. These researchers also reported that the ability of *n*TiO_2 to generate ROS was independent of its size. The impact of *n*TiO_2-A on oxidative stress and damage in spinach chloroplasts under UV-B radiation (500 $\mu mol\ m^{-2}\ s^{-1}$) was investigated by Lei et al. (2008). The spinach seeds was pre-treated with 0.25 % *n*TiO_2-A (4 h, 10 °C), washed with distilled water and planted in the soil. At four leaf stage, the spinach was sprayed once with *n*TiO_2-A and the chloroplasts were extracted. The chloroplasts were illuminated with UV-B light and the oxidative stress and damage were measured. Results showed that *n*TiO_2-A treatment significantly decreased the accumulation of $O_2^{\bullet -}$ and H_2O_2, which resulted in marked reduction of lipid peroxidation, in spinach chloroplasts under UV-B irradiation. This was attributed to the ability of Ti^{4+}/Ti^{3+} to oxidize/reduce $O_2^-/O_2^{\bullet -}$ to O_2/H_2O_2. *n*TiO_2-A (2, 5, 10 ppm) also caused reduction in electrolyte leakage in the leaves of both cold stress sensitive (ILC 533) and tolerant (Sel 11,439) chickpea (*Cicer arietinum* L.) genotypes under cold stress (4 °C), but the reduction in electrolyte leakage was vaguely attributed to the "increased tolerance mechanisms" induced by *n*TiO_2-A in chickpea (Mohammadi et al. 2013). On the other hand, duckweed (*Lemna minor*) exposed to *n*TiO_2-A (10–2,000 ppm) suspensions for 7 days, did not show lipid peroxidation at $\leq$200 ppm treatments but exhibited significant membrane damage at $\geq$500 ppm (Song et al. 2012). However, there was no mechanism given on how the *n*TiO_2-A modified the lipid peroxidation in duckweed. Similarly, altered *n*TiO_2-R (5–50 ppm) did not cause lipid peroxidation in *V. faba* even after 48-h exposure (Foltete et al. 2011). The lack of lipid peroxidation was probably due to biologically inert forms of *n*TiO_2-R internalized in the roots.

Nanoparticulate ZnO also possesses photocatalytic activity making it able to generate free radicals (Xia et al. 2008). Studies revealed that the photocatalytic activity and ROS generation by *n*ZnO are related to its morphology: greater exposure of polar faces leads to higher surface oxygen vacancy that could trap electrons and produce free radicals like $HO^{\bullet}$ (Li et al. 2008; Mclaren et al. 2009). Phytotoxicity studies on *n*ZnO are inconclusive on whether the NPs or NP-released ions are contributing to the observed toxic responses. For example, ROS production in *Allium cepa* exposed to *n*ZnO were attributed to both NPs and NP-released Zn ions (Kumari et al. 2011). In contrast, oxidative stress in *A. cepa* and buckwheat (*Fagopyrum esculentum*) was attributed to *n*ZnO (Ghodake et al. 2011;

Lee et al. 2013), while that in green algae *Pseudokirchneriella subcapitata* was attributed to dissolved free zinc ions (Lee and An 2013).

Reactive oxygen species generation in tomato roots treated with *n*NiO (0.25, 0.5, 1.0, 1.5, and 2.0 mg/mL) has been investigated by Faisal et al. (2013). The researchers found that *n*NiO caused ROS generation in tomato roots with a very sharp increase observed at higher *n*NiO concentrations (1.0, 1.5 and 2.0 mg/mL). They also found high levels of ROS in the protoplasts extracted from tomato roots. As a consequence, lipid peroxidation in the treated tomato roots was greatly elevated by 39.3–49.5 %, relative to the control. However, it is not clear if ROS generation was induced by *n*NiO or Ni ions since both Ni species were detected in the tomato root cells. It has already been established that Ni ions generates $HO^{\bullet}$ radical in plant cells through the Haber-Weiss cycle; however there is no clear mechanism on how *n*NiO induces ROS production in plants. Similarly, studies on *n*CuO are inconclusive on whether ROS generation and oxidative damage in plants was due to NPs or NP-released Cu ions (Shi et al. 2011; Lee et al. 2013; Nair and Chung 2014). For example, an experiment on *n*CuO with appropriate soluble copper control revealed that increased lipid peroxidation in plants could apparently be attributed to *n*CuO because of its limited dissolution in growth media (Shi et al. 2011; Lee et al. 2013). However, some researchers believe that *n*CuO gets dissolved inside the plant releasing Cu ions that may undergo redox reactions between Cu^{2+} and Cu^{+} and cause oxidative damage (Hoshino et al. 1999; Shi et al. 2011). However, some studies also showed that phytotoxicity could be induced by both *n*CuO and NP-released Cu ions (Dimpka et al. 2013).

Elodea densa exposed to *n*CuO (0.025, 0.25, 0.5, 1.0, and 5.0 mg/L) for 3 days also manifested significantly higher lipid peroxidation in the leaves at 0.25, 1.0, and 5.0 mg/L concentrations compared to the control (Nekrasova et al. 2011). The enhanced lipid peroxidation was attributed to the involvement of *n*CuO as polyvalent metals in ROS generation via the Fenton reaction (Fubini et al. 2007). The membrane damage, as measured by K^{+} leakage assays, in maize exposed to *n*CuO (10 and 100 mg/L) for 15 days in a hydroponic setup has also been reported (Wang et al. 2012). Results demonstrated that *n*CuO compromised the membrane integrity in roots than the shoots in both 10 and 100 mg/L concentrations, relative to the control, which was obviously due to the direct exposure of roots to the *n*CuO solution. It is interesting to note that the membrane damage at 100 mg/L *n*CuO was concomitant with the significant reduction in water content. The researchers hypothesized that water deficit due to blocking of water channels by *n*CuO lead to the inhibition in respiration rate that resulted in ROS generation and oxidative damage (Wang et al. 2012). *n*Fe_3O_4 (30 and 100 mg/L) also greatly enhanced the degree of lipid peroxidation in the roots, but not in the shoots, of ryegrass and pumpkin (Wang et al. 2011). The increase in lipid peroxidation was also attributed to *n*Fe_3O_4 blocking the aquaporins and disturbance in the respiration rate in the root. Similarly, the massive deposition of cobalt (II, III) oxide nanoparticles on root surface caused the oxidative damage in *A. cepa* (Ghodake et al. 2011).

1.2.2.2 Other Nanoparticles

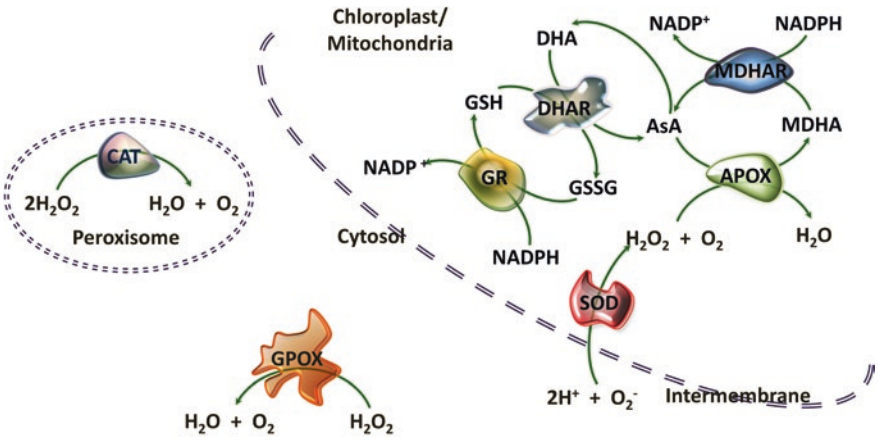
Sharma et al. (2012a) determined the effect of 7-day treatment of *n*Ag (25–400 ppm) on the H_2O_2 generation and lipid peroxidation in *B. juncea*. Interestingly enough, they found a significant reduction in H_2O_2 accumulation and lipid peroxidation at 25 and 50 ppm *n*Ag-treated plants. These researchers hypothesized that *n*Ag increased the efficiency of redox reactions, based on the ability of *n*Ag to act as electron relay center that improves the efficiency of catalytic activity in redox reactions (Mallick et al. 2006).

Begum et al. (2011) performed toxicity study in cabbage, tomato, and red spinach exposed to graphene (500, 1000, and 2000 mg/L) for 20 days. Results showed a graphene concentration-dependent increase in H_2O_2 production, cell death and electrolyte leakage in graphene-treated leaves. The negative impact of graphene was attributed to its aggregation on root surface. Studies in rice suspension cells exposed to 20 mg/L sonicated multi-walled carbon nanotubes (S-MWCNTs) revealed a time-dependent increase in ROS content, which reached up to 3.5 times higher than the untreated, and decreased cell viability at increased S-MWCNTs concentrations (20–80 mg/L) (Tan et al. 2009). An increased ROS generation and enhanced degree of membrane damage in tobacco BY-2 cells incubated in 0.01 mg/mL water-soluble carboxyfullerenes for 3 days has also been observed (Liu et al. 2010). Similarly, ROS accumulation and lipid peroxidation were attributed to the association of CNTs with the cell walls of both rice and tobacco BY-2 suspension cells. On the contrary, a significant reduction in ROS concentration and absence of lipid peroxidation in root tips of *A. thaliana* seedlings germinated in agar treated with 0.01 mg/mL water-soluble fullerene malonic acid derivative (FMAD) were reported (Liu et al. 2013). Here, neither cell wall nor membrane damage was observed, which led the researchers to conclude that auxin disruption, abnormal cell division, and microtubule disorganization resulted in reduced mitochondrial activity and lower ROS generation. These findings are in agreement with those reported by Boghossian et al. (2013); they found that fullerenol and SWCNT had no ROS scavenging ability.

1.2.3 Antioxidative Defense System

Figure 1.4 displays the different enzymes and low molecular weight antioxidants that comprise the antioxidative defense system in plants. The enzymes include catalase (CAT), guaiacol peroxidase (GPOX), ascorbate peroxidase (APOX), superoxide dismutase (SOD), glutathione reductase (GR), and dehydroascorbate reductase (DHAR). Thiols (GSSG or GSH) and ascorbate are the common low molecular weight antioxidants. As shown in the figure, CAT and GPOX quench both ROS and peroxy radicals while SOD catalyzes the dismutation of O_2^- to H_2O_2.

The APOX, DHAR, and GR are involved in a network of redox reactions in the Halliwell-Asada pathway (ascorbate-glutathione cycle) that control ROS. The



Enzymes	NPs with enzyme-like activity
Catalase	CeO_2, Fe_3O_4, Co_3O_4
Peroxidase	CeO_2, Fe_3O_4, Co_3O_4, CuO, MnO_2, Au
Superoxide dismutase	CeO_2, Pt, Fullerene

Fig. 1.4 *Upper part* The antioxidative enzyme defense system in plants. Reprinted with permission from Rico et al. (2013a). Copyright 2013 American Chemical Society. *Bottom part* Nanoparticles mimicking the activity of natural enzymes (Wei and Wang 2013)

APOX directly reduces the H_2O_2 generated by SOD into H_2O. The DHAR regenerates ascorbate that is utilized by APOX for the reduction of H_2O_2. The GR generates reduced glutathione that is utilized by DHAR to regenerate ascorbate. Wei and Wang (2013) reviewed the antioxidant ability of nanoparticles, and their mechanisms, that mimic the activity of natural enzymes. They found that various nanoparticles exhibit enzyme-like activities: *n*CeO_2, *n*Fe_3O_4, *n*Co_3O_4 mimic catalase; *n*CeO_2, *n*Fe_3O_4, *n*Co_3O_4, *n*MnO_2, *n*CuO, and *n*Au exhibit peroxidase activity; *n*CeO_2, *n*Pt, and fullerene demonstrate superoxide dismutase property. Unfortunately it is difficult, if not impossible, to detect these mimetic activities in experiments using NPs exposure to whole individual plant. Despite the numerous nanophytotoxicity studies showing the disturbances in enzyme activities in plants exposed to NPs, there is no evidence that could correlate the former to the chemical properties of NPs. There is no way to ascertain that the observed changes in enzyme activities were due to the enzyme interactions with the NPs. In fact, studies showed irregular and unpredictable effects of NPs on enzyme activities. For example, *n*TiO_2-A enhanced the activities of SOD, CAT, APOX, and GPOX

in spinach (Lei et al. 2008) and GPOX, SOD, CAT in *L. minor* (Song et al. 2012), but decreased the GR and APOX activities in *V. faba* (Foltete et al. 2011). This makes it difficult to conclude which NPs affect which enzymes. Earlier reviews have shown that the type, concentration, properties, and exposure media of NPs are the important factors affecting the toxicity responses, including oxidative stress and antioxidative defense system in plants. Further, it is generally assumed that the alterations in enzyme activities in exposed plants are responses to modulations in ROS concentration (Fig. 1.1). Thus, the role of NPs chemical attributes on the modulation of antioxidant defense system in plants is not clear, and a topic needed to be explored.

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Chapter 2
Role of Nanoparticles in Plants

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Abstract Nanotechnology opens a large scope of novel application in the fields of biotechnology and agricultural industries, because nanoparticles (NPs) have unique physicochemical properties, i.e., high surface area, high reactivity, tunable pore size, and particle morphology. Nanoparticles can serve as "magic bullets", containing herbicides, nano-pesticide fertilizers, or genes, which target specific cellular organelles in plant to release their content. Despite the plenty of information available on the toxicity of nanoparticles to plant system, few studies have been conducted on mechanisms, by which nanoparticles exert their effect on plant growth and development. Therefore, the present review highlights the key role of nanoparticles in plants. Moreover, nanoscience contributes new ideas leading us to understand the suitable mode of action of nanoparticles in plants. The appropriate elucidation of physiological, biochemical, and molecular mechanism of nanoparticles in plant leads to better plant growth and development.

Keywords Plant nutrition · Plant growth and development · Nanoparticles · Photosynthesis

2.1 Introduction

Nanotechnology, a new emerging and fascinating field of science, permits advanced research in many areas, and nanotechnological discoveries could open up novel applications in the field of biotechnology and agriculture. In the field of electronics, energy, medicine, and life sciences, nanotechnology offers an expanding research, such as reproductive science and technology, conversion of agricultural and food

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wastes to energy and other useful byproducts through enzymatic nanobioprocessing, chemical sensors, cleaning of water, disease prevention, and treatment in plants using various nanocides (Carmen et al. 2003; Nair et al. 2010). Although fertilizers are very important for plant growth and development, most of the applied fertilizers are rendered unavailable to plants due to many factors, such as leaching, degradation by photolysis, hydrolysis, and decomposition. Hence, it is necessary to minimize nutrient losses in fertilization, and to increase the crop yield through the exploitation of new applications with the help of nanotechnology and nanomaterials. Nanofertilzers or nano-encapsulated nutrients might have properties that are effective to crops, released the nutrients on-demand, controlled release of chemicals fertilizers that regulate plant growth and enhanced target activity (DeRosa et al. 2010; Nair et al. 2010). Higher plants, as sessile organisms, have a remarkable ability to develop mechanism to perform better under suitable and unsuitable conditions. Nowadays scientists/researchers want to develop new techniques that could be suitable for plants to boost their native functions. Nanoparticles have unique physicochemical properties and the potential to boost the plant metabolism (Giraldo et al. 2014). According to Galbraith (2007) and Torney et al. (2007) engineered nanoparticles are able to inter into plants cells and leaves, and also can transport DNA and chemicals into plant cells. This area of research offers new possibilities in plant biotechnology to target specific genes manipulation and expression in the specific cells of the plants. The researchers have augmented plants' ability to harvest more light energy by delivering carbon nanotubes into chloroplast, and also carbon nanotubes could serve as artificial antennae that allow chloroplast to capture wavelengths of light which is not in their normal range, such as ultraviolet, green, and near-infrared (Cossins 2014; Giraldo et al. 2014). The engineered carbon nanotubes also boost seed germination, growth, and development of plants (Lahiani et al. 2013; Siddiqui and Al-Whaibi 2014). However, the majority of studies on NPs to date concern toxicity. Comparatively few studies have been conducted on NPs are beneficiary to plants. Research in the field of nanotechnology is required to discover the novel applications to target specific delivery of chemicals, proteins, nucleotides for genetic transformation of crops (Torney et al. 2007; Scrinis and Lyons 2007). Nanotechnology has large potential to provide an opportunity for the researchers of plant science and other fields, to develop new tools for incorporation of nanoparticles into plants that could augment existing functions and add new ones (Cossins 2014). In the present review, we discuss the recent developments in plant science that focuses on the role of nanoparticles (NPs) in plant growth and development and also on plant mechanism.

2.2 Effects of Nanoparticles on Plant Growth and Development

Nanoparticles interact with plants causing many morphological and physiological changes, depending on the properties of NPs. Efficacy of NPs is determined by their chemical composition, size, surface covering, reactivity, and most importantly the dose

at which they are effective (Khodakovskaya et al. 2012). Researchers from their findings suggested both positive and negative effects on plant growth and development, and the impact of engineered nanoparticles (ENPs) on plants depends on the composition, concentration, size, and physical and chemical properties of ENPs as well as plant species (Ma et al. 2010). Efficacy of NPs depends on their concentration and varies from plants to plants (Table 2.1). However, this review covers plausible role NPs in seed germination, roots, plant growth (shoot and root biomass) and photosynthesis.

2.2.1 Silicon Dioxide Nanoparticles

Plant growth and development starts from the germination of seeds followed by root elongation and shoot emergence as the earliest signs of growth and development. Therefore, it is important to understand the course of plant growth and development in relation to NPs. The reported data from various studies suggested that effect of NPs on seed germination was concentrations dependent.

The lower concentrations of nano-SiO_2 improved seed germination of tomato (Fig. 2.1; Siddiqui and Al-Whaibi 2014). According to Suriyaprabha et al. (2012) nano-SiO_2 increased seed germination by providing better nutrients availability to maize seeds, and pH and conductivity to the growing medium. Bao-shan et al. (2004) applied exogenous application of nano-SiO_2 on Changbai larch (*Larix olgensis*) seedlings and found that nano-SiO_2 improved seedling growth and quality, including mean height, root collar diameter, main root length, and the number of lateral roots of seedlings and also induced the synthesis of chlorophyll. Under abiotic stress, nano-SiO_2 augments seed germination. Haghighi et al. (2012), in tomato and Siddiqui et al. (2014) in squash reported that nano-SiO_2 enhanced seed germination and stimulated the antioxidant system under NaCl stress. Shah and Belozerova (2009) tested silica, palladium, gold and copper NPs in their study and found that all these NPs have a significant influence on lettuce seeds. Exogenous application of nano-SiO_2 and nano-titanium dioxide (nano-TiO_2) improves seed germination of soybean by increasing nitrate reductase (Lu et al. 2002) and also by enhancing seeds ability to absorb and utilize water and nutrients (Zheng et al. 2005). Under salinity stress, nano-SiO_2 improves leaf fresh and dry weight, chlorophyll content and proline accumulation. An increase in the accumulation of proline, free amino acids, content of nutrients, antioxidant enzymes activity due to the nano-SiO_2, thereby improving the tolerance of plants to abiotic stress (Kalteh et al. 2014; Haghighi et al. 2012; Li et al. 2012; Siddiqui et al. 2014). Wang et al. (2014) performed an experiment on rice plant treated with quantum dots (QDs), without QDs and with silica coated with QDs, and found silica coated with QDs promoted markedly rice root growth. Nano-SiO_2 enhances the plant growth and development by increasing gas exchange and chlorophyll fluorescence parameters, such as net photosynthetic rate, transpiration rate, stomatal conductance, PSII potential activity, effective photochemical efficiency, actual photochemical efficiency, electron transport rate and photochemical quench (Siddiqui et al. 2014; Xie et al. 2011).

Table 2.1 Beneficiary concentration(s) of nanoparticles for plants

Nanoparticle(s)	Beneficiary concentration(s)	Plant	Part of plant/process	Reference(s)
Graphene oxide	400 and 800 mg/L	*Vicia faba* L.	Germination	Anjum et al. (2014)
CNTs	40 µg/mL	*Lycopersicum esculantum*	Germination and seedling growth	Morla et al. (2011)
	75 wt% CNTs	*Medicago saliva, Triticum aestivum*	Root elongation	Miralles et al. (2012)
	75 wt% CNTs Impurities	*Medicago saliva, Triticum aestivum*	Root elongation	Miralles et al. (2012)
SWCNTs	9, 56, 315, and 1,750 mg/L	*Allium cepa, Cucumis sativus*	Root elongation	Cañas et al. (2008)
MWCNTs	25–100 µg/mL	*Hordeum vulgare* L., *Glycine max, Zea mays*	Germination	Lahiani et al. (2013)
	50 and 200 µg/mL	*Lycopersicon esculentum Mill*	Plant height and number of flowers	Khodakovskaya et al. (2013)
	5 up to 500 µg/mL	*Nicotiana tabacum*	Growth	Khodakovskaya et al. (2012)
o-MWCNTs	10–160 µg/mL	*Triticum aestivum*	Root growth, vegetative biomass	Wang et al. (2012a)
wsCNTs	6.0 µg/mL	*Cicer arietinum*	Growth rate	Tripathi et al. (2011)
MWCNTs, dMWCNT	40 µg/mL	*Lycopersicon esculentum Mill*	Uptake nutrients (K, Ca, Fe, Mn and Zn)	Tiwari et al. (2013)
Pristine MWCNTs	20 mg/L	*Zea Mays*	Nutrient transport, biomass	Tiwari et al. (2014)
ZnO NPs	400 mg/kg	*Cucumis sativus* fruit	Micronutrients (Cu, Mn and Zn)	Zhao et al. (2014)
	1.5 ppm (foliar spray)	*Cicer arietinum* L	Shoot dry weight	Burman et al. (2013)
	20 ppm (suspension, foliar spray)	*Vigna radiata*	Biomass	Dhoke et al. (2013)
	1,000 ppm	*Arachis hypogaea*	Germination	Prasad et al. (2012)
	1,000 ppm	*Arachis hypogaea*	Stem, root growth and Yield	Prasad et al. (2012)
	500, 1,000, 2,000 and 4,000 ppm	*Vigna radiate* L. Wilczek	Dry weight	Patra et al. (2013)

(continued)

Table 2.1 (continued)

Nanoparticle(s)	Beneficiary concentration(s)	Plant	Part of plant/process	Reference(s)
GNPs	10 and 80 µg/mL	*Arabidopsis thaliana*	Germination	Kumar et al. (2013)
	10 and 80 µg/mL	*Arabidopsis thaliana*	Root length	Kumar et al. (2013)
	10 µg/mL	*Arabidopsis thaliana*	Shoot and root system (longer), early flowering, yield	Kumar et al. (2013)
AgNPs	10–30 µg/mL	*Boswellia ovaliofoliolata*	Germination and seedling growth	Savithramma et al. (2012)
	60 ppm	*Phaseolus vulgaris* L., *Zea mays* L.	Root length	Salama (2012)
	60 ppm	*Phaseolus vulgaris* L., *Zea mays* L.	Shoot length	Salama (2012)
	60 ppm	*Phaseolus vulgaris* L., *Zea mays* L.	Dry weight of root and shoot	Salama (2012)
	100 µM	*Vigna radiata*	Antagonize inhibition by 2,4-dichlorophenoxyacetic acid (2,4-D) at 500 µM of plant growth	Karuppanapandian et al. (2011)
Sulfur NPs	500, 1,000, 2,000 and 4,000 ppm	*Vigna radiata*	Dry weight	Patra et al. (2013)
SiO_2NPs	15 kg/ha	*Zea mays L.*	Growth parameters	Yuvakkumar et al. (2011), Suriyaprabha et al. (2012)
TiO_2 NPs	400 mg/L	*Arabidopsis thaliana*,	Root length	Lee et al. (2010)
	60 ppm	*Foenicutum vulgare*	Germination	Feizi et al. (2013)
	lower than 200 mg/L	*Lemna minor*	Plant growth	Song et al. (2012)
	1,000 mg/L	*Triticum aestivum*	Chlorophyll content	Mahmoodzadeh et al. (2013)
	0.25 %	*Spinacia oleracea*	Hill reaction, non cyclic photophosphorylation, protect chloroplasts from aging	Hong et al. (2005a, b)
	0.05–0.2 g/L	*Lycopersicon esculentum Mill*	Net photosynthetic rate, conductance to H_2O, and transpiration rate, Regulation of photosystem II (PSII)	Qi et al. (2013)

(continued)

Table 2.1 (continued)

Nanoparticle(s)	Beneficiary concentration(s)	Plant	Part of plant/process	Reference(s)
Nano-anatase TiO_2	0.25 % (foliar spray)	*Spinacia oleracea*	Rubisco activase (rca) mRNA expressions,	Ma et al. (2008)
	0.25 % (foliar spray)	*Spinacia oleracea*	Oxygen evolution, Rubisco carboxylation, Rubisco Activase, rate of photosynthetic carbon reaction	Gao et al. (2006), Zheng et al. (2007), Gao et al. (2008), Ma et al. (2008)
	0.25 %	*Spinacia oleracea*	Several enzymes activities induction	Yang et al. (2006)
Aluminum oxide NPs	400–4,000 mg/L	*Arabidopsis thaliana,*	Root length	Lee et al. (2010)
Alumina NPs	10 mg/L	*Lemna minor*	Root length	Juhel et al. (2011)
	0.3 g/L	*Lemna minor*	Biomass accumulation	Juhel et al. (2011)
nZVI (nanoscale Zero- Valent Iron particles) Iron oxide NPs	0.5 g/L	*Arabidopsis thaliana*	Root elongation	Kim et al. (2014)
Iron oxide NPs	0.5–0.75 g/L	*Glycine max*	Yield and quality	Sheykhbaglou et al. (2010)
	50 ppm (foliar spray)	*Vigna radiata*	Biomass	Dhoke et al. (2013)
ZnFeCu-oxide NPs (suspension)	50 ppm (foliar spray)	*Vigna radiata*	Biomass	Dhoke et al. (2013)
CeO_2 NPs	250 ppm	*Arabidopsis thaliana*	Biomass	Ma et al. (2013)
CO_3O_4 NPs	5 g/L	*Raphanus sativus* L.	Root elongation	Wu et al. (2012)
CuO NPs	500 mg/kg (sand culture)	*Triticum aestivum*	Biomass	Dimkpa et al. (2012)
Hydroxyapatite suspension	100–2,000 mg/L	*Lactuca sativa*	Root length	Wang et al. (2012b)

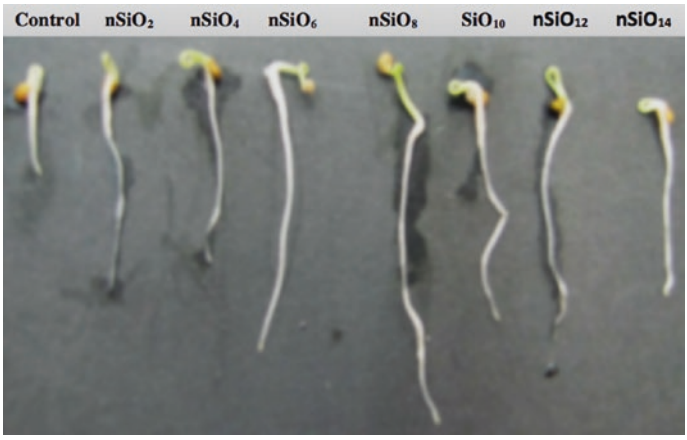

Fig. 2.1 Effect of $nSiO_2$ on seedlings growth of tomato. *1* SiO_0 (control), *2* SiO_2, *3* SiO_4, *4* SiO_6, *5* SiO_8 and *6* SiO_{10}, *7* SiO_{12}, *8* SiO_{14} [the concentration (in g L^{-1} for $nSiO_2$ is indicated as a subscript)]. (Siddiqui and Al-Whaibi 2014). Copyright © 2014 King Saud University

2.2.2 Zinc Oxide Nanoparticles

In many studies, increasing evidence suggests that zinc oxide nanoparticles (ZnONPs) increase plant growth and development. Prasad et al. (2012) in peanut; Sedghi et al. (2013) in soybean; Ramesh et al. (2014) in wheat and Raskar and Laware (2014) in onion reported that lower concentration of ZnONPs exhibited beneficial effect on seed germination. However, higher dose of ZnONPs impaired seed germination. The effect of NPs on germination depends on concentrations of NPs and varies from plants to plants. de la Rosa et al. (2013) applied different concentrations of ZnONPs on cucumber, alfalfa and tomato, and found that only cucumber seed germination was enhanced. Raliya and Tarafdar (2013) reported that ZnONPs induced a significant improvement in *Cyamopsis tetragonoloba* plant biomass, shoot and root growth, root area, chlorophyll and protein synthesis, rhizospheric microbial population, acid phosphatase, alkaline phosphatase and phytase activity in cluster bean rhizosphere. It is evident from the correlative light and scanning microscope, and inductive coupled plasma/atomic emission spectroscopy that seedling roots of *Vigna radiata* and *Cicer arietinum* absorbed ZnONPs and promoted the root and shoot length, and root and shoot biomass (Mahajan et al. 2011). Nano ZnO supplemented with MS media promoted somatic embryogenesis, shooting, regeneration of plantlets, and also induced proline synthesis, activity of superoxide dismutase, catalase, and peroxidase thereby improving tolerance to biotic stress (Helaly et al. 2014).

2.2.3 Carbon Nanotubes

Among the NPs, carbon nanotubes (CNTs) have acquired an important position due to their unique mechanical, electrical, thermal and chemical properties. The available data reveal that studies on CNTs have mainly focused on animals and humans (Ke et al. 2011; Tiwari et al. 2014). Comparatively, there has been scant information available on CNTs and their relation with plants cells and plant metabolism. Due to the unique properties of CNTs, they have the ability to penetrate the cell wall and membrane of cells and also provide a suitable delivery system of chemicals to cells. The single-walled-CNTs (SWCNTs) act as nanotransporters for delivery of DNA and dye molecules into plants cells (Srinivasan and Saraswathi 2010). However, in various studies researchers have reported that multi-walled-CNTs (MWCNTs) have a magic ability to influence the seed germination and plant growth, and work as a delivery system of DNA and chemicals to plants cells. MWCTs induce the water and essential Ca and Fe nutrients uptake efficiency that could enhance the seed germination and plant growth and development (Villagarcia et al. 2012; Tiwari et al. 2014). MWCNTs added to sterile agar medium stimulated seed germination of three important crops (barley, soybean, corn) due to the ability of MWCNTs to penetrate the seed coats as the nanotube agglomerates were detected inside the seed coats using Raman Spectroscopy and Transmission Electron Microscopy (Lahiani et al. 2013). Also, they reported that MWCNTs regulated genes expression encoding several types of water channel proteins in soybean, corn and barley seeds coat. The maximum germination rate in tomato, hybrid Bt cotton, *Brassica juncea*, *Phaseolus mungo* and rice was observed with MWCNTs (Morla et al. 2011; Nalwade and Neharkar 2013; Mondal et al. 2011; Nair et al. 2010; Gajanan et al. 2010). Also, many researchers confirmed the positive role of CNTs in seed germination and plant growth and development. Khodakovskaya et al. (2012) reported that MWCNTs act as regulators for seed germination and growth, and they demonstrated that MWCNTs have the ability to augment the growth of tobacco cell culture by upregulating the marker genes for cell divisions (*CycB*), cell wall formation (*NtLRX1*) and water transport (aquaporin, *NNtPIP1*). Wang et al. (2012a) reported oxidized-MWCNTs significantly enhanced cell elongation in the root system and promoted dehydrogenase activity. However, some researchers reported that MWCNTs do not exhibit a positive influence on seed germination in many plants even when they received high concentration of MWCNTs (Husen and Siddiqi 2014; Lin and Xing 2007). MWCNTs improve the root and stem growth and peroxidase and dehydrogenase activity may be due to primary uptake and accumulation of MWCNTs by roots followed by the translocation from roots to leaves (Smirnova et al. 2012) that could induce genes expression (Khodakovskaya et al. 2012; Lahiani et al. 2013; Wang et al. 2012a). Tripathi and Sarkar (2014) confirmed the presence of water soluble CNTs inside the wheat plants using Scanning Electron and Fluorescence Microscope, and they reported that CNTs induced the root and shoot growth in light and dark conditions. Also, MWCNTs improve water retention capacity and

biomass, flowering and fruit yield and increase medicinal properties of plants (Khodakovskaya et al. 2013; Husen and Siddiqi 2014). However, inhibitory effect of MWCNTS on plants growth has been reported by many researchers (Tiwari et al. 2014; Ikhtiar et al. 2013; Begum and Fugetsu 2012; Begum et al. 2014). Thus, the effect of NPs on plants varies from plant to plant, their growth stages, and the nature of nanoparticles.

2.2.4 Gold Nanoparticles

Few studies have been done on the interaction of gold nanoparticle (AuNPs) with plants. Some researchers found AuNPs induce toxicity in plants by inhibiting aquaporin function, a group of proteins that help in the transportation of wide range of molecules including water (Shah and Belozerova 2009). However, Barrena et al. (2009) in lettuce and cucumber, Arora et al. (2012) in *Brassica juncea*; Savithramma et al. (2012) in *Boswellia ovalifoliolata* and Gopinath et al. (2014) in *Gloriosa superba* reported that AuNPs improve seed germination. AuNPs improve the number of leaves, leaf area, plant height, chlorophyll content, and sugar content that lead to the better crop yield (Arora et al. 2012; Gopinath et al. 2014). Christou et al. (1988) introduced neomycin phosphotransferase II gene into soybean genome through DNA-coated gold particles. The positive effect of AuNPs therefore needs further study to explore the physiological and molecular mechanism. Kumar et al. (2013) reported AuNPs have a significant role on seed germination and antioxidant system in *Arabidopsis thaliana* and altered levels of microRNAs expression that regulates various morphological, physiological, and metabolic processes in plants.

2.2.5 Silver Nanoparticles

According to available data a large number of studies on silver nanoparticles (AgNPs) have been documented on microbial and animal cells; however, only a few studies were done on plants (Krishnaraj et al. 2012; Monica and Cremonini 2009). As we know, NPs have both positive and negative effects on plant growth and development. Recently, Krishnaraj et al. (2012) studied the effect of biologically synthesized AgNPs on hydroponically grown *Bacopa monnieri* growth metabolism, and found that biosynthesized AgNPs showed a significant effect on seed germination and induced the synthesis of protein and carbohydrate and decreased the total phenol contents and catalase and peroxidase activities. Also, biologically synthesized AgNPs enhanced seed germination and seedling growth of trees *Boswellia ovaliofoliolata* (Savithramma et al. 2012). AgNPs increased plants growth profile (shoot and root length, leaf area) and biochemical attributes (chlorophyll, carbohydrate and protein contents, antioxidant enzymes) of

Brassica juncea, common bean and corn (Salama 2012; Sharma et al. 2012). However, Gruyer et al. (2013) reported AgNPs have both positive and negative effect on root elongation depending on the plants species. They reported that root length was increased in barley, but was inhibited in lettuce. Also, Yin et al. (2012) studied the effects of AgNPs on germination of eleven wetland plants species (*Lolium multiflorum, Panicum virgatum, Carex lurida, C. scoparia, C. vulpinoidea, C. crinita, Eupatorium fistulosum, Phytolaca americana, Scirpus cyperinus, Lobelia cardinalis, Juncus effusus*) and found AgNPs enhanced the germination rate of one species (*E. fistulosum*). AgNP induces root growth by blocking ethylene signaling in *Crocus sativus* (Rezvani et al. 2012). The impact of AgNPs on morphology and physiology of plants depends on the size and shape of NPs. Syu et al. (2014) studied the effect of 3 different morphologies of AgNPs on physiological and molecular response of *Arabidopsis* and suggested that decahedral AgNPs showed the highest degree of root growth promotion (RGP); however, the spherical AgNPs had no effect on RGP and triggered the highest levels of anthocyanin accumulation in *Arabidopsis* seedlings. The decahedral and spherical AgNPs gave the lowest and highest values for Cu/Zn superoxide dismutase, respectively. The three different size and shape of AgNPs regulated protein accumulations such as, cell-division-cycle kinase 2, protochlorophyllide oxidoreductase, and fructose-1,6 bisphosphate aldolase and also induced genes expression involved in cellular events; for example AgNPs induced the gene expression of indoleacetic acid protein 8 (IAA8), 9-cis-epoxycarotenoid dioxygenase (NCED3), and dehydration-responsive RD22. Also, AgNPs activated the aminocyclopropane-1-carboxylic acid (ACC)-derived inhibition of root elongation in *Arabidopsis* seedlings, as well as reduced the expression of ACC synthase 7 and ACC oxidase 2, suggesting that AgNPs acted as inhibitors of ethylene perception and could interfere with ethylene biosynthesis.

2.2.6 Titanium Dioxide Nanoparticles

Similar to AgNPs, a number of researches have focused on the impact of titanium dioxide nanoparticles (TiO_2NPs) on bacteria, algae, plankton, fish, mice, and rats, but research focusing on the realization of the effects of TiO_2NPs on plant remains incomplete. TiO_2NPs enhanced seed germination and promoted radicle and plumule growth of canola seedlings (Mahmoodzadeh et al. 2013). Jaberzadeh et al. (2013) reported that TiO_2NPs augmented wheat plant growth and yielded components under water deficit stress condition. TiO_2NPs regulates enzymes activity involved in nitrogen metabolism such as nitrate reductase, glutamate dehydrogenase, glutamine synthase, and glutamic-pyruvic transaminase that helps the plants to absorb nitrate and also favors the conversion of inorganic nitrogen to organic nitrogen in the form of protein and chlorophyll, that could increase the fresh weight and dry weight of plant (Yang et al. 2006; Mishra et al. 2014). TiO_2NPs acts as a photocatalyst and induces an oxidation-reduction reaction (Crabtree 1998).

TiO_2NPs noticeably promotes aged seeds' vigor and chlorophyll formation and stimulates Ribulose 1, 5-bisphosphate carboxylase (Rubisco) activity and increases photosynthesis, thereby increasing plant growth and development (Yang et al. 2006). TiO_2NPs increases light absorbance, hasten the transport and conversion of the light energy, protect chloroplasts from aging, and prolong the photosynthetic time of the chloroplasts (Yang et al. 2006). It may be due to TiO_2NPs protects the chloroplast from excessive light by augmenting the activity of antioxidant enzymes, such as catalase, peroxidase, superoxide dismutase (Hong et al. 2005a).

2.3 Role of Nanoparticles in Photosynthesis

We know that photosynthesis is a key process for plants on earth that changes light energy to chemical energy. Plants convert only 2–4 % of the available energy in radiation into new plant growth (Kirschbaum 2011). Nowadays, scientists are trying to improve this low efficiency of vascular plants by manipulating techniques and gene manipulations. For speed-up of plant photosynthesis and turbocharged crops, scientists are working with Rubisco, an important enzyme for photosynthesis process to catalyze the incorporation of carbon dioxide into biological compounds. Recently, Lin et al. (2014) developed new tobacco plants by replacing the Rubisco gene for carbon-fixing in tobacco plant, with two genes of cyanobacterium *Synechococcus elongates*; these new engineered plants have more photosynthetic efficiency than native plants. Also, in the field of nanobiotechnology, researchers want to develop bionic plants that could have better photosynthesis efficiency and biochemical sensing. Giraldo et al. (2014) reported that embedded SWCNTs in the isolated chloroplast augmented three times higher photosynthetic activity than that of controls, and enhanced maximum electron transport rates, and SWCNTs enabled the plants to sense nitric oxide, a signaling molecule. They suggested that nanobionics approach to engineered plants would enable new and advanced functional properties in photosynthetic organelles. Also, they said that still extensive research would be needed to see the impact CNTs on the ultimate products of photosynthesis such as sugars and glucose. Also, Noji et al. (2011) reported that a nano mesoporous silica compound (SBA) bound with photosystem II (PSII) and induced stable activity of a photosynthetic oxygen-evolving reaction, indicating the light-driven electron transport from water to the quinone molecules, and they suggested that PSII-SBA conjugate might have properties to develop for photosensors and artificial photosynthetic system. SiO_2NPs improves photosynthetic rate by improving activity of carbonic anhydrase and synthesis of photosynthetic pigments (Siddiqui et al. 2014; Xie et al. 2012). Carbonic anhydrase supplies CO_2 to the Rubisco, which may improve photosynthesis (Siddiqui et al. 2012).

Nano-anatase TiO_2 have a photocatalyzed characteristic and improves the light absorbance and the transformation from light energy to electrical and chemical energy, and also induces carbon dioxide assimilation. TiO_2NPs protect chloroplast from aging for long time illumination (Hong et al. 2005a, b; Yang et al. 2006).

Nano-anatase TiO_2 enhances the photosynthetic carbon assimilation by activating Rubisco (complex of Rubisco and Rubisco activase) that could promote Rubisco carboxylation, thereby increasing growth of plants (Gao et al. 2006). Ma et al. (2008) studied the impact of nano-anatase on molecular mechanism of carbon reaction and suggested nano-anatase-induced marker gene for Rubisco activase (rca) mRNA and enhanced protein levels and activities of Rubisco activase resulted in the improvement of the Rubisco carboxylation and the high rate of photosynthetic carbon reaction. The exogenous application of TiO_2NPs improves net photosynthetic rate, conductance to water, and transpiration rate in plants (Qi et al. 2013). According to Lei et al. (2007) nano-anatase promoted strongly whole chain electron transport, photoreduction activity of photosystem II, O_2-evolving and photophosphorylation activity of chlorophyll under both visible and ultraviolet light.

According to Govorov and Carmeli (2007), metal nanoparticles can induce the efficiency of chemical energy production in photosynthetic systems. The chlorophyll in photosynthetic reaction center binds to the AuNPs and Ag nanocrystals, thereby forming a novel hybrid system that may produce ten times more excited electrons due to plasmon resonance and fast electron-hole separation. The enhancement mechanisms may help in the design of artificial light-harvesting systems.

2.4 Conclusion and Future Prospects

No doubt, nanotechnology is an evolutionary science and has introduced many novel applications in the field of electronics, energy, medicine, and life science. However, due to their unique properties, a number of researches have been done on the toxicological effect of NPs on plants, yet research focusing on the realization of the beneficial effects of NPs on plant remains incomplete. Few studies have shown positive effect of NPs on plant growth and development (Table 2.1). It is evident from compiled information that effect of NPs varies from plant to plant and depends on their mode of application, size, and concentrations. This chapter reveals that the research on NPs, essentiality for plants, is in the beginning; more rigorous works are needed to understand physiological, biochemical, and molecular mechanisms of plants in relation to NPs. Also, more studies are needed to explore the mode of action of NPs, their interaction with biomolecules, and their impact on the regulation of gene expressions in plants.

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Chapter 3
Implications of Nanotechnology on Plant Productivity and Its Rhizospheric Environment

Sanjog T. Thul and Bijaya K. Sarangi

Abstract Nanotechnology requires the ability to understand the materials and precisely manipulate it to nanoscale in a useful way. Nanotechnology emerged as a new broad science of diverse fields such as basic sciences, materials science, and engineering to assemble at the nanoscale. In contrast to conventional or other contaminants, nanoparticles are posing some new environmental challenges for scientists and environmentalists worldwide. Being a new area of science, nanotechnology will leave no field untouched including agriculture and allied sectors. So far, the use of nanotechnology in agriculture has been mostly theoretical, but it has begun to have a significant effect in the main areas of agrochemical industry. Nanoparticles finding great potential as delivery systems to specific targets in living organisms and is being used in medical sciences. In plants, the same principles can be applied for a broad range of uses, particularly to tackle phytopathological infections, nutrition supplement and as growth adjuvant. Nanoparticles can be tagged to agrochemicals or other substances as delivery agent to plant system and tissues for controlled release of chemicals. Doing so, the negative effects of nanomaterials on plant productivity and soil microbes and environment must not be overlooked, such as toxicity generated by free radicals leading to lipid peroxidation and DNA damage. Key focus of the chapter particularly relates the use of nanoparticles on agricultural crops and its toxic implications to plants and microbes naturally present in soil and generation of nanowaste in agroecosystem.

Keywords Nanoformulations · Phytotoxicity · Nanowaste · Agroecosystem

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3.1 Introduction

The developments in nanotechnology and nanotechnology-based industries and products are tremendously growing. Recent estimates till October 2013, the nanotechnology-based consumer products inventory grows to 1,628 products or product lines (Fig. 3.1). The use of nanomaterials (NMs) in biomedicine (Zhang et al. 2008) and in agriculture (Joseph and Morrison 2006) is one of the most intensely studied areas in nanotechnology. Nanoscale materials have shown to be taken up by bacteria (Liu et al. 2009b), and also have the ability to penetrate plant cells (Liu et al. 2009a) and induce phytotoxicity at high doses (Stampoulis et al. 2009). Nanotechnology-based agrochemical researches have motivated a number of scientists and environmentalists worldwide to consider the use of nanotechnology for agricultural crops.

Practically, nanotechnology permits broad advantages in agricultural research, such as disease prevention and treatment in plants using various nanocides (Carmen et al. 2003) and nutrient management of agriculture field using nanofertilizers (Priester et al. 2012). Various kinds of nanomaterials such as; metal, nonmetal, carbon nanotubes, quantum dots, magnetic particles, polymers, etc. have been studied for their use and possible effect in different areas. Each of these nanomaterials exerts its positive and negative effects mostly depending on its size and interaction with the plant tissues or microbes. However, the current level of knowledge does not convey any clear evidence of the benefits and/or risks (Kah et al. 2013).

The route of entry of these nanomaterials in food chain may be from direct application on land or biosolids treated in conventional wastewater treatment plants (WWTPs) (Brar et al. 2010). However, manufactured nanomaterials (MNMs) although measurable in WWTP systems (Kiser et al. 2009) are neither monitored nor regulated. Though there are scientific reports on measurement and detection of such material and contaminants using sophisticated instruments (Khodakovskaya et al. 2011), but the use of such high cost monitoring tools seems to be nonfeasible on routine basis. Despite the success of nanotechnology, the lack of scientific knowledge concerning the potential health and environmental risks needs to be addressed well in advance.

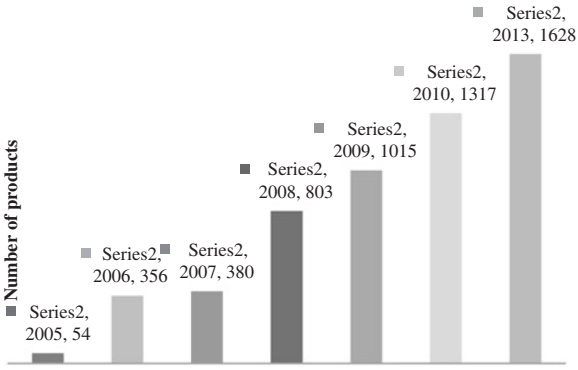

Fig. 3.1 Year wise inventory update for total nanotechnology-based products (*Source* Project on Emerging Nanotechnologies (2014))

3.2 Nanomaterial as Delivery Systems

Agrochemicals are in general applied to crops in the form of suspension/solution by spraying. Due to problems such as leaching of chemicals, degradation by photolysis, hydrolysis, and by microbial degradation, most of the chemicals is lost. Hence, repeated application is necessary to have an effective control which on the other hand results in deterioration of soil and water quality. In this context, nanoencapsulated agrochemicals need to be designed in such a way that they possess all necessary properties such as effective concentration (with high solubility, stability, and effectiveness), time controlled release in response to certain stimuli, enhanced targeted activity and less toxicity (Boehm et al. 2003; Green and Beestman 2007; Wang et al. 2007). Tsuji (2001) reported the control of parasitic weeds with properly designed functional nanocapsulated herbicides which have better penetration through cuticle to controlled release of active constituents and to reduce the phytotoxicity of herbicides on crops.

Likewise, use of surface modified hydrophobic nanosilica to control a range of agricultural insect pests (Rahman et al. 2009) and surface functionalized mesoporous silica nanoparticles (MSNs) to precisely manipulate gene expression at single cell level by delivering DNA and its regulators in a controlled fashion is reported (Torney et al. 2007). Magnetic nanoparticles have shown very specific localization to release their load, which is of great interest in the study of nanoparticulate delivery for plants with no toxicity (Zhu et al. 2008). Quantum dots (QDs) of CdSe/ZnS conjugated with glycine, mercaptosuccinic acid, cysteine, and amine were reported to be visibly transported to a limited extent in the vasculature of ryegrass, onion, and chrysanthemum plants when cut stems were placed in aqueous QD solutions. However, they were not seen to be taken up at all by rooted whole plants (Al-Salim et al. 2011).

Single-walled carbon nanotubes (SWCNTs) were reported to enhance root elongation in onion and cucumber (Canas et al. 2008). Similarly, Khodakovskaya et al. (2009) reported the effects of multiwalled carbon nanotube (MWCNT) on the seed germination and growth of tomato plants. Also, Lin and Xing (2007) reported positive effects of MWCNTs on radish, rape, rye grass, lettuce, corn, and cucumber. These results showed significant and encouraging effects on growth and development processes of plants.

The use of polymer matrix that is subject to swelling and dissolution was found to influence the diffusion pathways and thus alter the release behavior (Kaunisto et al. 2013). Examples of polymers used include nanospheres of polyethyleneglycol (Yang et al. 2009) or polyvinylpyrrolidone (Botts et al. 2006). Such materials are often used because they are well established from medical applications. The use and preparation of nanopolymer such as liposomes as delivery system for the slow release of insecticide was first described by Bang et al. (2009). Since then, two reports (Hwang et al. 2011; Kang et al. 2012) highlighted insecticidal efficacy of liposome based formulations. Kang et al. (2012) described that nanoformulation of pyrifluquinazon had its best lethal efficiency for 14 days after treatment

compare to pure compound which lasts for 2 days. Similarly, Xiang et al. (2013) used cellulose based polymer and demonstrated that increasing the cellulose nanocrystal content in the fibers, increases the rate of fiber degradation and release of thiamethoxam herbicide.

3.3 Nanomaterial in Agro-system

3.3.1 Nanopesticide and Herbicides

Conventional methods to control the pathogens and pests have affected both the environment and economy of farmers, as 90 % of the applied pesticides are lost to the air during application and as runoff. Additionally, indiscriminate usage of pesticide increases pathogen and pest resistance, reduces soil biodiversity, diminishes nitrogen fixation; contributes to bioaccumulation of pesticides, pollinator decline and destroys habitat for birds (Ghormade et al. 2011). Nanoscaled delivery system with active compound (pesticide and or herbicide) can be applied only when necessary in the field (Gruere et al. 2011).

Avermectin, a pesticide which is known to block neurotransmission in insects by inhibiting chloride channel. It is inactivated by ultraviolet on the fields with half-life of 6 h only, whereas, slow release of encapsulated avermectin by the nanoparticles (NPs) carrier was reported for about 30 days (Ghormade et al. 2011). Similarly, a commercial product 'Karate® ZEON' is a quick release microencapsulated formulation containing lambda-cyhalothrin which breaks open upon contact with leaves. In contrast, the gutbuster microencapsules containing pesticide that breaks open to release its contents upon coming in contact with alkaline environments, including the stomach of certain insects (Lyons et al. 2011).

A series of polyethylene glycol (PEG) based insecticide formulations found to release active compounds at slower rate compare to commercial formulations comprising imidacloprid (Adak et al. 2012), carbofuran (Pankaj et al. 2012), and thiram (Kaushik et al. 2013). The release of insecticide was noted to be dependent on PEG molecular weight. The release of β-cyfluthrin from the nanoformulation was recorded over a period that ranged from 1 to 20 days (Loha et al. 2011), whereas release from a commercial formulation was found within 4–5 days (Loha et al. 2012). In another report, a nanofiber network composed of poly (lactic acid) and cellulose nanocrystals loaded with thiamethoxam were efficient against whitefly over a 9 day period in a glass house experiment, at 50 % of the recommended dosage of thiamethoxam (Xiang et al. 2013). Active compounds conjugated in nanoformulations for agricultural use found to be more effective compared to their conventional counterparts (Table 3.1).

Nanoformulations containing glyphosate was found to increase the bioavailability of the herbicide while avoiding a number of the adjuvant present in current glyphosate formulations, which have been associated with toxicity to nontarget organisms (Piola et al. 2013). Kanimozhi and Chinnamuthu (2012) used

Table 3.1 Nanomaterial based formulations for agricultural use

Nanoformulations	Materials used	Active compounds	References
Herbicide	Zn–Al	2-4-dichlorophenoxyacetate	Hussein et al. (2005)
Pesticide	SiO_2	Validamycin	Liu et al. (2006)
Pesticide	Polymer	Bifenthrin	Liu et al. (2008)
Insecticide	TiO_2/Ag	Dimethomorph	Guan et al. (2010)
Pesticide	PEG	Carbofuran	Shakil et al. (2010)
Insecticide	TiO(2)	Avermectins	Guan et al. (2011)
Insecticide	Chitosan	Etofenprox	Hwang et al. (2011)
Insecticide	Polymer	Thiamethoxam	Sarkar et al. (2012)
Insecticide	Polymer	β-cyfluthrln	Loha et al. (2012)
Insecticide	A1	Nanoalumina	Stadler et al. (2012)
Insecticide	SiO_2	Chlorfenapyr	Song et al. (2012)
Pesticide	SiO_2	1-naphthylacetic acid	Ao et al. (2013)
Insecticide	Sodium alginate	Pyridalyl	Saini et al. (2014)
Herbicide	Polymer	Atrazine	Pereira et al. (2014)

manganese carbonate as core material coated with water soluble polymers such as sodium Poly Styrene Sulfonate and Poly Allylamine Hydrochloride. Further, Manganese carbonate core materials were etched out to form hollow-shell particles which were loaded with herbicide pendimethalin for field application.

3.3.2 Nanofertilizers

Soil fertility mainly depends upon its organic and inorganic components such as salts of sodium, potassium, and phosphorous; oxides of nitrogen and sulfur, etc. The soil organic matter provides the energy and nutrients for soil microbes which ensure high yields of healthy crops due to their enzymatic action. Thus, it is mandatory to conserve it for efficient physical, chemical, and biological soil functioning (Six et al. 2002).

Millan et al. (2008) reported the use of urea-fertilized zeolite chips, for slow release of nitrogen fertilizers. Ammonium-charged zeolite has shown its capacity to raise the solubilisation of phosphate minerals and thus goes to improved phosphorus uptake and yield of crop plants. In this line, Jinghua (2004) showed that application of a nanocomposite consists of N, P, K, micronutrients, mannose, and amino acids enhance the uptake and use of nutrients by grain crops. In an interesting strategy, Kottegoda et al. (2011) reported sustained release of nitrogen into the soil using urea-modified hydroxyapatite nanoparticle which were encapsulated under pressure into cavities of the soft wood of *Gliricidia sepium*. In this study, nanofertilizer showed an initial burst and a subsequent slow release up to day 60 compared to the commercial fertilizer, which released heavily at beginning followed by low and nonuniform quantities until around 30 day.

3.4 Phytotoxicity of Nanomaterials

To date research on interaction of nanoparticles that results into phytotoxicity is negligible. Apart from detrimental effect upon direct contact of NPs, these can also diffuse into the intercellular space, the apoplast, and be adsorbed or incorporated into the membranes (Nowack and Bucheli 2007). Plant cells carry a negative surface charge, which allows the transport of negatively charged compounds into the apoplast. The casparian strip poses a barrier to the apoplastic flow and transport, and only symplastic transport is possible into the xylem. However, this barrier is not perfect and compounds can enter the xylem through holes or damaged cells without ever crossing a cell membrane and be further transported to the shoots. This process is found to be a dominant process for the uptake of metal complexes with chelators such as EDTA and their translocation to the shoots (Tandy et al. 2006). This indicates that negatively charged NP could enter the apoplasm of the root cortex and eventually also the xylem, but are not taken up by the cells.

In one of the study, Lee et al. (2008) demonstrated the effects of copper nanoparticles (CuNPs) on the seedling growth of mung bean and wheat wherein mung bean was found to be more sensitive to CuNPs than wheat. Transmission electron microscopy (TEM) images confirmed the entry of CuNPs across the cell membrane. Bioaccumulation of NPs increased with its concentration in growth media and their bioavailability to the test plants was estimated by calculating the bioaccumulation factor. Also, studies on the effects of CuNPs on the growth of zucchini plants showed reduced length of emerging roots (Stampoulis et al. 2009) and modulation of ascorbate-glutathione cycle, membrane damage, in vivo ROS detection, foliar H_2O_2 and proline accumulation and reduced seed germination percentage in rice (Shaw and Hossain 2013).

It is also important to mention that the phytotoxicity due to bioaccumulation, biomagnification, and biotransformation of engineered nanoparticles in food crops are still not well understood. Few studies have been reported on the accumulation of engineered nanomaterials in crop plants such as rape, radish, lettuce, corn, and cucumber (Rico et al. 2011). The carbon-based fullerenes (C_{70} and fullerols $C_{60}(OH)_{20}$) and most of the metal-based nanomaterials (titanium dioxide, cerium oxide, magnetite, zinc oxide, gold, silver, copper, and iron) were reported to be accumulated in the plants (Rico et al. 2011). Moreover, accumulated nanomaterials in the plants can be the part of biological food chain. As a part, positive effects of metal-based nanomaterial on plant encouraged for some crops, on the other hand, significant negative effects were also observed, such as reduced germination, root growth, and shoot length (Thul et al. 2013).

The seed germination of rye grass and corn was reported to be inhibited by nanoscale Zn (35 nm) and ZnO (15–25 nm), respectively. Root growth was found to be significantly inhibited, however, such an inhibition for seed was not detected when soaked in nano-ZnO suspension due to the selective permeability of seed coat (Lin and Xing 2007). Not only the size of NPs, but reduced length of shoot and root of wheat was observed in a dose-dependent manner (Dimkpa et al. 2013).

In another study, uptake of ZnONPs causes damage of epidermal and cortical cells and transport from one cell to other through plasmodesmata (Lin and Xing 2008). Similarly, the evidence for the entrapment of AgNPs by the cuticle, and penetration into the leaf tissue through stomata, and oxidation of AgNPs and complexation of Ag^{+} by thiol-containing molecules was reported by Larue et al. (2014). Furthermore, the cytotoxic and genotoxic impacts of AgNPs were reported in root tips of onion (Kumari et al. 2009). Similar effects of chromosomal aberrations and DNA damage were also observed with TiO_2 (Pakrashi et al. 2014). Recently, TiO_2NPs were reported to affect the molecular expression profiles of microRNAs (Frazier et al. 2014).

3.4.1 Metal Nanoparticle Induced Predictive Physiological and Biochemical Changes in Plant

The manifestation of the metal and their nanoparticles interaction and accumulation in plant systems could be responsible for changes in vegetative growth, development and differentiation, onset of senescence, dormancy, abscission, flowering and fruit setting, and other ecological productivity (Gardea-Torresdey et al. 2004; Vernay et al. 2008). It has also been reported that nanomaterials can generate ROS, affect lipid peroxidation (Cabiscol et al. 2000). This has significant biochemical and molecular effect on the membrane permeability and fluidity, making cells more susceptible to osmotic stress and failure to nutrient uptake. It is known that the stress is perceived through the growth matrix, i.e., soil and water and a series of metabolic activities (Viswanathan et al. 2004; Sarangi et al. 2009) are triggered to alleviate the metal stressors (Verbruggen et al. 2009). In order to deal with the situation; in the first step plants modulate their action actively to prevent metal entry through the expense of energy. In the second step, further entry of the metal into the cytosol is prevented by modulation of transporters in the plasma membrane so that intracellular buildup of metal ions does not exceed the threshold concentration. In order to prevent metal ion buildup, the plant system have developed several well synchronized system to efflux the ions from the cellular milieu (Lin et al. 2006). In case of failure in these strategies, plants actively chelate the metal particles through specific low and moderately large sized molecules such as; phytochelatins (Cobbett and Goldsbrough 2002), metallothionins (Maitani et al. 1996; Guo et al. 2008), and other thiol rich compounds which act as chaperons to maintain the cellular homeostasis (Nelson 1999). In the extreme case of failure of the above mentioned strategies, plants try to compartmentalize the metal particles into vacuoles. All such metabolic processes are active processes in the expense of energy from metabolites (Bertrand and Poirier 2005). Expense of the metabolites is a penalty on the plant; which are otherwise required for growth and development to complete its annual or perennial lifecycle. Although, the concentration of nanoparticles affecting the biochemical and physiological processes of biological organisms is a matter of debate, it needs to be worked out through systematic

investigation. However, it is predicted that the reactivity of a particular metal nanoparticles would depend on the niche; biochemical and physiological alterations in crops and plant systems that impact on crop yield and ecological productivity.

3.5 Influence of Nanomaterials on Rhizospheric Environment

The effect of specific metal nanoparticles on soil microflora could be conspicuous. The germicidal properties of Ag and Cu nanoparticles are well documented. Uptake of manufactured nano-CeO_2 nanomaterials into roots and root nodules found to eliminate N_2 fixation potentials and impaired soybean growth (Priester et al. 2012). Also, Fan et al. (2014) observed the impact of nano TiO_2 on *Rhizobium*–legume symbiosis using garden peas and *Rhizobium leguminosarum* bv. *viciae* 3,841, and found that nano TiO_2 exert morphological changes in bacterial cells. Further, it was noticed that the interaction between these two organisms was disrupted in the form of root nodule development and the subsequent delay in onset of nitrogen fixation. The alteration of bacterial communities was reported to be in a dose-dependent manner, with some taxa increasing as a proportion of the community, whereas more taxa decreasing that resulted in reduced diversity (Ge et al. 2012).

The direct application of NPs on land or treated biosolids containing mobile NPs may come in contacts with the soil microbes (Fig. 3.2). These microbes are also efficient to adsorb and accumulate one or other form of nanomaterials, which in turn initiates the mobilization of nanomaterials through food chains and can alter communities comprising multiple populations (e.g., plant, fish, bacteria) within food webs (Holden et al. 2013). Plants generally depend on soil bacteria and fungi to help mine nutrients from the soil. A study finds that the popular microbicidal AgNPs negatively impacts on the growth of plants and kills the soil microbes that sustain them (Zeliadt 2010). Not only microbes, but activity

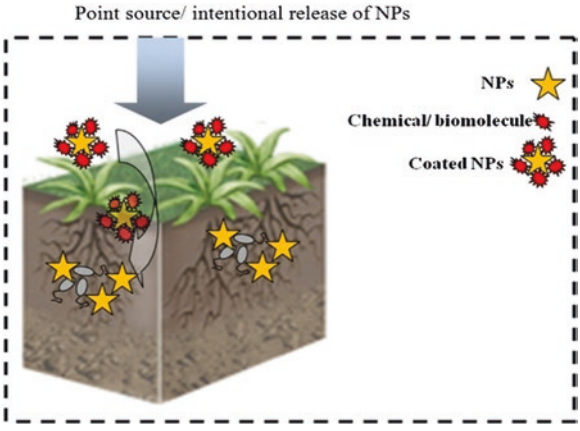

Fig. 3.2 Fate and sink of NPs in agricultural ecosystem (Thul et al. 2013)

of several soil enzymes such as soil protease, and catalase, and peroxidase were found to be significantly reduced by ZnO and TiO_2NPs (Du et al. 2011).

Moreover, inorganic TiO_2, SiO_2, and ZnO were found to exert toxic effect on bacteria. The toxicity of these elements further significantly enhanced in presence of light (Adams et al. 2006). A range of studies has been reviewed and focused on nanoparticles—microbial interactions to correlate the physicochemical properties of engineered metal and metal oxide NPs and their biological response. Further, it has been concluded that the species specific toxicity can be attributed to nanoparticles' size and shape. However, the surface coating of the material, which can be altered significantly by environmental conditions, can ameliorate or promote microbial toxicity (Suresh et al. 2013). Studies on ecologically relevant bacterial species such as *E. coli, Bacillus subtilis, Pseudomonas putida*, and other have clearly indicated that NPs can be taken up by microbes (Table 3.2).

Table 3.2 Nanotoxicity on diverse microbes

Microbes	Toxicity	Nanomaterials	References
E. coli	Inhibition of bacterial growth, bactericidal action	Ag	Pal et al. (2007)
Pseudomonas putida	Inhibition of bacterial growth	ZnO	Li et al. (2011)
B. subtilis, E. coli	Mild toxicity due to ROS production	TiO_2, SiO_2, ZnO	Adams et al. (2006), Sapkota et al. (2011), Li et al. (2011)
E. coli, P. aeruginosa, Staphylococcus aureus, and *Salmonella typhimurium*	Antibacterial activity	Ag	Sahu et al. (2012)
Nitrogen fixing root nodules	Decrease of N_2 fixation potentials	CeO_2	Priester et al. (2012)
Rhizobiales, Bradyrhizobiaceae, Bradyrhizobium, Methylobacteriaceae	Decline in bacterial communities and reduced diversity	TiO_2, ZnO	Ge et al. (2012)
AMF (*Trifolium repens*)	Reduced mycorrhizal clover biomass	FeO, Ag	Feng et al. (2013)
Proteobacteria and *Verrucomicorbia*	Decrease in community abundance	MWCNTs	Khodakovskaya et al. (2013)
B. cereus, *P. stutzeri*	Decreased microbial transcriptional response	Ag, Al_2O_3	Fajardo et al. (2014)
P. stutzeri	Increased oxidative stress	nZVI	Sacca et al. (2014)
Gram-positive and Gram-negative bacteria, and fungi	Reduced biomass	SWCNT	Jin et al. (2014)
Rhizobium leguminosarum	Morphological changes to the bacterial cells	TiO_2	Fan et al. (2014)

Most of the microbes have developed effective molecular mechanisms and operated specific biochemical pathways to efflux, detoxify, and accumulate the metals ions much before it was learnt by the plants. Further, microbes are also capable to volatilize some of the metal ions to get rid of its acute toxicity (De Souza et al. 1999). Although microbes have developed resistance and avoidance mechanism, but more targeted studies are needed in regards to beneficial soil microbes such as N_2 fixing, phosphate solubilizers, AM fungi to establish the uptake mechanisms and consequences in soil and microbes.

3.6 Fate of Nanomaterials and Generation of Nanowaste

3.6.1 Accumulation in Plants

So far, very few nanoparticles and plant species have been studied with respect to the accumulation and subsequent availability of nanoparticles in food crops (Yin et al. 2011). The transfer of NPs into the food chain through edible plants is of great concern. The fruits of one such food plant *Cucumis sativus* L. which is a freshly consumed as garden vegetable analyzed using synchrotron μ-XRF and μ-XANES, showed root-to-fruit translocation of TiO_2 without biotransformation (Servin et al. 2013). Similarly, bioaccumulation of Ce and Zn was confirmed by μ-XRF images, suggesting that Ce moves between tissues with water flow during transpiration (Zhao et al. 2013b). Likewise, modified ultra-small TiO_2 (anatase) surface with Alizarin red S, and sucrose is found to accumulate in *Arabidopsis thaliana*. This study demonstrated that nanoconjugates traversed cell walls, entered into plant cells, and accumulated in specific subcellular locations (Kurepa et al. 2010). Microscopic observation reported by Ma et al. (2013) for plant seedlings of cattail (*Typha latifolia*) and hybrid poplars (*Populous deltoids* × *Populous nigra*) indicated that large amount of nZVI coated on plant root surface as irregular aggregates and some penetrated into several layers of epidermal cells of poplar root cells. Shi et al. (2014) investigated the phytotoxicity and accumulation of CuO NPs to *Elsholtzia splendens* (a Cu-tolerant plant) under hydroponic conditions is dose-dependent. Cu K-edge X-ray absorption near-edge structure analysis revealed CuO NPs-like deposits in the root and leaf cells. Similarly, Hu et al. (2014) have reported that aggregation and dissolution of ZnONPs are responsible for zinc accumulation in leaves and roots of *Salvinia natans* after 7 days of exposure. In another study, Zhai et al. (2014) observed that uptake and presence of AuNPs in cytoplasm and various organelles of root and leaf cells of poplar plant by transmission electron microscopy and measured by inductively coupled plasma mass spectrometry (ICP-MS).

3.6.2 Aggregation in Soil and Water Bodies as Nanowaste

Quantitative data related to concentrations of nanoparticles in natural water have not been reported so far. However, a recent report using a simplified box model

and their known uses (Boxall et al. 2007), Klaine et al. (2008) has suggested environmental concentrations of approximately 1–100 μg L^{-1} as compared to typical dissolved and colloidal organic matter in freshwaters which may be found at 1–10 mg L^{-1} concentrations.

Soils and water are likely to be increasingly at receiving end of NPs due to growing consumer products that contains NPs. The level of NPs in soil and water is increasing due to the growing consumer products that contained NPs. Investigation on waste streams revealed the occurrence of NPs (Biswas and Wu 2005; Bystrzejewska-Piotrowska et al. 2009), indicating the necessity of further systematic investigation into the fate and bioavailability of nanoparticles in soils. Retention of NPs in soils was studied by Cornelis et al. (2012), wherein the dominant properties that determine the retention of AgNP in natural soil was correlated to negatively charged AgNP which was found to be adsorbed preferentially at positively charged surface sites of clay-sized minerals. The high organic carbon content in the agricultural soil likely contributed to an organic surface coating and resulted in NPs mobility through the soil. Further, Cornelis et al. (2014) have thoroughly reviewed the fate and bioavailability of engineered nanomaterials in soils, wherein author concluded that salinity, texture, pH, concentration and nature of mobile organic compounds, and degree of saturation determine ENM bioavailability.

The surface properties of the nanoparticles are known to be one of the most important factors that govern their stability and mobility as colloidal suspensions, or their adsorption or aggregation and deposition. Zhao et al. (2013a) observed coexistence of ZnONPs with Zn dissolved species were continuously released into the soil solution to replenish the Zn ions or ZnONPs scavenged by roots as compared to soil treated with alginate which promotes the bioaccumulation of Zn in corn plant tissues. In another interesting study, the fate of Cu and ZnONPs was monitored over 162 days and it was observed that both NPs traveled through the soil matrix at differential rates. CuNPs reported to be retained in the soil matrix at a higher rate compared to ZnONPs. Leaching of Cu and Zn ions from the parent NPs was also observed as a function of time (Collins et al. 2012). Physicochemical characteristics of NPs (e.g., shape, size, and surface charge) and soil (e.g., pH, ionic strength, organic matter, and clay content) affect physical and chemical processes, resulting in NPs dissolution, agglomeration, and aggregation. The combined results reported in the literature, suggests that metallic CuNPs can be considered the least mobile as compared to Fe_3O_4, CuO, TiO_2 and ZnONPs (Ben-Moshe et al. 2010). The behavior of NPs in soil controls their mobility and their bioavailability to soil organisms which may interact with beneficial soil microbes (Fig. 3.2) and extend the impact on their survival.

Failure to address the concerns of leftover of leachates from excess and after use NPs ultimately finds the way and accumulates over a time period in the form of aggregates and colloids in soil and water bodies. These aggregates and colloids containing NPs will generate an additional anthropogenic waste (nanowaste) in the agroecosystem (Fig. 3.3). This needs continuous monitoring of the fate of nanoproducts vis-a-vis the left over nanowaste and soil composition.

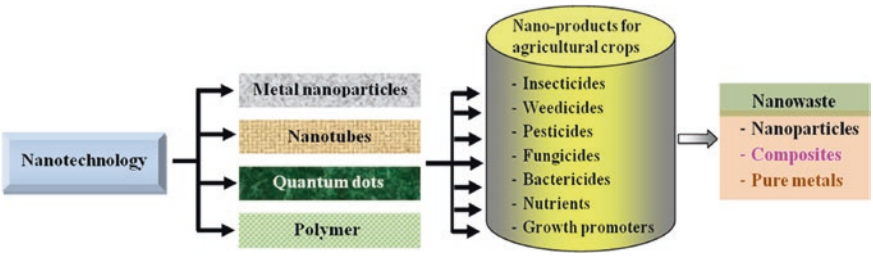

Fig. 3.3 A schematic pathway of nanotechnology to nanowaste in agroecosystem (Thul et al. 2013)

3.7 Conclusions

Recent rapid advances in understanding, synthesis, and manipulation of nanoparticles undoubtedly will continue with phenomenal growth of nanomaterial encompassed products. Use and its application in the field of agriculture, for improved crop growth, have shown significant promising potency and active uptake of necessary ingredients and absorbents. However, due to the very small size, reactivity, and efficient penetration ability, metal nanoparticles could reach many intracellular and extracellular sites of plants. This may trigger a set of physiological processes such as senescence affecting plant growth, crop yield, and ecological productivity. Moreover, there are major concerns on the use of NMs due to the toxicity to microbial systems present in the soil environment. The nanoparticle interactions with bacteria can vary. Scientific reports suggest that metal and metal oxide NPs of small size are more toxic. The long-term deposition of nanomaterials in the form of aggregates and colloids, not only threaten the security of soil and water resources, but, may prove to be impossible to remediate. In view of the foreseeable use of NPs based products, there is a need for systematic study to evaluate the effects of nanoparticles on crop plants and their environmental consequences.

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Chapter 4
Nanoparticles in Sustainable Agricultural Crop Production: Applications and Perspectives

Allah Ditta, Muhammad Arshad and Muhammad Ibrahim

Abstract For the ever-increasing population of the world, an increasing demand for more and more food is required. To cope with this alarming situation, there is a dire need for sustainable agricultural production. In agriculture, management of optimum plant nutrients for sustainable crop production is the priority-based area of research. In this regard, much advancement in the area of plant nutrition has come forward and nano-nutrition is one the most interesting areas of research for sustainable agriculture production. Nanotechnology has revolutionized the world with tremendous advancements in many fields of science like engineering, biotechnology, analytical chemistry, and agriculture. Nano-nutrition is the application of nanotechnology for the provision of nano-sized nutrients for the crop production. Two sources of nanoparticles (NPs) have been used; biotic and abiotic. The abiotic form of nutrients or NPs is prepared from inorganic sources like salts but it is not safe because many of these are non-biodegradable. While the biotic ones are prepared from organic sources which are definitely the biodegradable and environment friendly. So, a few studies/attempts have been made in the field of nano-nutrition and a lot more are expected in the near future because this field of plant nutrition is sustainable and efficient one. Using nano-nutrition we can increase the efficiency of micro- as well as macronutrients of the plants. In this chapter, the focus has been made on the importance of nano-nutrition in the sustainable agricultural production and its future scenario so that it could be possible to apply this knowledge on a large scale without any concern regarding environment.

Keywords Nanotechnology · Agricultural production · Applications · Nanoscience · Nanofertilizers

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4.1 Introduction

The world agriculture is facing many challenges like changing climate, urbanization, sustainable use of natural resources, and environmental issues like runoff, and accumulation of pesticides and fertilizers. These problems are further intensified by an alarming increase in food demand that will be needed to feed an estimated population of 6–9 billion by 2050 (Chen and Yada 2011). Further, the petroleum resources of the world are decreasing, there will be an additional demand on agricultural production as agricultural products and materials will soon be viewed as the foundation of commerce and manufacturing. At one fell swoop, there are new opportunities emerging, e.g., generation of energy and electricity, from agricultural waste but pending workable economics and encouraging policy (Fakruddin et al. 2012). The above-mentioned scenario of rapidly developing and complex agriculture system is the greatest challenge that will be posed to the developing countries, as in the developing countries, agriculture is the backbone of the national economy. It faces many critical issues like lack of new arable soil, reduction of the current agricultural land due to competing economic development activities, commodity dependence, poverty, and malnutrition which are needed to be solved on sustainable basis. Profound structural changes in the agricultural sector has occurred due to the fast development in the technological innovations but these also pose challenges like sustainable production considering food security, poverty reduction, and public health improvement. For developing countries, advancement in science and technology can offer potential solutions for discovering value addition in their current production systems.

Many technologies have been developed that have the potential to increase farm productivity and also reduce the environmental and resource costs related with agricultural production. These technologies have the ability to conserve land and water by increasing yields through the application of the same or fewer inputs ultimately conserve environment (Prasad et al. 2012a). However, it will be very critical to support them as these may not be commercially profitable and may also result in increase in the disparity between developing and developed countries. So their social and ethical implications should be considered. However, need of an hour is to consider their efficiency in some fields while these may not provide a solution to the existing problems associated with food production and its distribution round the world. Therefore, the developing countries should actively participate in research and development of these technologies while considering their ability to utilize these new technologies (Prasad et al. 2014).

In this regard, nanotechnology has been a novel scientific approach that makes use of the manipulation of materials for their novel, physical as well as chemical properties at nano-scale. About 2/5th of the population depends on agriculture for their livelihood in the developing countries of the world and hence agriculture, in these countries, is regarded as the backbone of the country (Brock et al. 2011).

From the literature, it has been clear that nanotechnology has the potential to revolutionize the agricultural and food industry with novel tools for enhancing the productivity of the crop plants through efficient nutrients in the form of nanofertilizers, nanopesticides, or nanoherbicides by the plants (Tarafdar et al. 2013). The agricultural productivity could be enhanced by the use of NPs as nutrient elements for enhanced germination, formulation of nanofertilizers, nanoporous zeolites for slow release, and efficient dosage of water and fertilizer, nanocapsules for herbicide delivery and vector and pest management and nanosensors for pest detection (Scrinis and Lyons 2007; Scott 2007). These applications would definitely be helpful for the solutions of the limitations and challenges facing large scale, chemical, and capital intensive farming systems. So far, the nanotechnology is at its nascent stage and many success stories have been documented especially from the crop production point of view. This chapter is focused on reporting the latest advancements in the field of agricultural production through nanotechnology and its future perspectives in sustainable agriculture.

4.2 What Is Nanotechnology?

Nanotechnology, the vast field of twenty-first century, has a very significant impact on world's economy, industry, and people's life (Gruère et al. 2011; Scott and Chen 2003a). It deals with the physical, chemical, and biological properties of matter considered at nanoscale (1–100 nm) and their implications for the welfare of human beings (Holdren 2011). According to US EPA (US Environmental Protection Agency), nanomaterial is an ingredient containing particles with at least one dimension that approximately measures 1–100 nm. It has the ability to control and/or manufacture matter at this scale which results in the development of innovative and novel properties like increase in the surface area of the particles (Table 4.1) that can be utilized to address numerous technical and societal issues (Fig. 4.1).

Table 4.1 Size of different organism and biomolecules on micro- and nano metric scale (Ditta 2012)

Sr. No.	Nature of organism and different biomolecules	Size range (μm)	Size (nm)
1	Plant, animal cell	10–100	10,000–100,000
2	Bacteria	≤1–10	≤1,000–100
3	Virus	0.03–0.1	30–100
4	Simple molecules (proteins, DNA turns)	0.001–0.01	1–10
5	Atoms (DNA "base")	0.0001–0.001	0.1–1

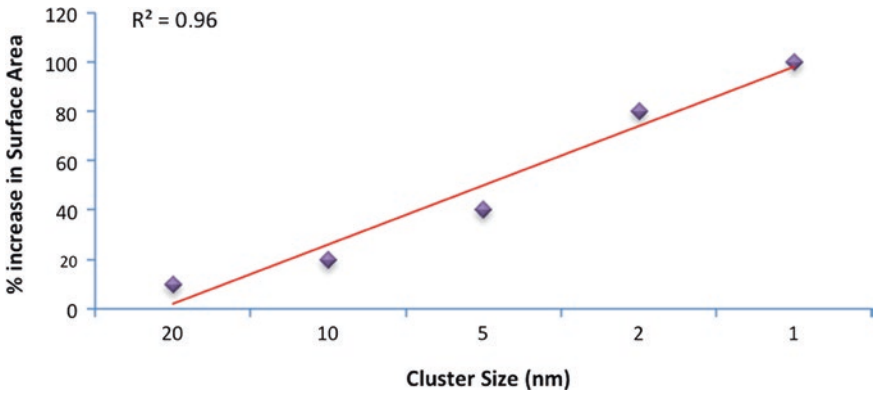

Fig. 4.1 Relationship between cluster size (nm) and surface area (%) [Modified from Ditta (2012)]

4.3 Overview of the Applications of Nanoparticles in Agriculture

Applications of nanotechnology, in materials sciences and biomass conversion technologies applied in agriculture are the basis of providing food, feed, fiber, fire, and fuels (Fig. 4.2). In the future, demand for food will increase tremendously, while the natural resources such as land, water, and soil fertility are limited. The cost of production inputs like chemical fertilizers and pesticides is expected to increase at an alarming rate due to limited reserves of fuel like natural gas and petroleum (Prasad et al. 2012a). In order to overcome these constraints, the precision farming is a better option to reduce production costs and to maximize the output, i.e., agricultural production. Through the advancement

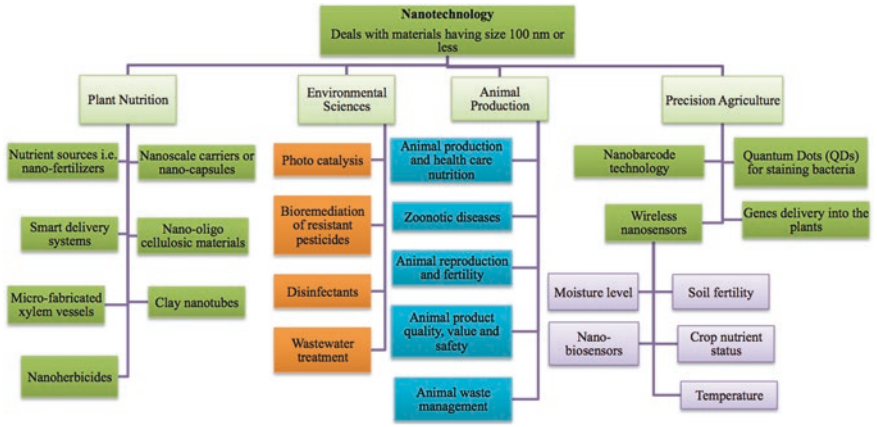

Fig. 4.2 Diagram showing the general applications of nanotechnology in agriculture

in nanotechnology, a number of state-of-the art techniques are available for the improvement of precision farming practices and will allow a precise control at nanometer scale (De et al. 2014; Ngô and Van de Voorde 2014). The detailed description of the applications of nanotechnology in sustainable agricultural crop production is given in the following section of this chapter.

4.3.1 Effect of NPs on Seed Germination and Growth of Different Crop Plants

Nanomaterials (NMs) have great implications in sustainable agricultural crop production and many studies reported their positive impact on various crops (Table 4.2). Mainly, germination of various crops has been reported to be improved in these reports. For example, by the application of $nSiO_2$ in maize (*Zea mays* L.) and tomato (*Lycopersicum esculentum* Mill.) (Suriyaprabha et al. 2012a, b; Siddiqui and Al-Whaibi 2014), carbon nanotubes in tomato (*L. esculentum* M.), mustard (*Brassica juncea*), black gram (*Phaseolus mungo*) and rice (*Oryza sativa* L.) (Khodakovskaya et al. 2009; Nair et al. 2010; Ghodake et al. 2010), $nTiO_2$ in spinach (*Spinacia oleracea*) and wheat (*Triticul aestivum* L.) (Zheng et al. 2004; Hong et al. 2005; Yang and Watts 2005; Yang et al. 2006; Lei et al. 2008; Feizi et al. 2012; Larue et al. 2012), Al_2O_3 in *Arabidopsis thaliana* and *Lemna minor* L. (Lee et al. 2010; Juhel et al. 2011), Nano Si, Pd, Au, Cu in lettuce (*Lactuca sativa*) (Shah and Belozerova 2009), SiO_2 and TiO_2 in soybean (*Glycine max*) (Lu et al. 2001), the germination was improved. Moreover, by the application of SiO_2–Ag, powdery mildew of pumpkin (*Cucurbita pepo*) was controlled (Park et al. 2006). An increase in the germination rate of the above stated crops is an important aspect of the NMs however, the application of these NMs as a nutrient source for the entire growth cycle of two crop plants needs to be explored yet. So, the evaluation of these materials as a nutrient source, their critical concentration, and their phytotoxic effects, if any need, to be explored in future.

4.3.2 Purification of Irrigation Water

Irrigation water could be purified by employing the process of nanofiltration instead of traditional methods of using UV light or chemicals (Hillie and Hlophe 2007). Nanofiltration makes use of the nanofilters with nanopores which have not only the ability to remove the water borne pathogens but also heavy metals like lead, uranium, and arsenic (Gao et al. 2014; Zhu et al. 2014). For this purpose, fused mesh carbon nanotubes have been employed successfully and has been proved to be the economical one. The microbial endotoxins, genetic materials, pathogenic viruses, and micro-sized particles have been successfully removed by the use of nanoceram filter having positive charge on their surface (Gibbons et al. 2010).

Table 4.2 Effect of different NPs on germination and growth of different crops

Nanoparticle	Crop	Comments	References
ZnO	Peanut (*Arachis hypogaea*)	Improved growth and yield	Prasad et al. (2012b)
	Clusterbean (*Cyamopsis tetragonoloba* L.)	Improved shoot-root growth, chlorophyll (photosynthetic pigment), total soluble leaf protein content, rhizospheric microbial population, and P nutrient-mobilizing enzymes (phytase, acid and alkaline phosphatase)	Raliya and Tarafdar (2013)
SiO_2	Maize (*Zea mays* L.)	Enhanced plant dry weight and levels of organic compounds such as proteins, chlorophyll and phenols	Suriyaprabha et al. (2012a, b)
	Tomato (*Lycopersicum esculentum* Mill.)	Improved seed germination	Siddiqui and Al-Whaibi (2014)
Carbon nanotubes	Tomato (*L. esculentum* M.)	Improved seed germination and root growth	Khodakovskaya et al. (2009)
	Wheat (*Triticum aestivum* L.)	Enhanced root growth	Wang et al. (2012)
	Mustard (*Brassica juncea*) and black gram (*Phaseolus mungo*)	Improved germination and seedling growth	Ghodake et al. (2010), Mondal et al. (2011)
	Rice (*Oryza saliva* L.)	Improved germination of seeds	Nair et al. (2010)
	Common gram (*Cicer arietinum*)	Enhanced overall growth rate	Tripathi et al. (2011)
	Tobacco (*Nicotiana tabacum*) cells	Activated carbon (AC) stimulated cell growth (16 % increase) only at low concentrations (5 μg/mL)	Khodakovskaya et al. (2012)

(continued)

Table 4.2 (continued)

Nanoparticle	Crop	Comments	References
TiO_2	Spinach (*Spinacia oleracea*)	Accelerated the germination of the aged seeds, promoted photosynthesis and nitrogen metabolism, and improved growth, decreased oxidative stress caused by UV-B radiation	Zheng et al. (2004), Hong et al. (2005), Yang and Watts (2005), Yang et al. (2006), Lei et al. (2008)
	Wheat (*Triticum aestivum* L.)	Accelerates the germination of the aged seeds, increased root elongation	Feizi et al. (2012), Larue et al. (2012)
Al_2O_3	*Arabidopsis thaliana*	Showed no toxic effect on root elongation and its development	Lee et al. (2010)
	Lenina minor L.	Substantially increased biomass	Juhel et al. (2011)
Si, Pd, Au, Cu	Lettuce (*Lactuca sativa*)	Improved seed germination	Shah and Belozerova (2009)
SiO_2 and TiO_2	Soybean (*Glycine max*)	Enhanced nitrate reductase activity in, and apparently hastened its germination and growth	Lu et al. (2001)

The magnetic properties of certain metals like Fe in the form of mono-disperse magnetite (Fe_3O_4) could be utilized for the separation of heavy metals, e.g., arsenic (As) from the irrigation water (Yavuz et al. 2006). This has been possible due to the use of magnetic NPs and magnetic separations at very low magnetic field gradients. For this purpose, a simple handheld magnet could be used to remove nanocrystals and arsenic from water and this treatment could be used for irrigation water filtration process (Faria et al. 2014).

4.3.3 Zeolites for Water Retention

Zeolite is a complicated silicate mineral with spacious pores and channels within its crystal structure which makes it different from other silicate minerals. It has a unique property of high cation exchange capacity (CEC), as it requires other positively charged accessory cations to become form electrically neutral and stable mineral. It can combine with other cations like Na^+, K^+, Ca^{2+}, etc. (Dana 1977; Navrotsky et al. 1995). Generally, it has high CEC (ten times more than that of soil), large amount of free water in the structural channels and high adsorption ability with surface area of about 1150.5 $m^2\ g^{-1}$ (Sand and Mumpton 1978). Due to these properties, these have been used in inorganic membrane science and technology (Burggrafand and Cot 1996; Yardley 2000) for improving water quality (Pirtola et al. 1998) and ameliorating soil (Genxing et al. 1991; Booker et al. 1996; Haidouti 1997). Xiubin and Zhanbin (2001) reported that zeolite could increase infiltration by 7–30 % on gentle slope land and more than 50 % on steep slope land. Moreover, the treated soil could increase soil moisture by 0.4–1.8 % in the extreme drought condition and 5–15 % in general situation. Overall, they suggested that their use could reduce overland flow (surface runoff) and protect the soil from erosion which ultimately helps in the regulation of water supply for crops in severe drought conditions. Thus, zeolite could be potentially applied for dry land farming but some technological aspects like the characterization of Bronsted and Lewis acid centers, the available deposits in each country, to determine whether zeolites could be used to reduce the nitrate leaching, to develop methodologies for nano-organo-zeolite fertilizers, their nutrient release pattern, their physical stability in a variety of soils and to determine their long-term effects on soil flora and fauna need to be explored (Ramesh et al. 2010).

4.3.4 Nanoscale Carriers and Nanofertilizers

Nanoscale carriers could be utilized for the efficient delivery of fertilizers, pesticides, herbicides, plant growth regulators, etc. (Prasad et al. 2012a). The mechanisms employed by these carriers in the efficient delivery, better storage and controlled release include encapsulation and entrapment, polymers and

dendrimers, surface ionic and weak bond attachments, and others (Sawant et al. 2006). These mechanisms help to improve their stability against degradation in the environment and ultimately reduce the amount to be applied which reduces chemicals run off and alleviates environmental problems.

Nanofertilizers have proved to be another landmark in the history of crop production through nanotechnology. There are many issues with the use of traditional chemical fertilizers however, low use efficiency is the prominent one, which not only increases the cost of production but also causes environmental pollution (Wilson et al. 2008). Nanomaterials with large surface area could solve this problem due to their nanosize. These could be utilized as nanocoatings, e.g., sulfur nanocoating ($\leq$100 nm layer), ensuring their controlled release, surface protection, and ultimately boosting up their use efficiency (Brady and Weil 1996; Santoso et al. 1995). Nanofertilizers have been proved more efficient compared to the ordinary fertilizers as these reduce nitrogen loss due to leaching, emissions, and long-term incorporation by soil microorganisms (Liu et al. 2006a). Moreover, controlled release fertilizers may also improve soil by decreasing toxic effects associated with over-application of traditional chemical fertilizers (Suman et al. 2010). There are also reports about the use of nanoencapsulated slow-release fertilizers (DeRosa et al. 2010). Recently, biodegradable, polymeric chitosan NPs (~78 nm) have been used for controlled release of the NPK fertilizer sources such as urea, calcium phosphate and potassium chloride (Corradini et al. 2010). Other NMs like kaolin and polymeric biocompatible NPs could also be utilized for this purpose (Wilson et al. 2008).

4.3.5 Plant Hormones

Nanotechnology researchers have studied the regulation of plant hormones like auxin which is responsible for proper root growth and seedling organization and how plant roots acclimatize to their environment, particularly to marginal soils (McLamore et al. 2010). In this regard, the world's largest agrochemical corporation, Syngenta has formulated a product, the Primo MAXX®, a plant growth regulator which has been found to induce resistance in turf grass against biotic and abiotic stress and allow it to withstand ongoing stresses throughout the growing season (Pérez-de-Luque and Rubiales 2009).

4.3.6 Nanoparticles and Plant Protection

Nanotechnology has also been applied in the field of plant protection against insects and pests. The nanoparticles could be effectively utilized in the preparation of new formulations like pesticides, insecticides, and insect repellants (Barik et al. 2008; Gajbhiye et al. 2009). As mentioned in the later section, NMs like nanosilica

has been successfully used for the transfer of targeted genes into the cells (Torney 2009) and this technique could also be used in the formulation of pesticides, insecticides, and insect repellants (Barik et al. 2008; Gajbhiye et al. 2009). Moreover, it has also been reported that nanoemulsions like oil in water could be used for the formulation of pesticides against various insect pests (Wang et al. 2007). For example, poly-ethylene glycol-coated NPs loaded with garlic essential oil has been successfully tested against store-product pests like *Tribolium castaneum* insect (Yang et al. 2009). Porous hollow silica nanoparticles (PHSNs) loaded with validamycin (pesticide) have been successfully employed as an efficient and controlled release formulation for water soluble pesticides (Liu et al. 2006b). Moreover, nanosilica has been utilized as a sole nanoinsecticide and its insecticidal property was suggested due to its absorption ability into the cuticular layer of insect pests which otherwise acts as a barrier for protection of insect pests against pesticides (Barik et al. 2008). Moreover, due to their small size (∼3–5 nm), modified surface charge and hydrophobicity could be successfully utilized to manage a variety of ecto-parasites of animals and agricultural insect pests (Ulrichs et al. 2005). In addition to nanosilica, the insecticidal properties of silver, aluminum oxide, zinc oxide, and titanium dioxide NPs have been successfully utilized in the management of rice weevil and grasserie disease in silk worm (Goswami et al. 2010). Nanosilver is the most studied and utilized nano particle for biosystem due to its strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities (Young 2009). Its strong inhibitory and bactericidal effects compared to the bulk are suggested due to the high surface area and high fraction of surface atoms (Suman et al. 2010). Moreover, nanosilver has been suggested to change the biochemistry of plasma membrane (Pal et al. 2007) and prevents the expression of proteins associated with ATP production (Yamanka et al. 2005). The exact mechanisms behind this control are still unknown and need to be explored in future studies. It has been effectively used as an anti-fungal agent on potato dextrose agar (PDA) and 100 ppm of AgNPs (Kim et al. 2012).

Teodoro et al. (2010) reported the insecticidal activity of nanoalumina against two insect pests viz. *S. oryzae* L. and *Rhyzopertha dominica* (F.) of stored food supplies. Zinc oxide (ZnO) and magnesium oxide (MgO) nanoparticles have been proved effective antibacterial and anti-odor agents (Shah and Towkeer 2010) and are proposed to be utilized as an antimicrobial preservative for food products (Aruoja et al. 2009; Huang et al. 2005; Sharma et al. 2009).

Another remarkable feature of nanotechnology is the introduction of annoencapsulation of chemicals like fertilizers, insecticides, and herbicides. It is the process through which the nanochemicals are released into the plant body in a controlled way for improving their efficiency (Scrinis and Lyons 2007). This process is similar to that of the transfer of genes using nanocarriers which ensure not only the delivery of the chemical into the target system but also helps its release in a controlled passion (Torney 2009). The controlled release of the nanochemicals is caused by the processes like that of the diffusion, dissolution, biodegradation, and osmotic pressure with specific pH (Ding and Shah 2009; Vidhyalakshmi et al. 2009). Nanoencapsulation has revolutionized the use of pesticides and herbicides.

So nanocapsules could facilitate the successful incursion of herbicides through cuticles and tissues, allowing slow and regular discharge of the active substances and could act as magic bullets (Pérez-de-Luque and Rubiales 2009).

So these materials have been proved efficient carrier materials for immediate as well as prolonged delivery of pesticides to the crop plants. Moreover, compared to commercially available insecticides, these NPs may provide an eco-friendly and cost effective for the control of pathogenic microbes (Park et al. 2006; Kumar and Yadav 2009; Prasad et al. 2011; Swamy and Prasad 2012; Prasad and Swamy 2013) and such studies may expand the frontiers for nanoparticle-based technologies in pest management.

4.3.7 Micro-fabricated Xylem Vessels

We are able to study the physicochemical and biological interactions between plant cell bodies and various disease causing organisms, i.e., pathogens through the advancement in nanofabrication and characterization tools. These tools have helped us in understanding the mechanisms involved and ultimately improved the strategies for the treatment of these diseases (Cursino et al. 2009). For example, in the past, to study xylem inhabiting bacteria, changes in bacterial populations were monitored through destructive sampling techniques at different distances from inoculation sites but it doesn't provide the information about colonization, film development, and subsequent movement and re-colonization at new areas because the same sample site cannot be followed temporarily. It has only been possible through the discovery of micro-fabricated xylem vessels with nano-sized features that we are able to study the above stated mechanisms which otherwise were not possible through traditional methods (Zaini et al. 2009).

4.3.8 Clay Nanotube

Another achievement in the field of plant protection is the development of clay nanotubes (Halloysite). These have been developed as carriers of pesticides for low cost, extended release and better contact with plants and it will reduce the amount of pesticides by 70–80 %, hence it will reduce the cost of pesticide and also the impact on water streams (Murphy 2008).

4.3.9 Nanobarcode Technology

In our daily life, identification tags have been applied in wholesale agriculture and livestock products. Due to small size, NPs have been applied in many fields ranging from advanced biotechnology to agricultural encoding. Nanobarcodes

(>1 million) have been applied in multiplexed bioassays and general encoding because of their possibility of formation of large number of combinations that made them attractive for this purpose. UV lamp and optical microscope is used for the identification of micrometer sized glass barcodes which are formed by doping with rare earth containing a specific type of pattern of different fluorescent materials (Mathew et al. 2009). The particles to be utilized in nanobarcodes should be easily encodable, machine-readable, durable, sub-micron sized taggants particles. For the manufacture of these nanobarcode particles, the process is semi-automated and highly scalable and involves electroplating of inert metals (Gold, Silver etc.) into templates defining particle diameter, and then resulting striped nano-rods from the templates are released. These nanobarcodes have the following applications.

4.3.9.1 Biological Applications of Nanobarcodes

Nanobarcodes have been used as ID tags for multiplexed analysis of gene expression and intracellular histopathology. Improvement in the plant resistance against various environmental stresses such as drought, salinity, diseases, and others has been only possible through the advancement in field of biotechnology at nanoscale. In the near future, more effective identification and utilization of plant gene trait resources is expected to introduce rapid and cost effective capability through the advances in nanotechnology-based gene sequencing (Branton et al. 2008). It has been proved economically proficient, rapid, and effortless technique in decoding and recognition of diseases as multiple pathogens in a farm could be tagged and detected at a time using any fluorescent-based tools through this technique (Li et al. 2005).

4.3.9.2 Nonbiological Applications of Nanobarcodes

The nanobarcodes serve as uniquely identifiable nanoscale tags and have also been applied for non-biological applications, e.g., authentication or tracking in agricultural food and husbandry products. This nanobarcode technology will enable us to develop new auto-ID technologies and for tagging of items previously not practical to tag with conventional barcodes (Han et al. 2001).

4.3.10 Nanotechnology for Crop Biotechnology

Nanomaterials have also been employed in the field of crop biotechnology for the improvement of the crops. These NMs have served as the magic bullets for an efficient delivery system of genes (Pérez-de-Luque and Rubiales 2009). For example, Mesoporous nanosilica particles have been chemically coated and served as

the gene carriers for their delivery into the tobacco and corn plants (Torney et al. 2007). These particles are absorbed through the cell wall and the target genes are efficiently delivered to the plant system in a non-toxic way. Moreover, carbon nanotubes (CNTs) have proved to be an effective nanocargo to deliver DNA and small molecules into tobacco cells (Liu et al. 2006a, b).

4.3.11 Nanosensors

Nanotechnology has also enabled us an efficient use of agricultural natural assets like water, nutrients, and chemicals during farming, as nanosensors have been developed, and these have been proved to be user friendly.

4.3.11.1 Controlling the Level of Soil Nutrients

Nanosensors have not only been used as nanobiosensors but also for the control of soil nutrients and these have helped in the reduction of fertilizer consumption and environmental pollution (Ingale and Chaudhari 2013).

4.3.11.2 Nanobiosensors

Several nano-based biosensors have been developed to detect contaminants, such as crystal violet or malachite green concentrations in seafood and parathion residues or residues of organophosphorus pesticides on vegetables (Amine et al. 2006). These instruments are able to reduce the time required for lengthy microbial testing and immunoassays. Applications of these instruments include detection of contaminants in different bodies like water supplies, raw food materials, and food products. A variety of characteristic volatile compounds are produced by the microorganisms that are useful as well as harmful to human beings, e.g., fermentation makes use of yeasts, while alcohol is produced as a byproduct when bacteria eats sugar. For the rapid growth of a wide range of microorganisms, dairy products, bakery products, and other food products represent ideal media. The most common causal organisms of food rotting are bacteria. Foul odor is a clear indication of food rotting. Human nose can detect and distinguish a large number of odors but sometimes it may be impractical and a further cause for poisoning. However, it is more sensible to use an instrument like rapid detection biosensors for the detection of these odors. Many researchers around the world have reported about the efficacy of NPs in different fields like drug delivery, biosensing, etc. (Panyam and Labhasetwar 2003; Zanello et al. 2006; Harrison and Atala 2007).

4.3.11.3 Enzymatic Biosensors

Enzymes can act as a sensing element as these are very specific in attachment to certain biomolecule (Le Goff et al. 2011; Sassolas et al. 2012). According to Su and Li (2004), enzymatic biosensors based on immobilization surface are classified into four groups (1) controlled-pore glass beads with optical transducer element, (2) polyurethane foam with photothermal transducer element, (3) ion-selective membrane with either potentiometric or amperometric transducer element, and (4) screen-printed electrode with amperometric transducer element.

4.3.11.4 Electronic Nose (E-Nose)

This device has been successfully used to identify different types of odors and is based on the operation of the human nose. It uses a pattern of response across an array of gas sensors. It can identify the odorant, estimate the concentration of the odorant, and find characteristic properties of the odor in the same way as that might be perceived by the human nose. It mainly consists of gas sensors which are composed of NPs, e.g., ZnO nanowires (Patel 2002; Hossain et al. 2005). Their resistance changes with the passage of a certain gas and generates a change in electrical signal that forms the fingerprint pattern for gas detection. This pattern is used to determine the type, quality, and quantity of the odor being detected. It also has an improved surface area which helps in better absorption of the gas. E-nose technology combined with both metabolic and biomass parameters can altogether represent reliable indicators of the metabolic status of soil ecosystems (De Cesare et al. 2011).

4.3.12 Gold Nanoparticles

Man has been fascinated by gold for a long time. It is one of the most widely studied and abundantly used NPs like bulk gold. Due to several qualities, it has remained valuable both as a medium of exchange and for decorative use as jewelry throughout history. The gold nanoparticles (GNPs), commercially used as rapid testing arrays for pregnancy tests and biomolecule detectors, are based on the fact that the color of these colloids depends on the particle size, shape, refractive index of the surrounding media, and separation between the NPs. A quantifiable shift in the Surface Plasmon Response (SPR) absorption peak results due to a small change in any of these parameters. Rhodamine B-covered gold nanoparticle (RB-AuNP)-based assay with dual readouts (colorimetric and fluorometric) has been developed for detecting organophosphorus and carbamate pesticides in complex solutions (Liu et al. 2012). The GNPs-based assay for assessing antioxidant activity of chrysanthemum extracts and tea beverages in vitro based on the sample-mediated generation and growth of GNPs is feasible and thus offers great promise for estimating the antioxidant activity of chrysanthemum extracts, tea beverages, and other plant-related food (Liu et al. 2012).

We can make these NPs attach to specific molecules by carefully choosing the capping agent for stabilizing gold NPs. These specific molecules are adsorbed on the surface of these NPs where these change the effective refractive index (RI) of the immediate surroundings of the NPs (Sugunan et al. 2005). A few NPs will be adsorbed if the detecting molecules (bio-macromolecules) are larger than the gold NPs and result in the formation of lumps after agglomeration. Ultimately, the color of gold NPs is changed due to the shift in SPR that results from the reduction of particle spacing.

4.3.13 Smart Dust

Smart dust sensors could be used in determining the amount of pollutants and dust in the air (Scott and Chen 2003b). We can use the "smart dust" technology for monitoring various parameters like temperature, humidity, and perhaps insect and disease infestation to create distributed intelligence in vineyards and orchards.

4.3.14 ZigBee a Mesh Networking Standard

ZigBee, a wireless mesh networking standard with low cost and utilizes low power. It has given the concept of "Smart Fields" and "Soil Net." It consists of one or more sensors for environmental data (temperature, humidity etc.), a signal conditioning block, a microprocessor/microcontroller with an external memory chip, and a radio module for wireless communication between the sensor nodes and/or a base station. It can be used for the identification and monitoring of pests, drought or increased moisture levels in order to counterbalance their adverse effects on crop production (Nath and Chilkoti 2004). Through this wireless sensor technology with nanoscale sensitivity, we can control plant viruses and level of soil nutrients as the plant surfaces can be changed at nanoscale with specific proteins. This technology is important in realizing the vision of smart fields in particular. Wireless network sensor technology can also be used for monitoring the optimal conditions for mobile plants biotechnology (Van Dam and Langendoen 2003; Lu et al. 2004; Jha et al. 2011).

4.4 Conclusion and Perspectives

Nanotechnology has great potential in improving the quality of life through its applications in various fields like agriculture production and food system. The nanomaterials have been applied as nutrients for the crop plants in the form of nanofertilizers and as crop protectants in the form of nanopesticides and nanoherbicides. Nanosensors have played a remarkable role in precision

agriculture. Nanocarriers could be designed in such a way that these can anchor the plant roots or to the surrounding soil structure and organic matter. This can only be possible through the understanding of molecular and conformational mechanisms between the delivery nanoscale structure and targeted structures and matters in soil (Johnston 2010). These advances will help in slow uptake of active ingredients thereby reducing the amount of inputs to be used and also the waste produced.

Of course, nanotechnology has great potential in various walks of life but we must be very careful about any new technology to be introduced for its possible unforeseen related risks that may come through its positive potential. However, it is also critical for the future of a nation to produce a trained future workforce in nanotechnology. In this process, to inform the public at large scale about its advantages is the first step which will result in tremendous increase in the interest and discovery of new applications in all the domains. The theme of the book chapter is based on the provision of basic knowledge about the applications of nanotechnology in agriculture and their prospects in near future with reference to the current situation around the world. In this chapter, some of the potential applications of nanotechnology in agricultural production for the welfare of humans and for sustainable environment, challenges, and opportunities for developing countries have been described. The nanomaterials have greatly influenced the crop production in the form of nanofertilizers, nanopesticide, nanoherbicides, and precision farming techniques round the world. However, there is still a research gap regarding their mechanism of action and their potential risks after entering into the food chain and needs to be explored in future.

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Chapter 5
Interactions Between Engineered Nanomaterials and Plants: Phytotoxicity, Uptake, Translocation, and Biotransformation

Peng Zhang, Yuhui Ma and Zhiyong Zhang

Abstract The interactions between engineered nanomaterials (ENMs) and plants are of particular importance, as plants directly interact with soil, water, and the atmosphere, and serve as a potential pathway of ENMs exposure for higher species through the food chain. The aim of this chapter is to extend our current understanding about interactions between ENMs and plants, including phytotoxicity, uptake, translocation, and biotransformation of ENMs in plant systems. The mechanisms underlying ENMs phytotoxicity and bioavailability are not well understood. It is clear that more investigations are urgently required in the area of ENMs–plants interactions, as well as the development of novel techniques for in vivo characterization of ENMs to enable these fields to keep pace with the sustainable implementation of nanotechnology.

Keywords Engineered nanomaterials · Phytotoxicity · Uptake · Translocation · Biotransformation

5.1 Introduction

With the rapid development and wide application of nanotechnology, increasing amount of manufactured engineered nanomaterials (ENMs) will be inevitably discharged into the environment, which may pose a threat to ecological species (Nel et al. 2006; Maynard et al. 2006; Oberdörster et al. 2005). As a novel contaminant, the environmental significance and biological effects of ENMs have attracted much attention. Most studies about the toxicity of ENMs have focused

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on their influence on animals and human cells, but relatively scant attention has been paid to the effects of ENMs on plants. Plants represent the largest interface between the environment and the biosphere. As the end receivers of environmental contaminants, they will not only be affected directly by ENMs but also affect transformation and fate of ENMs and, via bioaccumulation through the food chain, constitute a main route of exposure for higher species (Holbrook et al. 2008; Judy et al. 2011; Zhu et al. 2008). The interactions between plants and ENMs can shed light on the environmental consequences of nanotechnology. However, studies about the interaction between ENMs and plants have been largely ignored. Most of the available studies on nanophytotoxicity have focused mainly on toxicity symptoms of plants, and relatively few studies examined the mechanisms of ENMs phytotoxicity, uptake, translocation, and bioaccumulation. Therefore, it is necessary to have a systematic review of the published researches in this field. Collaborations among materials scientists, biologists, and toxicologists are needed to optimize the applications of ENMs, while minimizing their health and environmental impacts.

5.1.1 Interactions of ENMs and Plants

Nanomaterials are powders and materials that measure <100 nm in at least one dimension and that have been specifically engineered for various applications (Colvin 2003). These materials differ from bulk counterparts in that they have larger specific surface areas, greater reactivity, and are subject to quantum confinement (Service 2003). Engineered nanomaterials (ENMs) mainly include the following types: (1) Carbon nanomaterials (NMs), including carbon nanotubes (CNTs), fullerenes (C_{60}), and graphene. (2) Metal-based nanoparticles (NPs), including zero-valent metal (such as Au, Ag, and Fe NPs, etc.), metal oxide (such as nano-ZnO, -TiO_2, and -CeO_2, etc.), and metal salts (such as nano silicates and ceramics, etc.); (3) quantum dots (QDs, such as CdSe, CdTe, etc.); (4) Nanopolymers (such as dendrimers, polystyrene, and latex, etc.); and so on. During their manufacture and use, ENMs can be released into the environment deliberately (Liu et al. 2009; Mauter and Elimelech 2008; Torney et al. 2007) or accidentally (Barnard 2010). The rapidly increasing applications of ENMs have raised questions concerning potential adverse effects on environmental and human health. To support sustainable development of nanotechnology, possible risk assessment must be evaluated based on sound research to elucidate all relevant aspects of this concern.

5.1.2 Phytotoxicity of Engineered Nanomaterials

Early studies of ENM–plant interactions focused mostly on the phytotoxicology of ENMs. The phytotoxicity of ENMs has been shown to differ depending on the type of ENMs and plant species. Phytotoxicity assays are generally performed at two stages

of plant development: (i) during germination, when the germination rate and root elongation are measured, and (ii) during seedling growth, in which root/shoot elongation and dry weight are frequently used to assess exposure effects. The common endpoints are most useful for comparison among plants and ENMs. Recently, the number of leaves (Lee et al. 2010) and the chlorophyll content (Parsons et al. 2010) of exposed plants, as well as the cytotoxicity and genotoxicity of ENMs (Ghosh et al. 2010; Khodakovskaya et al. 2011, 2012; Lopez-Moreno et al. 2010a; Wang et al. 2011) have been included as novel endpoints for phytotoxicity assays.

To date, a wide variety of effects of ENMs on plants have been observed. The most frequently used test species in phytotoxicity assays are chosen according to the U.S. EPA (1996) or OECD (2003) guidelines, including monocotyledonae and dicotyledonae crop species, as well as economically or ecologically important noncrop species. Species that have been used in phytotoxicity, uptake and bioaccumulation studies include wheat (*Triticum aestivum*), pumpkin (*Cucurbita pepo*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), soybean (*Glycine max*), corn (*Zea mays*), tomato (*Lycopersicon esculentum*), rice (*Oryza sativa*), cabbage (*Brassica oleracea*), etc. The most frequently tested ENMs are those widely produced and used, including carbon NMs and metal-based NPs. Several reviews have inventoried the studies of ENM effects on terrestrial plants (Ma et al. 2010a; Miralles et al. 2012a; Peralta-Videa et al. 2011). Herein, we introduced some more recent results and related phytotoxicity mechanisms.

5.1.2.1 Carbon Nanomaterials

Carbon NMs have attracted significant interest due to their remarkable physicochemical characteristics (Mauter and Elimelech 2008). A number of investigators have demonstrated phytotoxicity of carbon NMs to a range of plant species, but the contradictory results may come from the different experimental conditions and plant species. There was a consensus that a high degree of CNT functionalization leads to a dramatic reduction in toxic effects. For example, Cañas et al. (2008) investigated the effects of functionalized and nonfunctionalized single-walled carbon nanotubes (fCNTs and CNTs) on root elongation of six crop species (cabbage, carrot, cucumber, lettuce, onion, and tomato). They found that phytotoxicity varied between CNTs and fCNTs, with CNTs affecting more species. By using SEM, they found CNTs were adsorbed onto the root surface, but not accumulated in plants. Stampoulis et al. (2009) showed that 1,000 mg/L MWCNTs had no effect on the germination rate, but reduced the biomass to 60 % of the control in zucchini (*C. pepo*) under hydroponic conditions. Liu et al. (2010) found that ($C_{70}(C(COOH)_2)_{4-8}$) can inhibit root elongation in Arabidopsis (*Arabidopsis thaliana*) and induce abnormal root gravitropism. Fluorescence imaging at the molecular level revealed that the internalization of a C_{70} fullerene malonic acid derivative in roots interrupted the transport of the plant hormone auxin. Lin et al. (2009) found that NOM-mediated MWCNTs and C_{70} delayed rice flowering by up to 1 month; an effect attributed to the likely interference of

carbon NMs with nutrient and water uptake. Alternatively, Lin and Xing (2007) found that 2,000 mg/L multiwalled carbon nanotubes (MWCNTs) had no obvious effects on the germination and root growth of six higher plant species. Similarly, Ma and Wang (2010) reported that 2–15 mg/L fullerenes had no impact on cottonwood growth. These findings are in agreement with the results of De La Torre-Roche et al. (2012), where no toxicity was reported upon exposure to C_{60} under vermiculite-based conditions. The same research group further reported that zucchini and tomato growth were unaffected by MWCNTs or C_{60} exposure, but C_{60} reduced corn and soybean biomass by 36.5–45.0 % at 500 mg/kg (De La Torre-Roche et al. 2013). In a recent study, Liu et al. (2013) investigated the change of cell wall of tobacco plant cell (*Nicotiana tobacum* L. cv. Bright Yellow) under the repression of water-soluble carboxyfullerenes ($C_{70}(C(COOH)_2)_{2-4}$). The adsorption of this NM on cell wall led to cell growth inhibition, with disruption of cell wall and membrane. Results of AFM ligand-receptor binding force measurement and confocal imaging revealed an increase of the glycosyl residue on the cell wall of carboxyfullerene-treated cells and accompanied by the elevated reactive oxygen species (ROS). This study provided direct evidence on the change of the living plant cell wall composition under the repression of fullerenes. In another study, Avanasi et al. (2014) assessed the soil sorption, degradation, and plant uptake of fullerene using ^{14}C-labeled C_{60} solutions, indicating that C_{60} released to the environment will not be highly bioavailable for plants (~7 %), but will likely persist in soil for a period more than 1 year. Research on the risks of graphene, the most recently discovered carbon allotrope with exceptional properties, to the ecosystem is just beginning. Begum et al. (2011) reported that graphene significantly inhibited plant (cabbage, tomato, red spinach, and lettuce) growth and biomass compared to a control. The mechanisms of phytotoxicity involved oxidative stress.

On the other hand, the positive effects of carbon NMs on plants have also been reported. For instance, Miralles et al. (2012b) demonstrated that 2,560 mg/kg of industrial-grade MWCNTs enhanced germination and root elongation of alfalfa and wheat. Remarkably, the catalyst impurities, not solely the CNTs, also enhanced root elongation in alfalfa seedlings as well as wheat germination. Raman mapping showed that CNTs were adsorbed onto the root surfaces of alfalfa and wheat without significant uptake or translocation. However, Khodakovskaya et al. (2013) found that MWCNTs can penetrate the seed coat and improve the water delivery, which was the main reason for the enhanced germination rates and biomass of tomato in MWCNT-amended medium. Similarly, another study showed that the presence of MWCNTs in the growth medium increased tobacco (*Nicotiana tabacum*) cell growth by upregulating the expression of water channel genes and aquaporin (Khodakovskaya et al. 2012). More recently, Tiwari et al. (2013) also found that pristine MWCNTs could benefit the germinative growth and biomass of maize seedlings at low concentrations by enhancing water and nutrient transport, but that their potency could be diminished by high concentrations of ions/polar species in the medium. They suggested a potential utilization of CNTs for optimizing water transport in arid-zone agriculture and of improving crop biomass yields. Hu and Zhou (2014) reported a novel and biocompatible hydrated graphene

ribbon (HGR) could promote aged (2 years) wheat seed germination, increase seed germination, and enhance resistance to oxidative stress. The metabonomics analysis indicated that HGR could upregulate carbohydrate, amino acid, and fatty acids metabolism that determined secondary metabolism, nitrogen sequestration, cell membrane integrity, permeability, and oxidation resistance. Anjum et al. (2013, 2014) assessed the germinating faba bean (*Vicia faba L.*) seedlings tolerance to different concentrations (0, 100, 200, 400, 800, and 1,600 mg/L) of single-bilayer graphene oxide sheet (GO; size: 0.5–5 μm) and underlying potential mechanisms. They revealed both positive and negative concentration-dependent GO-effects on *V. faba*. Significant negative impacts of GO concentrations (ordered by magnitude of effect: 1,600 > 200 > 100 mg GO L^{-1}) were indicated by decreases in growth parameters and the activity of redox enzyme systems, as well as by increases in the levels of electrolyte leakage (EL), H_2O_2, and lipid and protein oxidation. The positive impacts of GO (in order of impact: 800 > 400 mg/L) included significant improvements in *V. faba* health status indicated by decreased levels of EL, H_2O_2, and lipid and protein oxidation, as well as by increased redox enzyme activity, proline and seed-relative water content (Anjum et al. 2013, 2014). These findings demonstrate the complex interactions of carbon NMs with terrestrial plant species and highlight the need for further investigation.

5.1.2.2 Metal-Based Nanomaterials

Terrestrial plants interact directly with the soil, water, and atmospheric environmental compartments, all of which can be routes of ENMs exposure. Various types of metal-based oxide ENMs with different properties are designed for applications in biotechnology, industry or agriculture, and their transport and bioaccumulation through the food chain is plausible. Metal-based ENMs have different effects on plants, with both positive and negative effects have being reported. The results of phytotoxicity were dependent on the properties of ENMs, plant species, as well as experimental conditions. There were even conflicting results of the same kind of ENM in some cases. For example, several articles have shown that nano-TiO_2 had a positive effect on growth of spinach, with improving light absorbance, increasing the activity of activity Rubiso activase enzymes, or decreasing the oxidative stress to chloroplast caused by UV-B radiation (Gao et al. 2008; Lei et al. 2008; Yang et al. 2007). However, some other studies reported that nano-TiO_2 had genotoxicity. Ghosh et al. (2010) reported that 4 mM L^{-1} nano-TiO_2 could induce micronuclei and DNA laddering in the root cells of *Album cepa*. Wang et al. (2011) found that the penetration of nano-TiO_2 into *A. thaliana* cells triggers the disassembly of their microtubular network, causing an overload of the proteasome system and isotropic growth of the root epidermal cells. Clément et al. (2013) revealed that TiO_2 NPs in anatase crystal structure were more toxic than in rutile to flax (*Linum usitatissimum*). Because of the lipophilicity, the rutile TiO_2 NPs formed larger aggregates in aqueous medium, and thus a lower toxicity than the anatase.

Nano-CeO_2 is another representative of metal-based ENMs and is always considered as insoluble compound under environmental conditions (Johnson and Park 2012). Most of the studies showed that nano-CeO_2 was nontoxic to plants (Birbaum et al. 2010; Ma et al. 2010b; Schwabe et al. 2013; Zhao et al. 2013). Some other studies suggested that the nano-CeO_2 could affect the antioxidant defense enzyme activities (Rico et al. 2013a, b; Zhao et al. 2012a) and nutritional properties (Morales et al. 2013; Zhao et al. 2014) of plants, although the seedlings showed no visible signs of toxicity. In an early report, the root growth was significantly enhanced in corn (*Z. mays*) and cucumber (*C. sativus*) but retarded in alfalfa (*Medicago sativa*) and tomato (*Lycopersicum esculentum*) at the presence of nano-CeO_2. The shoot elongation was promoted by nano-CeO_2 in the four plant species at almost all concentrations (0–4,000 mg/L) (Lopez-Moreno et al. 2010b). The same group also demonstrated the genotoxic effects of nano-CeO_2 to soybean plants, with the appearance of new bands in the random amplified polymorphic DNA (RAPD) assay (Lopez-Moreno et al. 2010a). Priester et al. (2012) revealed that nano-CeO_2 could not only reduce the growth and yield, but also shut down nitrogen fixation of soybean plants gown in soil at high concentrations. Ma et al. (2013a) gave an example for concentration-dependent effects of nano-CeO_2 to *Arabidopsis*. Plant biomass was significantly increased at 250 ppm nano-CeO_2, but was decreased by up to 85 % at 500–2,000 ppm in a dose-dependent mode. Moreover, chlorophyll, anthocyanin, and MDA production were all affected at high concentrations. Wang et al. (2012a) documented the chronic phenotypic response of tomato plants to nano-CeO_2 at relatively low concentrations (0.1–10 mg/L). They showed that nano-CeO_2 had either an inconsequential or a slightly positive effect on plant growth and tomato production at the applied concentrations. In a separate study, this group investigated the transgenerational impact of nano-CeO_2 at the same low concentrations. The results indicated that second generation seedlings grown from seeds collected from treated parent plants with nano-CeO_2 (treated second generation seedlings) were generally accumulated much smaller biomass and were somewhat weaker than seedlings grown from seeds from untreated parent plants (Wang et al. 2013b).

The phytotoxicity mechanism of ENMs is not clear yet. A possible cause for phytotoxicity of metal-based ENMs is the release of toxic ions, especially for those easy to release heavy metal ions, which is one of the largest controversial problems in nanotoxicology study (Lubick 2008; Murashov 2006; Yang and Watts 2005). The dissolution of metal-based ENMs in the biological environment may require particular attention. Nano-ZnO is one of the typical samples. Some studies considered the toxicity of nano-ZnO all come from the released Zn^{2+} (Franklin et al. 2007; Lopez-Moreno et al. 2010a, b; Miller et al. 2010), while others thought the toxicity of ZnO NPs itself cannot be ignored (Lee et al. 2010; Lin and Xing 2007). There are similar cases for other metal-based NMs, such as Ag NPs (Ma et al. 2010a; Navarro et al. 2008; Yin et al. 2011), Cu NPs (Lee et al. 2008; Musante and White 2012), CuO NPs (Dimkpa et al. 2012; Wang et al. 2012b), and Al_2O_3 NPs (Lee et al. 2010; Poborilova et al. 2013), etc. Neither of these studies had clarified the difference in phytotoxicity between metal-based NMs and

released ions, nor the effects of ions adsorbed on NPs, so it needs further research on the toxic mechanism of soluble metal-based NMs NPs.

We studied the phytotoxicity of a series of rare earth oxide (REO) NMs and found that REO NMs did not affect the germination of seven higher plant species (radish, rape, tomato, lettuce, wheat, cabbage, and cucumber) (Ma et al. 2010b). 2,000 mg/L suspensions of nano-CeO_2 was also relatively innocuous during root elongation of six plants, except lettuce; whereas the same concentration of nano-La_2O_3, -Gd_2O_3, and -Yb_2O_3 severely inhibited root elongation in all tested species. On this basis, we further found that nano-CeO_2 had species-specific phytotoxicity, with inhibitory effect on the root growth of *Lactuca* plants. The results of X-ray absorption near edge fine structure (XANES) indicated that a small part of CeO_2 NPs were transformed from Ce(IV) to Ce(III) in roots of the plants, and the high sensitivity of *Lactuca* plants to the released Ce^{3+} ions caused the species-specific phytotoxicity of CeO_2 NPs (Zhang et al. 2013), which highlight the importance of test species in phytotoxicity studies of metal-based NMs. In two separate studies, we determined the internalization of REE in cucumber plant by transmission electron microscopy (TEM) and STXM when exposed to nano-La_2O_3 and nano-Yb_2O_3. They were present in the roots as RE phosphate and the observed phytotoxicity was mainly attributed to the released ions (Ma et al. 2011; Zhang et al. 2012a).

Recently, we evaluated the different phytotoxicity of CeO_2 and La_2O_3 NPs to cucumber plants and clarified the relation between physicochemical properties of NMs and their behaviors (Ma et al. 2014). The different distribution (Fig. 5.1) and speciation (Fig. 5.2) of Ce and La in the cucumber plants implied that La_2O_3 acted as its ionic form, while CeO_2 displayed the behavior of particles or particle–ion mixtures. The higher dissolution of La_2O_3 than CeO_2 NPs might be the reason for their significant difference in phytotoxicity and transporting behaviors in cucumbers.

5.1.3 Uptake and Translocation of ENMs in Plants

Compared with the phytotoxicity of ENMs, there was much less research on absorption, transport, and accumulation of ENMs in plant systems. ENMs might be absorbed by plants through roots or leaves exposure. For roots exposure, ENMs must penetrate the root epidermis and endodermis, entering into the xylem vessel, and then be transported to the aerial parts. While for leaves exposure, ENMs might be internalized through the leaf stoma, entering into the vascular system of leaves, and then be transported to other parts through the phloem. Currently, there is a dispute on whether the ENMs can be uptake by plants and transported in plants, but cellular penetration is the most accepted mode of action, though the exact uptake mechanisms are not fully understood. This section will focus on the uptake and transport of ENMs in plants, which can lead to the advancement of interaction between plants and ENMs.

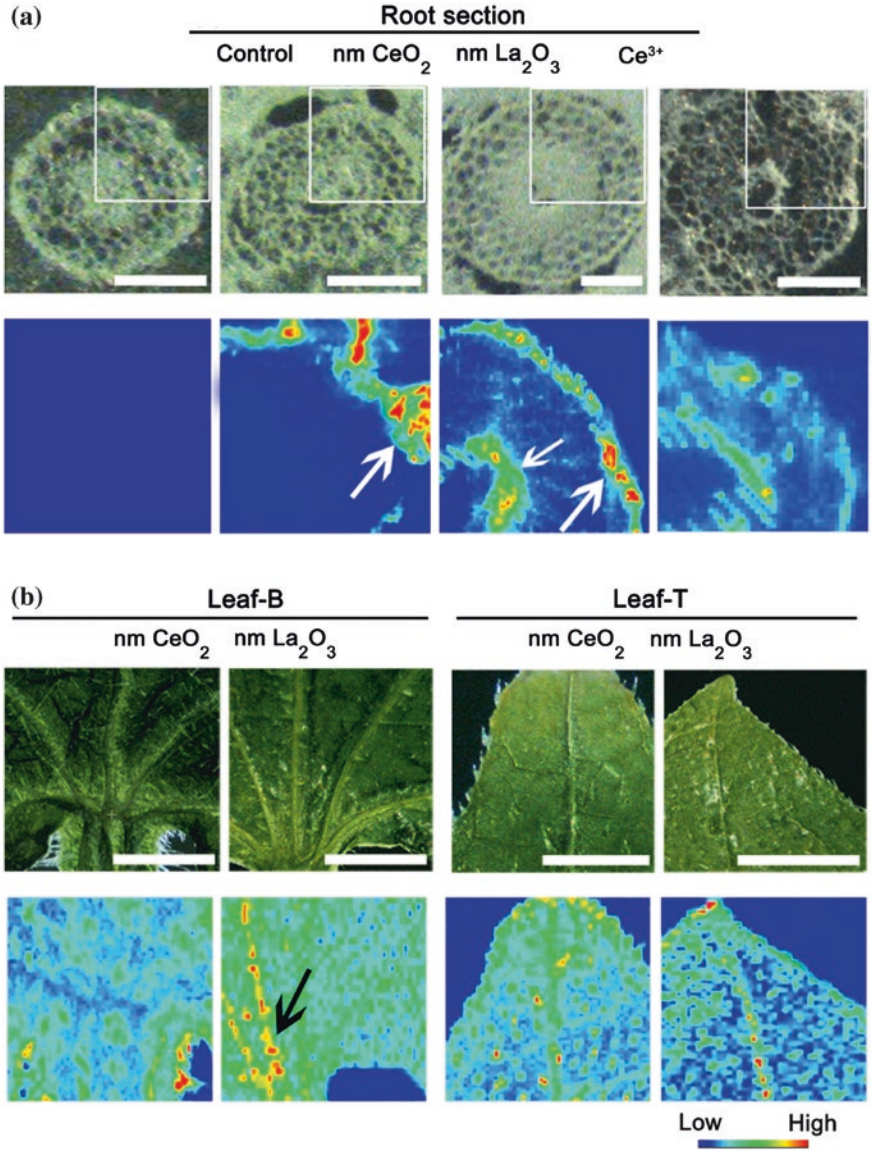

Fig. 5.1 SR-μXRF images of Ce or La in cucumber root sections (**a**) and leaves (*Leaf-B* means leaf base and *Leaf-T* means leaf tip) (**b**) under the control and different treatments. The images were normalized by the Compton scattering radiation and the *red colors* depicting elemental concentrations in each map are scaled to the maximum value for that map. A quarter of the root section was shown as denoted by *rectangles* in the LM images. The deposit of Ce or La in root sections and leaves were denoted by *arrows*. The *scale bars* in root sections represent 100 μm and in leaves represent 750 μm, respectively

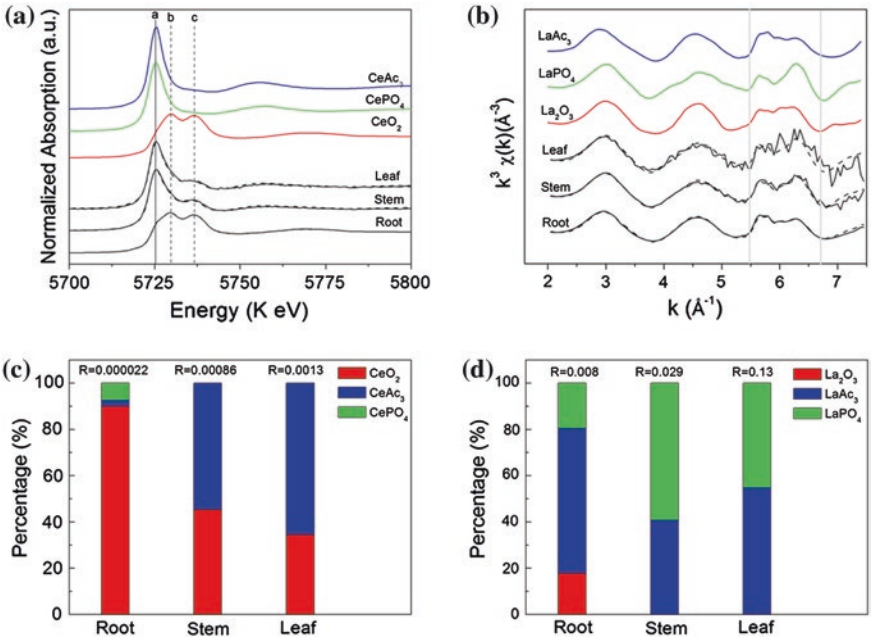

Fig. 5.2 Ce (**a**) L_{III}-edge XANES and La (**b**) EXAFS spectra (*solid line*) and LCF (*dashed line*) of cucumber plant tissues under treatments of 2,000 mg/L CeO_2 and La_2O_3 NPs for 14 days. **a**, **b** and **c** marked the feature of La(III), Ce(III) and Ce(IV), respectively. Percent contributions of each Ce (**c**) and La (**d**) standard spectrum to the fit obtained from LCF analysis. R factor of each sample is also listed in the figures

Different from animal cells, plant cells have cell walls, with almost no phagocytosis. Before entering plant cells, ENMs have to penetrate the cell wall and cytoplasm membrane. The pore sizes of plant walls are typically in the range of 3–8 nm, with the thickness of about 5–20 nm function as natural sieves (Carpita and Gibeaut 1993). ENMs with sizes smaller than the largest pore are expected to pass through and reach the plasma membrane, while the larger particle aggregates will not enter into plant cells. For instance, ultrasmall nano-TiO_2-Alizarin red nanoconjugates (about 3 nm) were capable of traversing cell walls, entering into plant cells, and accumulated in specific subcellular locations of *Arabidopsis* roots and leaves (Kurepa et al. 2010); whereas the aggregation of 25 nm TiO_2 on root surfaces of maize (*Z. mays* L.) seedlings hindered root hydraulic conductivity and water availability, and thus reduced transpiration and affected plant development (Asli and Neumann 2009). Sabo-Attwood et al. (2012) demonstrated that tomato seedlings have size selective absorption of Au NPs, with 3.5 nm Au NP spheres being uptake into the plants but 18 nm Au NPs remaining agglomerated on the root outer surfaces. Birbaum et al. (2010) suggested that no internalization or translocation was

found in live maize plants when exposed to 37 nm CeO_2 NPs either as aerosol or as suspension. On the other hand, some literature reported ENMs with larger size also can be absorbed and translocated in the plants. For example, 47 nm Fe_3O_4 was found to penetrate and transport in living pumpkin plants (Corredor et al. 2009). Using 45 nm upconversion nanoparticles (UCNPs) $NaYF_4$:Yb, Er as tracer, Hischemoller et al. (2009) proved that this NPs can be uptake and transported to vascular tissue, stems, and leaves of *A. thaliana*. The elongated shape of CNTs could hinder them penetrating tissue, while facilitating adsorption on root surfaces. The development of rice was retarded when exposed to a mixture of CNTs and natural organic matter despite that there was no evidence of internalization (Lin et al. 2009). Similarly, exposure to CNTs could increase seedling elongation of cucumber, or alter the morphology of and induce apoptosis of rice cells, without evidence of CNT internalization (Cañas et al. 2008; Tan et al. 2009).

Although the sample preparation protocols required for TEM of biological samples has been blamed for ENM loss, ENMs internalization in roots has been confirmed by TEM in some studies (Du et al. 2011; Lee et al. 2008, 2012; Lin and Xing 2008; Lin et al. 2009; Speranza et al. 2010). Some authors thought that ENMs may induce the formation of new and large-size pores, through which the larger ENMs were internalized. Optical and fluorescent microscopy has also been used to show ENM internalization (Liu et al. 2010; Wang et al. 2011; Wild and Jones 2009). Wild and Jones (2009) used two-photon excitation microscopy to observe MWCNTs piercing the cell wall of wheat roots and reaching the cytoplasm, though without wholly entering the cell. After root uptake and penetration of the epidermal cells of ENMs, further transport requires circulation across the root and to the xylem. ENMs may be transported through cell wall pores, the apoplastic pathway, or the symplastic pathway through plasmodesmata, channels approximately 40 nm in diameter that connect adjacent cells (Tilney et al. 1991). The translocation of ENMs from the roots to aerial parts of plants has also been determined. C_{70}-NOM could enter the vascular system of rice plants and is translocated to stems, leaves, and even to the progeny through seeds (Lin et al. 2009). Similarly, carbon-coated iron NPs were capable of penetrating living pumpkin tissues and migrating to different regions of the plant (Corredor et al. 2009). Zhang et al. (2011) tracked the spread of two types of ceria NPs in cucumber plants using a radiotracer method and discovered radioactive ^{141}Ce was throughout the plants. Cerium atoms were found primarily around the edges of younger leaves, and eventually spread throughout the leaves. This distribution pattern was distinct from that of aqueous Ce^{3+} ions, which accumulated preferentially along the veins (Fig. 5.3). Zhao et al. (2012b) investigated the uptake of bare and coated CeO_2 NPs by corn plants grown in soil and showed that surface coating and soil organic matter played important roles in the mobility and bioavailability of CeO_2 NPs. FITC-stained CeO_2 NPs were observed in cell walls of cortex and vascular cylinder, demonstrating that CeO_2 NPs can be taken up by plants. Hong et al. (2014) found that foliar applied atmospheric CeO_2 NPs can be taken up and distributed within cucumber plant tissues. Similarly, Larue et al. (2014) suggested that Ag NPs could be transferred in all types of tissues in lettuce plants through both stomatal and

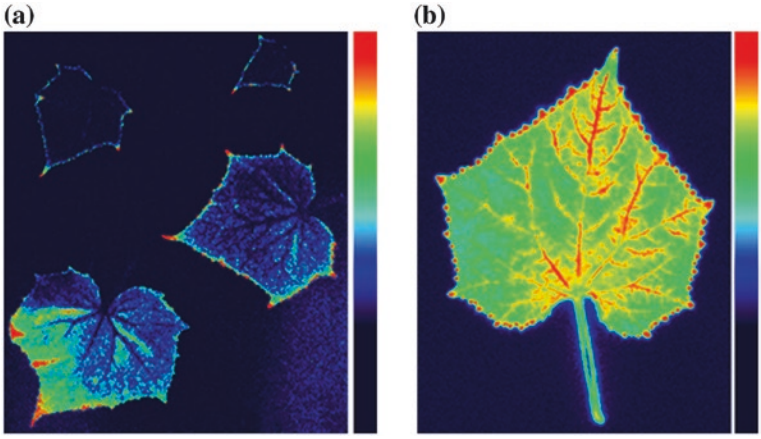

Fig. 5.3 Autoradiographs of Ce in cucumber leaves. **a** ceria NPs; **b** Ce^{3+} ions

cuticular pathways after foliar exposure. Using combination of confocal laser scanning microscopy (CLSM), TEM and proton-induced X-ray emission (micro-PIXE) elemental analysis, Sun et al. (2014) determined the location and quantification of 20 nm mesoporous silica nanoparticles (MSNs) in four plant species tissues and in cellular and subcellular locations. The results show that MSNs could penetrate into the roots via symplastic and apoplastic pathways and then via the conducting tissues of the xylem to the stems and leaves of the plants. Chen et al. (2010) thought that $C_{60}(OH)_{20}$, a water-soluble fullerene derivative, was transported through the apoplastic pathway in the plant tissue, because these ENMs was confined between the cell wall and the plasma membrane of *Allium cepa* cells. Wang et al. (2012b) combined TEM and energy dispersive spectroscopy (EDS) to detect CuO NPs in the xylem sap of maize plants, providing evidence that this ENM can penetrate the root system, reach the xylem, and be translocated to the aerial parts. Split-root experiments and high-resolution TEM observation further showed that CuO NPs could be translocated from shoots back to roots via phloem, and CuO NPs could be reduced from Cu (II) to Cu (I). Also observed by TEM, Zhai et al. (2014) found that Au NPs could be directly taken up by poplar (*Populus deltoides*) roots and translocated to stems and leaves, without dissolved gold ions. On the other hand, Au (III) ions were taken up and reduced into Au NPs inside whole plants. Au NPs were observed in the cytoplasm and various organelles of root and leaf cells. Such plant-induced biotransformation of ENMs has also been observed in other studies, which is critical to understanding the interaction between ENMs and plants and will be elaborated in the next section.

To date, the most accepted explanation for ENM translocation is that ENMs can move intra- and/or extracellularly through tissues until they reach the xylem. When considering transport across the root, special consideration needs to be given to the Casparian strip, a cell wall incrustation in the mature root endodermis

of most plants that prevent the apoplastic transport of external material from the cortex to the stele (Luttge 1971). The mechanism of ENMs passing through the Casparian strip and entering the xylem is yet to be studied in-depth, but the root apex or meristematic zone is a possible access (Fellows et al. 2003), where the Casparian strip is not fully developed. Once reaching the vascular system, an ENM could be translocated to the aerial parts of the plant along with the water transpiration and nutrient flow in transmission. Figure 5.4 illustrates the proposed uptake and translocation pathways, as well as biotransformation of ENMs in plants (Gardea-Torresdey et al. 2014).

The uptake and translocation of ENMs in plants are not only related to the particle composition, size, shape, surface properties, etc., but also to the type of plant species. For example, Zhu et al. (2012) showed that positively charged Au NPs were most readily taken up by plant roots, while negatively charged Au NPs were most efficiently translocated into stems and leaves from the roots. Radish and ryegrass roots generally accumulated higher amounts of the Au NPs than rice and pumpkin roots. Each of the Au NPs used in this study were found to accumulate to statistically significant extents in rice shoots, while none of them accumulated in the shoots of radishes and pumpkins. Similarly, the tissue level uptake and spatial distribution NPs in rice roots and shoots were affected by the surface charges of Au NPs (Koelmel et al. 2013). Au concentration in rice roots followed

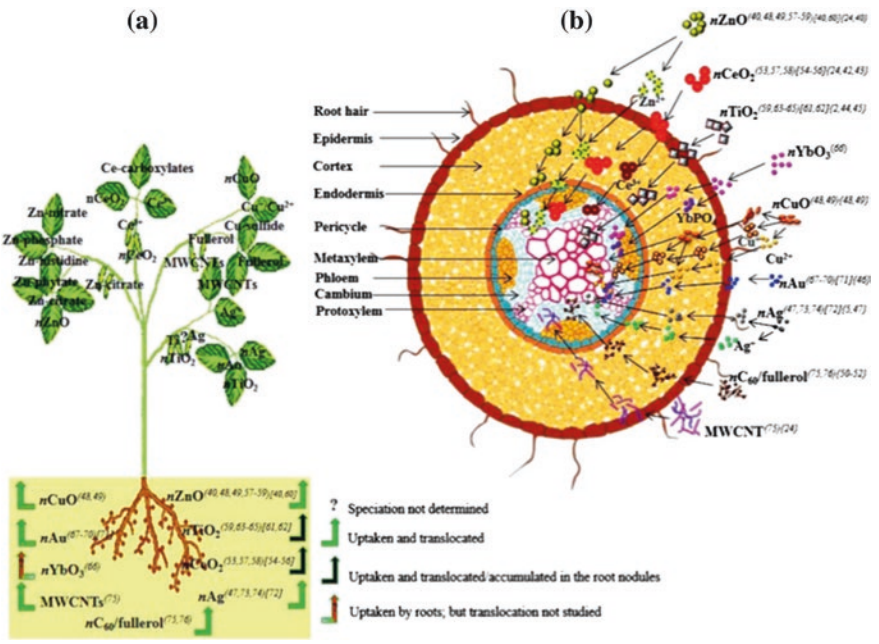

Fig. 5.4 Uptake, translocation, and biotransfomation of ENMS in a plant system. Images are adapted with permission from Gardea-Torresdey et al. (2014), Environ. Sci. Technol., copyright 2014 American Chemical Society

the order of AuNP(+) > AuNP(0) > AuNP(−) but with the reversed order for shoots, indicating preferential translocation of negatively charged Au NPs. Glenn et al. (2012) studied the uptake of Au NPs in three kinds of aquatic plants and found the absorption was size and species dependent. TEM and EDS indicated that 4- and 18-nm Au NPs were absorbed by *Azolla caroliniana*, whereas only 4-nm AuNPs were absorbed by *Myriophyllum simulans*. *Egeria densa* did not absorb Au NPs of either size. They thought the absorption of Au NPs by plants may relate to the salinity tolerance of each species. Ma et al. (2013b) also reported the uptake and accumulation of nanoscale zero-valent iron (nZVI) were plant species dependent, with the observed internalization of nZVI by poplar root cells while not by cattail (*Typha latifolia*) root cells. Upward transport from roots to shoots was not observed for both plant species. Schwabe et al. (2013) found that gum arabic (GA)- and fulvic acid (FA) -containing CeO_2 NPs could be translocated into pumpkin shoots while not into wheat plants. The presence of organic acids affected the amount of CeO_2 associated with roots.

5.1.4 Transformation of ENMs in Plants

ENMs are highly dynamic and reactive with comparison to their bulk counterparts due to their unique physicochemical properties. When in environmental and biological systems, ENMs will inevitably interact with the natural and biological components and undergo physicochemical changes, e.g., incidental coating by natural organic matters and biomolecules, dissolution, or redox reactions (Lowry et al. 2012). Typically, metal-based nanoparticles such as CuO, ZnO, and Ag may dissolve and release metal ions and chemically transform by reacting with inorganic (e.g., sulfide and phosphate) or organic substances which widely exist in the environment and living organisms (Dimkpa et al. 2012, 2013; Levard et al. 2012). ENMs may also physically interact with inorganic ions, biomolecules and natural organic matters, resulting in or decreasing the aggregation and alteration of surface chemistry properties (Nel et al. 2009; Quik et al. 2010; Zhang et al. 2009). Consequently, the behavior, fate, and toxicity of NMs will be undoubtedly altered or even determined by those transformation processes, rather than only by the "as manufactured" nanoparticles. Therefore, mechanisms and extents of these transformations must be understood for correctly understanding and forecasting the environmental and human health risks posed by these ENMs. However, to date, most of the global nanotoxicity researches have been focusing on the fate, distribution, and toxicity of pristine nanomaterials. Knowledge on the transformation type, rate, and extent of ENMs in the environmental and biological systems, and also the effects of transformation on their behavior and toxicity still remain largely unknown.

Phytotoxicity studies of ENMs have been carried out for nearly one decade but researches on biotransformation of NMs in plants remain untouched until recently. Lopez-Moreno et al. (2010a) reported that ZnO NPs transformed to Zn^{2+} as Zn

nitrite or Zn acetate in germinated soybean roots, while CeO_2 NPs remain unaltered (Lopez-Moreno et al. 2010a). However, several studies show contrary results that CeO_2 NPs can be transformed in plants (Hernandez-Viezcas et al. 2013; Zhang et al. 2012b, 2013). In plant system, ENMs may undergo different kinds of transformation with the assistance of plant components. Plant root can secrete large amount of exudates including inorganic ions, small molecular organic substances (e.g., phenols, aldehydes, amino acids, and organic acids) and high molecular pectin (e.g., polysaccharide and fatty acids), forming a microenvironment around the root called "rhizosphere" (Bais et al. 2006). It has been well known that root exudates in rhizosphere play a critical role in determining the behavior and toxicity of heavy metals. For example, organic acids and pectin in root exudates may form stable chelates with the heavy metals such as Pb^{2+}, Cu^{2+}, and Cd^{2+}, etc., limiting their uptake in roots (Morel et al. 1986). Likewise, ENMs may also undergo such kind of physicochemical transformation by interacting with root exudates since ENMs will directly contact with plant root in most cases. These transformations will affect the final fate and toxicity of ENMs in plants.

Most of the ENMs are easily adsorbed and aggregated on the root surface (Ma et al. 2013b; Zhang et al. 2011, 2012b). This physical transformation will limit the uptake of NPs in roots and its subsequent translocation. However, on the other hand, the adsorption of NPs onto root surface lead to a direct contact of NPs with root exudates and increase the possibility of NP transformation. For metal-based NMs, dissolution is the most common transformation process affecting their behavior and fate in plants. For example, biotransformation of ZnO nanoparticles has been studied by many recent studies (Dimkpa et al. 2013; Du et al. 2011; Hernandez-Viezcas et al. 2013; Priester et al. 2012; Wang et al. 2013a). All of these studies showed that no pristine ZnO are internalized in plants evidenced by synchrotron-based techniques (XANES), but mostly present as transformed Zn (II) species such as Zn citrate in soybean plants (Hernandez-Viezcas et al. 2013), Zn phosphate in sand cultured wheat (Dimkpa et al. 2012) and Zn citrate, histidine and phytate in soil cultivated cowpea plants (Wang et al. 2013a). Evidently, the toxic effects will be at least partially induced by the released Zn^{2+} cations. Organic acids excreted by plant roots play a critical role in the biotransformation of ENMs in plants by promoting the dissolution. We performed in-depth studies on the biotransformation of rare earth oxide (REO) NMs in plants and highlighted the critical role of root exudates in the transformation processes (Ma et al. 2011; Zhang et al. 2012a, b, 2013). A large amount of needle-like $LaPO_4$ clusters were found in intercellular regions (Fig. 5.5) as well as in vacuole and cytoplasm of germinated cucumber root with La_2O_3 NP treatment for 5 days, indicating a significant biotransformation of NPs in plants (Ma et al. 2011). A followed in vitro experiment suggests that root excreted organic acids play a critical role in the transformation process. Organic acids promoted the release of La^{3+} by forming the La carboxylate complexes, and then precipitated by phosphates which are widely existed in plants. Zhang et al. (2012a) reported that Yb_2O_3 NM treatment for 14 days reduced the biomass production of cucumber plant at concentration even as low as 0.32 mg/L. The authors also found that the Yb^{3+} concentrations

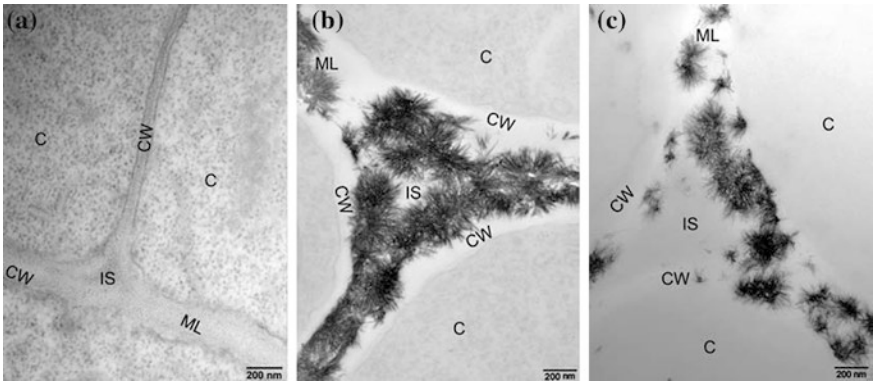

Fig. 5.5 TEM images of the cross section of cucumber root cells under control (**a**), 2,000 mg/L La_2O_3 NPs (**b**), and 200 mg/L $LaCl_3{\cdot}6H_2O$ (**c**) treatments for 5 days after germination. Cells wall (*CW*), cytoplasm (*C*), middle lamella (*ML*), and intercellular space (*IS*)

in the rhizosphere solution are much higher than that in the exposure solution. Organic acids in the rhizosphere greatly promoted the dissolution of Yb_2O_3. Nanoparticulate Yb_2O_3 are highly reactive and much easier to release Yb^{3+} ions than the bulk counterparts, resulting in the higher toxicity. It is worth noticing that ENMs cannot only transform outside the roots, but may also firstly enter into the roots and followed by transforming in plants. In cucumber roots with Yb^{3+} treatment, flocculent $YbPO_4$ was only found in intercellular regions; however, in that with Yb_2O_3 treatment, $YbPO_4$ was also found in vacuole and cytoplasm, indicating that Yb_2O_3 NP probably cross the cell wall, enter into the cytoplasm and vacuole and transformed to $YbPO_4$.

Reduction and oxidation are important reactions commonly occurred in soil–plant systems. Many ENMs containing metal elements with changeable valence states can undergo reduction, oxidation, and subsequent transformation by interacting with biogenic redox agents in plants (Wang et al. 2012b; Yin et al. 2011; Zhang et al. 2012b). Comprehensive studies on the environmental transformation of Ag.

NPs have been carried out and summarized by a recent review article (Levard et al. 2012). However, only one study was performed on the transformation of Ag NPs in plant (Yin et al. 2011). Metallic Ag NPs were oxidized as Ag_2S or Ag_2O in *Lolium multiflorum* roots. Wang et al. (2012b) found that CuO NPs could translocate from shoot back to root and partially reduced and transformed to Cu_2S and Cu_2O during the translocation (Wang et al. 2012b). Similar transformation of CuO NPs was also found in sand-grown wheat (Dimkpa et al. 2012).

CeO_2 NPs is among the most studied nanoparticles on their transformation in plants. CeO_2 NPs had been considered highly stable in environmental and biological surroundings and used as model nanoparticles for comparison with other unstable NPs (e.g., ZnO, Ag, etc.) which can be easily dissolved (Gaiser et al. 2009; Xia et al. 2008). However, Zhang et al. (2012b) found that CeO_2 NPs is not that stable and can be reduced and transformed to Ce(III) species (Zhang et al. 2012b). In the cucumber

roots with CeO_2 NPs treatment for 21 days, large amount of needle-like clusters were observed in intercellular regions and epidermis by TEM. Combined EDS analyses suggest that these clusters contained Ce and P with an atom ratio of about 1:1, indicating that these clusters may be $CePO_4$. This was further evidenced by STXM and XANES analyses, which provided a 2-D distribution and speciation of these clusters (Fig. 5.6). Bulk XANES studies suggested that Ce mostly presented as $CePO_4$ and CeO_2 in roots, but as Ce carboxylates and CeO_2 in stems and leaves (Fig. 5.7). Combining these results and further simulation study, the authors elaborated the transformation and translocation mechanism of CeO_2 NPs in cucumber plants. CeO_2 released Ce^{3+} with the assistance of the reducing agents and organic acids in the root exudates and subsequently transformed to $CePO_4$ and Ce carboxylates. The released Ce^{3+} ions were partially immobilized by the phosphates which are abundant in nutrient solution and plant tissues. The rest Ce^{3+} translocated from the roots to shoots or immobilized by carboxyl compounds in xylem during the translocation process. This study greatly favors us the understanding of the behavior of ENMs in plants. In another report by the same research group, biotransformation of CeO_2 NPs was also found in roots of *Lactuca* plants (Zhang et al. 2013). By interacting with root

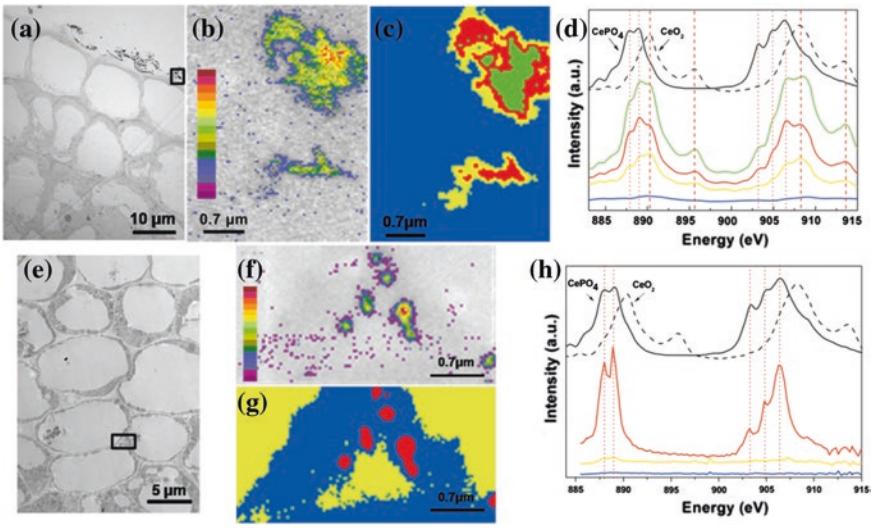

Fig. 5.6 **a** and **e** TEM images of root cells; **b** and **f** Ce maps of *rectangle area* in panels **a** and **e** obtained by a ratio of 886 and 888 eV images. *Color bar* values are estimated from Ce absorption coefficients and X-ray absorption measurements (in g/cm^2). The calculated surface densities are respectively between 1.1×10^{-5} to 6.4×10^{-5} and 2.4×10^{-6} to 2.8×10^{-5} g/cm^2; **c** and **g** *color-coded maps* of Ce components in panels **b** and **f** derived from an STXM Ce M edge stack analysis. The order of Ce contents is as follows: *green* > *red* > *yellow*; *blue color* represents the non-Ce regions; Panels **d** and **h** are, respectively, the XAFS spectra extracted from the image sequences of panels **c** and **g**. The *black line spectra* above belong to the standard compounds and the *colored spectra* below belong to the root samples. The *vertical red dotted lines* indicate the characteristic peaks of $CePO_4$ and the *dash lines* indicate the characteristic peaks of CeO_2 NPs

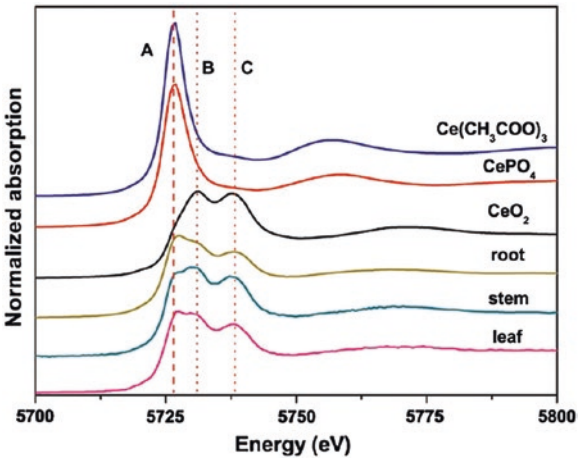

Fig. 5.7 XANES Ce LIII-edge spectra (5,723 eV) of root, stem, and leaf of cucumber plants treated with 2,000 mg/L CeO_2 NPs for 21 days. *Vertical dash line* and *dotted line* marked the feature of Ce(III) and Ce(IV) compounds, respectively

excreted organic acids, CeO_2 NPs released a small amount of Ce^{3+} (less than 6.2 % of total Ce in roots) in the roots of *Lactuca* plants. Interestingly, *Lactuca* plants are highly sensitive to the Ce^{3+}, resulting in the species-specific toxicity to CeO_2 exposure. Additionally, CeO_2 NPs with different sizes transformed to Ce carboxylates to different extents in the roots and induced different toxicity. Hence we can see that determining the transformation of ENMs is of critical importance when assessing their toxicity.

As for other kinds of nanoparticles (e.g., carbon-based nanomaterials and polymer nanomaterials), it is difficult to determine their transformation in plants due to high background of plant matrix and the lack of efficient detection methods. Despite there is no report on the biotransformation of these NMs in plants, the potential transformation cannot be neglected. Some in vitro studies have shown the possibility of transformation of carbon-based nanoparticles. For example, carbon nanotube can degrade with the existence of natural horseradish peroxide (Allen et al. 2008). Graphene oxide can be reduced via bacteria respiration (Salas et al. 2010).

5.2 Conclusions and Perspectives

Higher plants are susceptible to ENMs contamination through soil–plant system from incidental discharge or intended application of nanotechnology in agriculture and soil remediation. Phytotoxicity, accumulation, and potential biomagnification of ENMs through food chain have aroused great concerns not only on the environmental system but also on the human health. Although many efforts have been made to explore the phytotoxicity of ENMs, understanding on the toxicity mechanism and correlation with NP physicochemical properties is still limited.

Important questions that should be focused in the future research are noted as follows: (i) Comprehensive details about the physicochemical properties of experimentally used ENMs should be fully addressed. Environmental behavior and toxicity of ENMs are essentially affected by their physicochemical properties such as size, morphology, crystal structure, surface charge, etc. Results can be different when performed in different laboratories even for the same ENMs. Therefore, comprehensive characterization of ENMs before toxicity test is prerequisite for understanding the behavior and toxicity of ENMs in plants. (ii) More researches on the biotransformation of ENMs are needed. Nanomaterials cannot completely remain their original chemical forms and most of them undergo transformation at certain extents in plants. Complex environmental media and plant components will undoubtedly modify the physicochemical properties of ENMs, resulting in the transformation. Consequently, ENMs may accumulate in plant either as pristine or transformed forms. Data regarding the behavior and toxicity of pristine ENMs are not informative. To understand the behavior and toxicity mechanisms, whether the toxicity of ENMs that has been documented by many studies is induced by their pristine NP or transformed species need to be fully addressed. (iii) More realistic and holistic investigations are needed in future studies. Most of the current studies on ENM–plant interactions still performed by simple application mode especially the hydroponic cultivation. In addition, these studies mostly focused on the short-term behavior and effects of ENMs. It is undeniable that short-term studies provide a suitable way to exploring the mechanism of ENM behavior and toxicity in plants. However, long-term effect and persistence of ENMs in plants grown in natural habitat should be assessed for understanding the plant response to chronic exposure and life cycle of ENMs.

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Chapter 6
Toxicity of Nanomaterials to Plants

Kai-En Li, Zhen-Yi Chang, Cong-Xiang Shen and Nan Yao

Abstract Nanoparticles have many potential applications, especially in biomedical engineering and agriculture, but the toxicity of nanoparticles to plants has received little attention. Previously, we described an increase in the levels of reactive oxygen species (ROS) in rice (*Oryza sativa*) and *Arabidopsis thaliana* cells after nanoparticle treatments. We found that ROS resulted in programmed cell death and that the nanoparticles caused a dosage-dependent increase in cell death. Since then, accumulating data have indicated that nanomaterials cause toxicity in diverse organisms. Data from our lab and others indicate that we should critically examine the risks of nanoparticles, so that we can safely take advantage of the tremendous potential benefits of this new technology.

Keywords Nanoparticles · Nanomaterials · Plant toxicity · Reactive oxygen species

6.1 Introduction

With recent increases in nanomaterial production and usage in varied applications such as DNA delivery, medicine, and imaging, comes increased opportunities for organisms to be exposed to nanomaterials. Due to their small size and high surface reactivity, nanomaterials can potentially enter into the cell and interact with intracellular structures, which may produce toxicity by diverse mechanisms. Nanoparticles can inhibit the seed germination, reduce seedling, shoot and root growth, delay flowering, and decrease yield (El-Temsah and Joner 2012; Lee et al. 2008; Lin et al. 2009b). Additionally, nanomaterials can cause chromatin

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condensation, arrest mitosis, disturb metaphase, break cell walls, and specifically inhibit gene expression (Lin et al. 2009a, b; Kaveh et al. 2013; Kumari et al. 2009). This chapter will review current advances in the study of phytotoxicity of different nanomaterials and speculate on the mechanism of phytotoxicity.

6.2 Current Advances in Phytotoxicity of Nanoparticles

The phytotoxicity of nanomaterials, including carbon-based and metal-based types, is an emerging field and most studies have examined germination, cell cultures, and genetic effects. With the aid of detection techniques such as ion-coupled plasma-mass spectroscopy (ICE-MS), photothermal, and photoacoustic analysis (Khodakovskaya et al. 2011), and Raman spectroscopy, researchers have revealed the phytotoxicity of nanomaterials or nanoparticles.

6.2.1 Toxicity of Nanoparticles

Nanomaterials can be classified as carbon-based or metal-based (Maynard et al. 2011). The chemical characteristics and particle sizes differ between these two types of nanomaterials and thus, they have different negative and positive effects on plants (Table 6.1).

6.2.1.1 Carbon-Based Nanomaterials

Carbon-based nanomaterials include fullerene and carbon nanotubes, which can be single-walled carbon nanotubes and multi-walled carbon nanotubes. According to recent reports, carbon nanotubes and fullerene can cause damage to plants (Begum and Fugetsu 2012; Chen et al. 2010; Liu et al. 2010; Shen et al. 2010). Blossoming of rice plants incubated with C_{70} fullerene was delayed by at least 1 month and their seed-setting rate was reduced by 4.6 % compared to the controls (Lin et al. 2009b). Also, water-soluble fullerene inhibits plant growth and causes shortening of seedling roots and loss of gravitropism (Liu et al. 2010). These adverse effects may be caused by auxin disruption, abnormal cell division, and microtubule disorganization. Shen et al. (2010) found that certain amounts of single-walled carbon nanotubes can induce the production of reactive oxygen species (ROS), which eventually leads to programmed cell death, in *Arabidopsis* leaves, and protoplasts. In red spinach (*Amaranthus tricolor* L), phytotoxicity of multi-walled carbon nanotubes causes growth inhibition and cell death; multi-walled carbon nanotubes also cause ROS production and hypersensitive response-type necrotic lesions of leaf cells and tissues (Begum and Fugetsu 2012). Although plant cells and mammalian cells have different structures, such as the thick and rigid plant cell wall, chloroplasts and large

Table 6.1 The effect of nanomaterials on plants

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
SWCNT (FITC)		Tabacco BY-2 cells		Standard media	No apparent cell death	Liu et al. (2009)
SWCNT	1.19	Rice	400	1/2 MS	Delayed flowering, decreased yield	Lin et al. (2009b)
	1–2	Rice protoplasts	25	W5 media	Decreased viability	Shen et al. (2010)
			250	Injection leaf	Induced chromatin condensation	Shen et al. (2010)
	1–2	Arabidopsis	25	W5 media	Decreased viability	Shen et al. (2010)
		Protoplasts	250	Injection leaf	Induced chromatin condensation	Shen et al. (2010)
MWCNT		Zucchini	1,000	25 % Hoagland solution	Reduced biomass (38 %)	Stampoulis et al. (2009)
		Lettuce	2,000	Aqueous suspension	Reduced root length	Lin and Xing (2007)
	10–30	Rice	20, 40, 80	MS medium	Chromatin condensed inside the cytoplasm and cell death, plasma membrane detachment from cell wall, cell shrinkage	Tan et al. (2009)
	10–35	Tomato	50	MS medium	Upregulation of the stress-related genes	Khodakovskaya et al. (2011)
	11	Red spinach	125, 250, 500, 1000	Modified Hoagland medium	Exhibited growth inhibition, cell death	Begum and Fugetsu (2012)
	20	Tobacco cells	0.1, 5, 100, 500	MS medium	Induced 55–64 % increase of tobacco cell growth over control	Khodakovskaya et al. (2011)
Fullerene C_{60}		Arabidopsis		MS medium	Stress response of gene expression	Landa et al. (2012)
$C_{60}(OH)_{20}$	1.12–1.74 15.69–24.36	*Allium cepa* cell	10 110	Aqueous suspension	Plant cell damage	Chen et al. (2010)
$C_{70}(C(COOH)_2)_4$		Arabidopsis	100	1/2 MS agar	Retarded roots with shortened length and loss of root gravitropism	Liu et al. (2010)
Ag	<100	*Cucurbita pepo*	0, 100, 500	25 % Hoagland's solution	Reduced biomass and transpiration by 66–84 % when compared with bulk Ag	Musante and White (2012)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
Ag	<100	Onion	100	Aqueous suspension	Decreased mitosis; disturbed metaphase; sticky chromosome; cell wall broken	Kumari et al. (2009)
Ag (PVP)	<100	Castor	100, 200, 500, 1000, 2000	Aqueous suspension	No effect on seed germination, root growth, shoot growth	Yasur and Rani (2013)
Ag (PVP-coated)	20	Arabidopsis	1, 2.5, 5, 10, 20	1/2 MS	Biomass declined, upregulated genes associated with the response to metal and oxidative stress, downregulated genes associated with the response to pathogen and hormonal stimuli	Kaveh et al. (2013)
Ag (citrate-coated)	11.0 ± 0.7	Maize	73.4	Aqueous suspension	Inhibition of germination	Pokhrel and Dubey (2013)
		Cabbage	73.4	Aqueous suspension	Inhibition of early development	Pokhrel and Dubey (2013)
	20, 40, 80	Arabidopsis	66.84–534.72 $\times 10^{-3}$	1/4-strength Hoagland media	Less than 80 nm, are phytotoxic to root	Geisler-Lee et al. (2013)
Ag (carbon-coated)	25	Arabidopsis	0.01–100	1/4 strength Hoagland solution	Stimulated root elongation, fresh weight, and evapotranspiration of both plants at a narrow range of sublethal concentrations	Wang et al. (2013)
		Poplar	0.01–100	1/4 strength Hoagland solution	Stimulated root elongation, fresh weight, and evapotranspiration of both plants at a narrow range of sublethal concentrations	Wang et al. (2013)
Ag (PEG-coated)	5, 10	Arabidopsis	0.01–1	1/4 strength Hoagland solution	Stimulated root elongation, fresh weight, and evapotranspiration of both plants at a narrow range of sublethal concentrations	Wang et al. (2013)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
		Poplar	0.01–1	1/4 strength Hoagland solution	Stimulated root elongation, fresh weight, and evapotranspiration of both plants at a narrow range of sublethal concentrations	
Cu		Mungbean	<200	Agar culture media	Reduced seedling growth	Lee et al. (2008)
		Mungbean	800	Agar culture media	Reduced shoot growth	Lee et al. (2008)
		Wheat	<200	Agar culture media	Reduced root and seedling growth	Lee et al. (2008)
	50	Zucchini	1,000	1/4 Hoagland solution	Reduced biomass (90 %)	Stampoulis et al. (2009)
	50	Zucchini	1,000	Aqueous suspension	Reduced root growth	Stampoulis et al. (2009)
	<50	*Cucurbita pepo*	100, 500	1/4 Hoagland solution	Reduced growth and transpiration by 60–70 %	Musante and White (2012)
CuO	<100	Radish	10, 100, 500, 1000	NP suspension	Oxidative damage to DNA, inhibited root growth	Atha et al. (2012)
		Perennial Ryegrass	10, 100, 500, 1,000	NP suspension	Oxidative damage to DNA, inhibited root development	Atha et al. (2012)
Si	14	Arabidopsis	250, 1000	Hoagland's No. 2 Basalsalt mixture	No observed effect	Slomberg and Schoenfisch (2012)
	50	Arabidopsis	250, 1000	Hoagland's No. 2 Basal salt mixture	Reduced rosette diameter, biomass, and stem length	Slomberg and Schoenfisch (2012)
	200	Arabidopsis	250, 1000	Hoagland's No. 2 Basal salt mixture	Reduced rosette diameter, biomass, and stem length	Slomberg and Schoenfisch (2012)
	10	Zucchini	1,000	Aqueous suspension	Completely in inhibited germination	Stampoulis et al. (2009)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
SiO_2	42.8 ± 3.9	Arabidopsis	400, 2000, 4000	1/2 MS	Increased root length at 400 mg/L, but reduced root length at 2,000 and 4,000 mg/L	Lee et al. (2010)
Fe_3O_4	<50	Arabidopsis	400, 2000, 4000	1/2 MS	Reduced root length	Lee et al. (2010)
Fe_2O_3	10.2 ± 2.6	Mycorrhizal Clover	0.032, 0.32, 3.2 mg/kg	Sand culture (with Arbuscular mycorrhizal fungi)	Reduced biomass	Feng et al. (2013)
					Reduced he biomass	Feng et al. (2013)
Au(+)	2	Rice	1.6 for 5 days	Water suspension	None at this level of AuNP	Koelmel et al. (2013)
			0.14 for 3 months	Major nutrients aqueous suspension	None at this level of AuNP	Koelmel et al. (2013)
Au(–)	3	Rice	1.6 for 5 days	Water suspension	None at this level of AuNP	Koelmel et al. (2013)
			0.14 for 3 months	Major nutrients aqueous suspension	None at this level of AuNP	Koelmel et al. (2013)
Au(0)	4	Rice	1.6 for 5 days	Water suspension	None at this level of AuNP	Koelmel et al. (2013)
			0.14 for 3 months	Major nutrients aqueous suspension	None at this level of AuNP	Koelmel et al. (2013)
Au(–)	3.5	Tobacco	48	1/2 Hoagland's solution	Necrotic lesions observed by 14 days	Sabo-Attwood et al. (2012)
	18	Tobacco	76	1/2 Hoagland's solution	None outward signs of stress	Sabo-Attwood et al. (2012)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
NiO	23.34	Tomato	25, 50, 100, 250, 500, 1000, 1500, 2000	Aqueous suspension	Reduced root length and catalase (CAT), glutathione (GSH), superoxide dismutase (SOD) and lipid peroxidation (LPO) exhibit higher activities	(Faisal et al. 2013)
Pd entrapped in $Al(OH)_3$ matrix		Lettuce	0.013–0.06 %(w/w) $Al(OH)_3$ matrix	Aqueous suspension	No effect on germination	Shah and Belozerova (2009)
Al		Ryegrass	2,000	Aqueous suspension	Decreased root length	Lin and Xing (2007)
		Corn, lettuce	2,000	Aqueous suspension	Reduced germination and root length	Lin and Xing (2007)
Zn		Radish, rape, ryegrass, lettuce, corn, cucumber	2,000	Aqueous suspension	Highly reduced root growth	Lin and Xing (2007)
ZnO	9–37 (Mean: 19 ± 7)	Ryegrass	1,000	Hoagland solution	Reduced biomass, shrank root tips, epidermis and root cap broken, highly vacuolated and collapsed cortical cells	Lin and Xing (2008)
		Corn	2,000	Aqueous suspension	Reduced germination	Lin and Xing (2007)
		Radish, rape, ryegrass, lettuce, corn, cucumber	2,000	Aqueous suspension	Highly reduced root growth	Lin and Xing (2007)
	5	Zucchini	1,000	25 % Hoagland solution	Reduced biomass (78–90 %)	Stampoulis et al. (2009)
	8	Soybean	2000, 4000	Aqueous suspension	Decreased root growth	Lopez-Moreno et al. (2010)
	11.0 ± 0.7	Maize	0.01–1,000	Aqueous suspension	Did not inhibit seed germination	Pokhrel and Dubey (2013)
		Cabbage	0.01–1,000	Aqueous suspension	Dose-dependent inhibition of germination	Pokhrel and Dubey (2013)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
	<100	Arabidopsis	100	MS media	Stress response of gene	Landa et al. (2012)
	44.4 ± 6.7	Arabidopsis	400, 2000, 4000	1/2 MS	Heavily inhibited seed germination, reduced root length and leaf number	Lee et al. (2010)
	8	Soybean	500, 1000, 2000, 4000	Hoagland's solution	No effect on germination, increased root length at 500 mg/L (30 % over control) and decreased root length at 4,000 mg/L (40 % shorter than control)	Lopez-Moreno et al. (2010)
	44.46 ± 4.84	*Fagopyrum* Esculentum	1, 5, 100, 1000, and 2000	1/2 strength Hoaland's solution	Reduced biomass	Fan et al. (2014)
CeO_2	7	Alfalfa	1000, 2000		Slightly reduced shoot growth	Lopez-Moreno et al. (2010)
		Tomato	2,000	Aqueous suspension	Reduced shoot growth (30 %)	Lopez-Moreno et al. (2010)
		Cucumber	2,000	Aqueous suspension	Reduced shoot growth (20 %)	Lopez-Moreno et al. (2010)
		Maize	500, 1000, 2000	Aqueous suspension	Reduced shoot growth (30 %)	Lopez-Moreno et al. (2010)
		Alfalfa	500	Aqueous suspension	Significantly reduced biomass	Lopez-Moreno et al. (2010)
		Maize	500–2,000	Aqueous suspension	Reduced germination	Lopez-Moreno et al. 2010)
		Maize	4,000	Aqueous suspension	Reduced root growth	Lopez-Moreno et al. (2010)
		Tomato, cucumber	2,000	Aqueous suspension	Reduced germination	Lopez-Moreno et al. (2010)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
		Tomato	1,000–2,000	Aqueous suspension	Reduced root growth	Lopez-Moreno et al. (2010)
		Alfalfa	200–4,000	Aqueous suspension	Reduced root growth	Lopez-Moreno et al. (2010)
		Soybean	2,000	Aqueous suspension	Reduced germination	Lopez-Moreno et al. (2010)
		Alfalfa	4,000	NP suspensions	Reduced root growth	Lopez-Moreno et al. (2010)
		Corn	4,000	NP suspensions	Germination significantly reduced, root growth was prompted	Lopez-Moreno et al. (2010)
		Cucumber	4,000	NP suspensions	Germination significantly reduced 30 % at 2,000 mg/L, root growth was prompted	Lopez-Moreno et al. (2010)
		Tomato	4,000	NP suspensions	Germination significantly reduced 20 % at 2,000 mg/l, root growth was prompted	Lopez-Moreno et al. (2010)
		Cilantro	62.5–500 mg/kg	Organic potting soil	Large roots, longer shots	Morales et al. (2013)
		Arabidopsis	250	1/2 MS medium	Increased plant biomass	Huang et al. (2012)
			500–2,000	1/2 MS medium	Decreased plant growth by up to 85 %	Huang et al. (2012)
			1,000–2,000	1/2 MS medium	Reduced chlorophyll production by 60 and 85 %	Huang et al. (2012)
CeO_2 (uncoated)	17–100	Wheat	100	10 % Hoagland	No phenotypic response	Schwabe et al. (2013)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
	17–100	Pumpkin	100	20 % Hoagland medium	No phenotypic response	Schwabe et al. (2013)
Al_2O_3	13	Maize, cucumber, carrots, cabbage	2,000	Aqueous suspension	Reduced root growth	yang and Watts (2005)
		Corn	2,000	Aqueous suspension	Reduced root length	Lin and Xing (2007)
	~150	Arabidopsis	400, 2000, 4000	1/2 MS	Positive influence on root elongation	Lee et al. (2010)
	<50	Tobacco BY-2 cells	0.01-0.1	Liquid MS medium	Diminished cell viability, damaged plasma membrane, reduction in the activities of both dehydrogenase and oxidoreductase	Poborilova et al. (2013)
	8	Tomato	104, 315, 1750	Suspension (35:65CNT:poly-3-aminobenzoic acid)	Root reduction	Canas et al. (2008)
TiO_2	30	Maize	30, 1000	10 % strength Hoagland solution	Inhibited leaf growth, and transpiration	Asli and Neumann (2009)
	<150	Arabidopsis	100	MS medium	Little effect on gene expression	Landa et al. (2012)
		Arabidopsis	1×10^6	Soil	Increased green content	Lenaghan et al. (2013)
		Algae	1×10^6	Soil	Decreased green content	Lenaghan et al. (2013)
	35	Garden peas seed	100, 250, 500, 750, 1000	Suspension	No effect on the germination of peas grown, and the gross root structure	Fan et al. (2014)
	35	Garden peas	250, 500, 750	FP medium	Decreased the number of secondary lateral roots	Fan et al. (2014)
	5	Arabidopsis	0.25 %	Sprayed with suspension	Increased harvesting light energy	Ze et al. (2011)

(continued)

Table 6.1 (continued)

Nanomaterial	Particle size (nm)	Plant	Concentration (mg/L)	Growth condition	Effects	References
TiO_2 (colloidal)	5	Spinach	0.25 %	Aqueous suspension	Promoted antioxidant stress of spinach chloroplasts	Lei et al. (2008)
TiO_2 (Alizarin red S)	2.8 ± 1.4	Arabidopsis	20 μM	1/2 MS medium	Caused reorganization and elimination of microtubules	Wang et al. (2011)
TiO_2 (Alizarin red S)	2.8 ± 1.4	Arabidopsis	5 μM	Aqueous solution	Do not impose an excessive oxidative stress on the root tissues	Kurepa et al. (2010)
In_2O_3	20–70	Arabidopsis	25–2,000		No effect on biomass, 15 % on root elongation	Ma et al. (2013)
Quantum Dot	6.3 + 0.7	Arabidopsis	5.8 nM	1/4 strength	The ratio of reduced glutathione levels (GSH)	Navarro et al. (2012)
CdSe				Hoagland's solution	Relative to the oxidized glutathione (GSSG) in plants decreased	
MPA-linked CdSe QDs		Rice		Aqueous solution	Germination was arrested with quantum dots	Nair et al. (2011)
QD-COOH		WTK1 cells	25, 50, 100, 200, 400	Aqueous solution	Caused DNA damage	Hoshino et al. (2004)
QD-NH_2		WTK1 cells	50, 100, 200, 400	Aqueous solution	No significant effect	Hoshino et al. (2004)
QD-OH		WTK1 cells	50, 100, 200, 400	Aqueous solution	No significant effect	Hoshino et al. (2004)

central vacuoles, they show similar responses to fullerene (Chen et al. 2010). The bioaccumulation of dichlorodiphenyldichloroethylene, a persistent and estrogenic pollutant, in some food crops such as zucchini, soybean, and tomato, increased in the presence of C_{60} fullerene (De La Torre-Roche et al. 2012). Additional work investigated and found carbon nanotubes can penetrate the cell membrane of plant protoplasts (Serag et al. 2011a). Moreover, researchers (Avanasi et al. 2014) have measured plant uptake of C_{60} fullerene and found that ^{14}C-labeled C_{60} can be slowly absorbed by plants and will likely persist in soil for a long period.

6.2.1.2 Metal-Based Nanomaterials

Metal-based nanoparticles hold an important place due to their application prospect in the group of nanoparticles, and include metal oxide and metallic nanoparticles. Accumulating data indicate that metal-based nanoparticles usually inhibit development and can cause genetic damage in plants.

Atha et al. (2012) found that CuO nanoparticles can cause oxidative DNA damage in terrestrial plants such as radish, annual ryegrass, and perennial ryegrass. Landa et al. (2012) used microarray analysis to demonstrate that exposure of *Arabidopsis* to TiO_2, ZnO, and fullerene leads to distinct changes in the expression of stress genes. Also, the genes upregulated in response to nanoparticle treatments were primarily associated with the response to metals and oxidative stress, while downregulated genes were mainly involved in cell organization and biogenesis, indicating that phytotoxicity is highly dependent on the type of nanoparticle (Atha et al. 2012; Landa et al. 2012). Another report investigated the effects of cerium oxide (CeO_2) exposure on wheat and pumpkin by using hydroponic plant culture. CeO_2 nanoparticles have only minor effects and no growth reduction or toxic response was observed (Schwabe et al. 2013), but catalase and ascorbate peroxidase activity significantly increased.

Metallic nanoparticles release ionic salts, and the nanoparticles and ionic salts have similar effects; this prevents us from discerning whether metallic nanoparticles exert particle-specific toxicity. Ag nanoparticle toxicity in *Arabidopsis* is size and concentration dependent. Ag nanoparticles can be apoplastically transported in the cell wall and aggregate at plasmodesmata (Geisler-Lee et al. 2013). Research on *Lemna gibba* showed that the intracellular uptake of Ag directly involved Ag nanoparticles, and the induced oxidative stress was highly related to Ag accumulation inside plant cells (Geisler-Lee et al. 2013). Pokhrel and Dubey's observation of a 'tunneling-like effect' upon treatment with ZnO nanoparticles and varied metaxylem frequency with exposure to nanoparticles or free ions suggest that potential risks of metal nanoparticles, including their free ions, may affect the growth and development of agriculturally important plants such as maize and cabbage (Pokhrel and Dubey 2013). Research on Au nanoparticles shows that they are toxic to rice and can accumulate in the above-ground organs of the plant (Koelmel et al. 2013). Although currently we cannot eliminate the toxic response of ionic salts, the crucial effect of free ions released by metal-based nanoparticle should not be ignored.

6.2.1.3 Quantum Dots

Quantum Dots (QDs), which are often described as "artificial atoms," are one of the first nanotechnologies to be widely used in the biological sciences and are anticipated to eventually find applications in a number of commercial consumer and clinical products (Klimov 2007; Valizadeh et al. 2012). QDs exhibit unique luminescence characteristics and electronic properties, such as wide and continuous absorption spectra, narrow emission spectra, and high light stability (Bruchez et al. 1998). QDs demonstrate great potential for labeling cells, tracking particles, and harvesting solar energy. Nonetheless, before we take advantage of this new material, we should evaluate the risk of its toxicity to organisms. Nair et al. (2011) placed rice seeds in CdSe QDs and observed that the QDs inhibited germination. QDs can cause DNA damage and suppress the proliferation of cells in culture (Hoshino et al. 2004). Cell damage and even cell death can be induced by mercapto-undecanoic acid QDs (Shiohara et al. 2004). Although the exposure of single-walled carbon nanotubules to plants induced positive effects, the addition of QDs to the nanotubules dramatically changed the viability of the tomato plants by significantly accelerating leaf senescence and inhibiting root formation (Alimohammadi et al. 2011). Moreover, *Arabidopsis* root exposure to QDs could induce oxidative stress, as revealed by changes in the GSH/GSSG ratio (Navarro et al. 2012). Also, observation of QD transport and fate in soil, plants, and insects indicates that QDs may be transported across the environment (Al-Salim et al. 2011).

Unlike carbon-based nanomaterials, metal-based nanomaterials include many more types, and have more complex physical characteristics, which may lead to more complicated interactions between nanomaterials and plants. Given that metal-based nanomaterials have tremendous applications in industry, we should critically examine the toxicity of nanomaterials toward not only animals, but also plants.

6.2.2 Positive Effects of Nanoparticles

Although the phytotoxicity of nanomaterials in plants has been intensively reported, some studies of nanomaterials indicate that their phytotoxicity has limits, and some nanomaterials also can facilitate the growth and development of plants. For example, carbon nanotubes can enhance water uptake and growth in gram plants (*Cicer arietinum*) (Tripathi et al. 2011), and 500–4,000 mg/L ZnO can promote the root growth of soybean (Oberdorster 2010). A stimulatory effect was observed on root elongation, fresh weight, and evapotranspiration of both *Arabidopsis* and poplar at a narrow range of sublethal concentrations of Ag nanoparticles coated with both carbon and polyethylene glycol (Wang et al. 2013). Also, SiO_2 nanoparticles can increase root length at 400 mg/L (Lee et al. 2010) and CeO_2 nanoparticle suspensions promoted the root growth of corn and cucumber, even though the germination rate declined (Lopez-Moreno et al. 2010). Cilantro grown in organic potting soil had longer shoots and larger roots

with CeO_2 nanoparticles (Morales et al. 2013). *Arabidopsis* grown in MS media containing Al_2O_3 nanoparticles had longer roots (Lee et al. 2010) and TiO_2 nanoparticles can also increase the chlorophyll content of *Arabidopsis* (Lenaghan et al. 2013).

Khodakovskaya et al. (2011) discover that multi-walled carbon nanotubes can upregulate stress-related genes such as pathogen defense genes and water-channel genes. And their further research suggested that these nanotubes can enhance the growth of cultured tobacco cells by the activation of water channels and major gene regulators of cell division and extension, such as *NtPIP1, CycB*, and *NtLRX1* (Khodakovskaya et al. 2012). Researchers suggested the mechanism of the positive effect is the photocatalyzed character of nanomaterials and upregulation of water-channel protein in plants exposed to carbon nanomaterials (Ze et al. 2011). Recently, Giraldo et al. (2014) exploited the interaction between plant organelles and nonbiological nanostructures to augment photosynthesis and biochemical sensing in plants. This plant nanobionics approach can enhance the efficiency of photosynthesis and has the potential to detect real-time nitric oxide in chloroplast and leaves. The increase in absorption spectrum and electron transport rates caused by carbon nanotubes contributed to the enhancement of photosynthesis (Lee et al. 2010). Although several studies support the idea that electron transfer between carbon-based nanomaterials can increase photosynthesis, the specific mechanisms of various nanomaterials need further research (Boghossian et al. 2011; Giraldo et al. 2014; Ham et al. 2010).

6.2.3 Factors Affecting the Toxicity of Nanoparticles

It is difficult to determine the specific mechanism of phytotoxicity, because of uncertainty in the elements contributing to toxicity. Oberdorster (2010) compared nanoparticles with bulk particles, and concluded that twenty-two aspects could alter their biological effects. Several crucial factors affect the phytotoxicity of nanomaterials: the concentration of nanomaterials, the size and specific area of the particles, the physicochemical properties and stability of the particles, the species of plant and their developmental stage, the growth media, and the solution of the nanomaterials, etc.

Toxicity assessment of CeO_2 nanoparticles in cilantro grown in organic soil showed that the activity of catalyze and ascorbate peroxidase increased significantly only at a concentration 125 mg/kg (Morales et al. 2013). Four edible plants, including alfalfa, corn, cucumber, and tomato, show differential responses to CeO_2 nanoparticles (Lopez-Moreno et al. 2010). Treatment with 2,000 mg/L CeO_2 nanoparticles reduced the germination rate of corn, cucumber, and tomato, but did not cause significant reduction for alfalfa. Also, the root and stem growth of these four plants was differentially inhibited by CeO_2 nanoparticles. Although cucumber germination was not strongly affected by CeO_2 nanoparticles, its root and stem growth were significantly inhibited.

In addition, surface modification can change the cellular interactions of nanoparticles and modify their mechanism of toxicity. The toxic effects of CuO nanoparticles were found to be mainly driven by the solubilization of particles into toxic metal ions, while polymer coating of CuO nanoparticles changed the mechanism of nanoparticle toxicity to *L. gibba*, resulting in a more important contribution of ROS formation and decreasing plant growth even at a low concentration (Perreault et al. 2014). Surface modification of QDs changed their physicochemical properties. In addition, the cytotoxicity of QDs depends on their surface molecules. The properties of QDs are not related to those of the QD-core materials but to molecules covering the surface of QDs (Shiohara et al. 2004). According to the research on phytotoxicity of Si nanoparticles, the phytotoxic effect of Si nanoparticles is pH-dependent (Geisler-Lee et al. 2013). Furthermore, leaf necrosis caused by Au nanoparticles depends on particle size; 18 nm Au nanoparticles do not induce necrosis, but 3.5 nm Au nanoparticles do (Sabo-Attwood et al. 2012).

6.3 Mechanism of Phytotoxicity

The phytotoxicity of nanoparticles has been well documented, but their mechanism of phytotoxicity remains unclear. Dietz and Herth (2011) clearly put forward five models for the interaction between nanomaterials and organisms: (1) metal ions released by nanoparticles in solution produce a chemical effect; (2) the hard, spherical particles produce mechanical effects; (3) the nanoparticle surface produces catalytic effects; (4) the nanoparticle surface binds proteins, either by non-covalent or covalent mechanisms or causes oxidative effects; and (5) the nanoparticles change the chemical environment, especially the pH. According to the recent research, the mechanism of phytotoxicity may include elements of the five models mentioned above and other effects. The influence of nanoparticles on microorganisms can also play an important role in the environment.

6.3.1 Uptake of Nanomaterials

Assessing the toxicity and safety of nanoparticles requires an understanding of the uptake of nanoparticles. Most research has focused on determining the nature of the phytotoxicity of nanoparticles, but quantitative methods for measuring nanoparticles in plant tissues have not been established. Therefore, research on the uptake of nanoparticles by plants has not reached a conclusive verdict. According to the limiting pore sizes in cell walls (Carpita et al. 1979), nanoparticles smaller than 5 nm may have the capacity to traverse the intact cell wall efficiently. Carbon-based nanomaterials, such as fullerene C_{60} and fullerol, accumulate in plants, and most metal-based nanoparticles can be absorbed by plants and get accumulated in plant tissues (Rico et al. 2011).

Hitherto, several methods have been used directly to observe nanoparticles in plants, such as optical emission spectroscopy, X-ray absorption spectroscopy, Alizarin red S labeling, X-ray fluorescence, transmission electron microscopy (TEM), and fluorescein isothiocynate (FITC) labeling (Lin et al. 2009b; Lopez-Moreno et al. 2010; Kurepa et al. 2010; Serag et al. 2011a). Protoplast systems can also be useful to detect nanoparticles (Shen et al. 2010). For instance, Serag et al. (2011a) suggested that carbon nanotubes can traverse the plant cell membrane via a direct penetration mechanism, rather than endocytosis. However, other reports support the idea that endocytosis plays an essential role in the uptake of nanoparticles. Liu et al. (2009) pretreated the cells with wortmannin, an inhibitor of plant cell endocytosis, and the cellular fluorescence of single-walled carbon nanotubes stained with FITC decreased significantly, which implies that endocytosis functions as a main pathway for carbon nanotubes to enter the plant cell. Furthermore, end caps or carbon shells at the tips or nanotubes can facilitate the endocytosis of nanomaterials (Shi et al. 2011).

Additionally, using inhibitors such as probenecid (an inhibitor of carrier-mediated transport) and Exo1 (an inhibitor of ADP ribosylation factors) or changing the pH of the media can facilitate or inhibit the translocation and uptake of single-walled carbon nanotubes in *Catharanthus roseus*. Consequently, the trafficking of carbon nanotubes through the subcellular membranes of the plant cell involves a carrier-mediated transport (Serag et al. 2011b). Interestingly, Giraldo et al. (2014) reported that single-walled carbon nanotubes can passively transport and irreversibly localize in the lipid envelope of extracted plant chloroplasts, where they promote photosynthetic activity and enhance maximum electron transport rates (Giraldo et al. 2014). Taken together, the mechanism of the uptake of nanomaterials depends on the specific characteristics of the material; therefore, multiple mechanisms may be involved.

6.3.2 Metal Nanoparticles

Metal-based nanoparticles can release metal ions during exposure. Heavy metal ions, including redox active, (e.g., Cu, Fe) and non-redox active, (e.g., Cd, Ni) types, can induce ROS or perturb the redox balance in cells, thereby contributing to cell damage (Sharma and Dietz 2009). Many metals, such as Ag, Au, Fe, and Co, catalyze chemical reactions, especially reduction–oxidation reactions. Nanoparticles entering into cells can release metal ions that may alter proteins. Mechanical effects depend on the size, instead of the chemical properties of the particle (Dietz and Herth 2011). For example, the high concentration adsorption of hydrophobic nanoparticles onto the plant cell wall and their retention within the cell wall can cause cell damage and nanoparticles may clog pores on the cell wall, which can inhibit water uptake or cause physical damage to the cell wall (Chen et al. 2010). The ability to pass through the cell wall might not be a prerequisite for causing oxidative stress and toxicity. Some researchers suggest that despite

nanomaterials' inability to pass through the cell wall of plants, they can cause oxidative stress, and eventually lead to chromosome condensation (Shen et al. 2010). Similarly, CuO nanoparticles can also cause oxidative damage to plant DNA and can be detected in plant cells (Atha et al. 2012).

Surface effects have engaged a great deal of attention in the field of nanotoxicology. Particles with an oxic surface often form a layer of OH^- groups at the surface; these negatively-charged groups attract positively-charged side groups of proteins (Dietz and Herth 2011). Rice plants hydroponically exposed to positively, neutrally, and negatively-charged Au nanoparticles bioaccumulated Au nanoparticles and the organ level distribution depended on the surface charge of the nanoparticles, with negatively-charged, more toxic Au nanoparticles accumulating the most in above-ground organs (Koelmel et al. 2013). The cytotoxicity of QDs also depends on their surface molecules (Begum and Fugetsu 2012).

Experiments based on cell culture in Murashige and Skoog medium or Hoalgand's aqueous medium, or protoplast systems are not sufficient to provide information on the real toxicity of nanoparticles. The influence of nanoparticles on plants systems is more complicated than expected in soil-grown plants. Nanoparticles can affect microorganisms in the soil and then indirectly affect the growth of plants. For example, two metal nanoparticles affect arbuscular mycorrhizal fungi and therefore substantially alter plant growth (Feng et al. 2013). There are few reports of the interactions among plants, nanoparticles, and microorganisms, and further research can help us to identify actual risks of the utilization of nanoparticles.

6.3.3 Reactive Oxygen Species

Previously, we described an increase in levels of ROS in rice and *Arabidopsis* cells after nanoparticle treatments (Shen et al. 2010). We found that the stress of ROS resulted in programmed cell death and the effect of nanoparticles on cells was dosage-dependent. Similar to many abiotic and biotic stressors, the most common general stress symptom of nanoparticle toxicity appears to be the development of oxidative stress by enhanced production of ROS and peroxidative processes (Oberdorster et al. 2007). Hitherto, ROS is one of the crucial biomarkers of nanoparticle toxicity and can be measured by the direct quantification of ROS or by activity of anti enzymes such as superoxide dismutase (SOD), or catalase (CAT) (Begum and Fugetsu 2012; Faisal et al. 2013; Lin et al. 2012; Oukarroum et al. 2012; Perreault et al. 2014; Shaw and Hossain 2013; Thwala et al. 2013).

The work of Poborilova et al. (2013) in suspension cultures of tobacco BY-2 cells, showed that Al_2O_3 nanoparticles exhibited toxicity that was only connected with the generation of reactive oxygen and nitrogen in both concentration- and time-dependent ways. The protection against carbon nanotube-induced toxicity by the addition of ascorbic acid supports the idea that carbon nanotubes principally promote ROS generation (Begum and Fugetsu 2012). Ag nanoparticles cause

strong decreases in chlorophyll content and viable algal cells, and also increased ROS formation and lipid peroxidation in green algae (Oukarroum et al. 2012). Nano-CuO treatment causes oxidative damages to rice seedlings, as evident from high activity of ROS-scavenging antioxidant enzymes (Shaw and Hossain 2013).

A plausible mechanism of NiO nanoparticle-induced cellular toxicity in tomato root cells was put forward by Faisal et al. (2013). NiO-nanoparticles can traverse the cell wall and enter into the tomato cell and ROS are plausibly produced by surface effect and metal ions. After ROS are produced, they can translocate as a signal to the nucleus and mitochondria. ROS then cause peroxidation of MAPKs or PARP and perturb the balance of antioxidant defenses. These eventually lead to the cell death of tomato cells (Sharma and Dietz 2009). Our observations using cerium chloride detected by TEM also confirm that ROS production induced by nanoparticles on cell wall and plasma membrane directly contribute to downstream cell death (Shen et al. 2010). However, further studies should be done to search for general mechanisms of toxicity of nanoparticle-induced ROS production.

6.4 Summary

The application of nanomaterials in agricultural fields is currently limited. Recent reports (Feng et al. 2013; Landa et al. 2012; Shen et al. 2010) demonstrate the potential of nanomaterial treatments to increase defenses against pathogen attack. Nanomaterials can upregulate the expression of biotic stress genes and directly affect certain pathogens (Elumalai and Vinothkumar 2013). Moreover, some results demonstrate that spinach leaves remained green after treatment with nano TiO_2, even under culture in N-deficient conditions (Yang et al. 2007). Despite increasing excitement based on such examples, we must critically examine the risks of nanoparticles and then take advantage of the tremendous potential benefits of this new technology. For example, reduction of shoot growth is a commonplace phenomenon, even though the aerial organs of the plant do not directly touch the material (Slomberg and Schoenfisch 2012). How can nanomaterials affect the aerial organs of the plant? There are three speculations: first, nanomaterials can directly influence the growth of roots, and then influence the growth of the whole plant, including the shoot or rosette. Second, when the nanomaterials contact roots, an abiotic/biotic stress signal is produced and this signal may alter the aerial organs. Third, nanomaterials can cross the cell wall of root cells and move inside the plant, which may eventually influence the aerial organs. Since the aerial organs of plants provide important food sources for human beings, we need further research to evaluate the toxicity caused by nanomaterials.

So far, although nanotoxicology has attracted more attention and recent research achievements have improved our understanding of the mechanism of phytotoxicity, the following issues still puzzle us: (1) the effect of metal ions released by metal-based nanomaterials cannot be eliminated when we test phytotoxicity,

(2) The mechanism of transportation of nanomaterials through the cell wall remains unclear, and (3) The role of ROS in the interaction between nanomaterials and plants, whether as a signal or a direct cause of damage, also remains unclear.

Finally, the trend of the usage of nanomaterials cannot be halted because of their great potential applications and improvements in efficiency of both protein and DNA delivery to plant cells. However, due to their small size, nanoparticles can enter our life cycle and affect important elements in our environment, such as the atmosphere, aquatic environment, and soil. Plants are widespread and form the foundation of the food chain; therefore, the effects of nanoparticles on plant life cycle should be assessed. Further research on the phytotoxicity of nanomaterials can help us avoid the risk of damaging our environment.

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Chapter 7
Latest Developments of Nanotoxicology in Plants

Stefania Mura, Gianfranco Greppi and Joseph Irudayaraj

Abstract In the last years metal nanoparticles have been largely used for their unique physical, chemical, and biological properties, which differ from those of bulk materials. The wide number of applications has led to a significant diffusion of such particles in the environment and their absorption by plants. The aim of this chapter is to follow the metabolic pathway of nanoparticles (NPs) and nanomaterials inside the plant cells. In particular, the effects of different metal nanomaterials on seed germination, growth, chlorophyll concentration, biomass accumulation, root elongation, variation in the shoot/root ratio, photosynthetic characteristics, and antioxidant responses will be analyzed. Furthermore, the latest studies of phytotoxicity (including production of Reactive Oxygen Species (ROS), biomass reduction, stress levels, mitochondrial dysfunction, membrane damage, and release of toxic ions) will be presented. Along the chapter, the acute toxicity of nanomaterials in plants and the long-term effects to different generations will be investigated. Finally, the concentration of NPs in different parts of the plants and their uptake in plant foliar will be described with the choice of NPs that have less toxic and more useful effects in agriculture.

Keywords Plants · Nanoparticles · Nanomaterials · Phytotoxicity

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7.1 Introduction

Lately a significant progress on nanotechnology and nanoparticle research have been made, and a considerable interest has been directed toward the application of nanotechnology in agriculture, and the possible impact on the environment and food products (Mura et al. 2013). In fact, nanomaterials can be toxic either because of their catalytic properties, due to their large bioactive surface, and for their sizes that allow them to pass through the cell membranes and interact with cellular structures and biomolecules. A possible phytotoxicity can be due to interactions between nanomaterials and components of soil that can modify the uptake of nutrients by plants; furthermore, nanomaterials can be absorbed through roots and shoots by plants and transported and accumulated within plant body where interact with biomolecules and cells (Masarovicova and Kralova 2013). The last studies on phytotoxicity of different nanomaterials (ZnO, CeO_2, TiO_2, NiO, CuO, Ag, Au, SiO_2, nZVI, fullerenes, graphene oxide, carbon nanotubes) will be analyzed in detail in the next sections to understand the last advances in this topic.

7.1.1 Cerium Oxide NPs

Cerium oxide NPs are used as fuel additives, in chemical mechanical planarization, and in cosmetics as sunscreen. Thanks to their stability CeO_2 NPs seems to have a limited dissolution in soil and plant tissues, but it is still important to assess their interaction with plants. In a recent work (Rico et al. 2013), the impact of CeO_2 NPs on antioxidant defense system and oxidative stress of germinating rice seeds was examined. In particular, rice was chosen because is a food largely consumed by the world's population. The seeds were grown for 10 days in suspensions of CeO_2 NPs at different concentrations and an increase of their presence in tissues, proportional to CeO_2 concentration, was noted, without signs of toxicity. The higher concentration in the roots seems due to the direct exposure to the NPs suspension, compared to the shoots. In this case, the presence of NPs did not cause changes in the seedling growth (no signs of toxicity) or in germination rate of rice, and the unique variation observed was in the chlorophyll content without visible signs of phytotoxicity. Further studies were focused on monitoring reactive oxygen species, in fact, in plants, it is important to maintain a balance between production and removal of reactive oxygen species (ROS) because an excess of these species induces lipid peroxidation and oxidative stress. These studies evidenced as low concentrations of CeO_2 NPs led to a decrease of H_2O_2 content in root and shoot, while at high concentrations no significant variation of H_2O_2 was observed but only an alteration of the enzymatic activity, leading to a membrane damage and photosynthetic stress in the shoots. This behavior seems due to the ROS scavenging ability of CeO_2 NPs which increases with the decrease of NPs concentration and size. Finally, none of the concentration was observed to produce significant differences in lipid peroxidation. In this experiment, the seeds were

grown in suspensions but it is important to consider the real situation of nanoparticles released in the environment, where the organic matter, present in soils, can influence the NPs absorption.

This has been evidenced in another study of Schwabe et al. (2013) where pumpkin and wheat, two economically important plants, were exposed to CeO_2 NPs in hydroponic systems, with solutions containing gum arabic (GA) and fulvic acid (FA). The aim of the study was to characterize the effects initiated by plant root growth and natural organic matter on CeO_2 NPs behavior in a controlled hydroponic system. It was demonstrated as NPs translocated only into pumpkin shoots, without any toxic response. The organic matter affected the amount of NPs associated with roots (pure > FA > GA) but did not affect the translocation factor. In fact the addition of natural organic matter (NOM) influenced the amount of CeO_2 associated with the roots, because the adsorption of particles by the roots, stabilized with organic matter, was strongly reduced. FA and GA, therefore, did not only slow down agglomeration between particles, but inhibited the sorption of NPs by roots and, at the same time, reduced the uptake of NPs into pumpkin shoots.

Further investigation on the effect of organic soil, Morales et al. (2013) evidenced as CeO_2 NPs can be accumulated in plants of *Coriandrum sativum* L. without modification. Cilantro plants were germinated and grown in organic soil treated with 0–500 mg/kg CeO_2 NPs for 30 days, and analyzed by biochemical assays and spectroscopic techniques to determine the CeO_2 uptake, variations in macromolecules, and catalase (CAT) and ascorbate peroxidase (APX) activity (antioxidant enzymes that help plants to deal with oxidative stress or ROS). At concentrations of 125 mg/kg of CeO_2 NPs, plants produced longer roots and shoots, had a higher biomass production, and a significant increase of catalase activity in shoots and ascorbate peroxidase in roots. Furthermore CeO_2 NPs downregulated the production of these defensive enzymes, and changed the chemical environment of carbohydrates in shoots, suggesting that these NPs can change the nutritional properties of cilantro. Thus this study demonstrates that CeO_2 NPs have fertilizing effects and help plants to grow better. Although CeO_2 NPs produce stress in cilantro plants, but, at the same time have antioxidant activity. In fact CeO_2 NPs induce conformational changes within the plant (in the components of roots), visible in the spectra by vibrational shifting, but do not induce chemical reactions and substantial changes. From all these studies, it seems that CeO_2 NPs have only positive effects on the development of plants but what is their destiny for the second generation plants? The next work of Wang et al. (2013) will answer to this question.

Tomato plants were treated with low concentrations of CeO_2 NPs (10 mg/L) in their lifecycle and it was investigated if this treatment affected the seed quality and the development of second generation seedlings. The benefits obtained on the first generation, as illustrated in previous works, were lost in seedlings of the second generation. The results indicated that second generation seedlings, grown from seeds of treated plants, were smaller and weaker (smaller biomass, lower water transpiration, higher ROS content), furthermore they developed extensive root hairs compared with the control. This study provides the first evidence of the transgenerational impact of CeO_2 NPs on the development and growth of tomato

plants. These NPs in fact slightly improved the growth of wild plant (first generation seedlings) but, at the same time, weakened the capacity to respond to the fertilization effect of the CeO_2 NPs. The accumulation of CeO_2 NPs in plant seeds and fruit tissues suggests that they have a high impact that can influence subsequent generations. These results (Table 7.1) demonstrate that although the immediate results are positive there is the need to evaluate the long-term, multigenerational impact of NPs on plants.

7.1.2 Silver NPs

Silver is the most produced nanomaterial and is found in different commercial products for its properties of antimicrobial with inhibitory and bactericidal effects. The hazard of these NPs is due to the fact that it can be oxidized in water, can complex with anions, and finally, can be converted in heavy metals. The heavy metals can produce mutagenesis, carcinogenesis, and are toxic and dangerous because can accumulate in the edible parts of vegetables. For this reason, plants have an essential role in the fate and transport of NPs in the environment through their uptake and accumulation. To monitor these modifications Shams et al. (2013) suspended silver nanopowders of 50 nm in water with different concentrations and repeatedly sprayed them in different parts of cucumber plant to monitor their effects. The growth index and the concentration of silver in different parts of the plant were analyzed. Increasing the concentration of NPs, increased the concentration of silver heavy metal and the growth index, and at the same time the plant morphological characteristics were improved. The experiment considered cucumber because it is a famous and old vegetable and is widely cultivated in considerable amount. The cucumber fruit performance were significantly enhanced by increasing the concentration of AgNPs as the plant height and the number of leaves, while the treatments did not have significant effect on fruit pH and on the percentage of fruit dry matter but only on dry roots and leaves. The number, weight, and length of fruit per plant were significantly increased in plants sprayed with AgNPs and also the TSS (total soluble solids) was increased. Heavy silver accumulates mainly in roots and decreases in leaf, tissue, and skin of the fruit showing an improvement of silver heavy metal in various plant organs by increasing the concentration of AgNPs in a spray form. From these results, it can be evidenced that AgNPs can be used in the formulation of pesticides to improve the integrated pest management, obtaining a pathogen-free environment around the plant and inside the greenhouse because of AgNPs. In this way infestations of plant and diseases can be stopped, improving plant growth and crop production. The results of this study show as AgNPs are useful to control plant pathogens but it is important to monitor the amount of silver heavy metal in plants. This amount is less in fruit, the most edible part of the plant, but can be dangerous if consumed steadily, for this reason it is important to define the concentration limit for human body in the context of the consumed plants. Antifungal activity of AgNPs in plants' evaluations indicate that both silver ions and nanoparticles influence

Table 7.1 Effects of CeO_2 NPs on plants

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	ROS	Formulation used	References
CeO_2 NPs	Fuel additive; cosmetics	Higher in roots than in tissues and shoots		Chlorophyll content; low concentrations on NPs produce a decrease of H_2O_2 content	No sign of toxicity; no changes in seedling growth or in germination rate, no differences in lipid peroxidation	Scavenging activity of NPs that lead to an alteration of enzymatic activity, membrane damage and photosynthetic stress	Seeds in suspension with NPs	Rico et al. (2013)
CeO_2 NPs		Reduced uptake by pumpkin shoots, inhibition of the sorption by roots due to NOM			No toxic response		Plants exposed to NPs in hydroponic system with GA and FA	Schwabe et al. (2013)
CeO_2 NPs			Conformational changes within the plant (in the components of roots), but do not induce chemical reactions and substantial changes. Produce stress in cilantro plants, but, at the same time, have antioxidant activity	Downregulated the production of the defensive enzymes; changed the chemical environment of carbohydrates in shoots; change of the nutritional properties of cilantro	Longer roots and shoots; a higher biomass production; a significant increase of catalase activity in shoots and ascorbate peroxidase in roots; fertilizing effects and help plants to grow better		Cilantro plants were germinated and grown in organic soil with NPs	Morales et al. (2013)
CeO_2 NPs			Immediate positive results but need to evaluate the long-term, multigenerational impact	Second generation seedlings were smaller and weaker (smaller biomass, lower water transpiration); extensive root hairs	Benefits obtained on the first generation were lost in the second	Higher ROS content in the second generation	Tomato plants treated with NPs and studied the second generation seedlings	Wang et al. (2013)

formation of spores and progress of pathogenic fungi. The efficacy of AgNPs is much greater with preventative application, which may promote the direct contact of silver with spores and inhibit their viability (Ouda 2014). But are these effects due to the antibacterial properties of Ag or to nanomaterials? This was investigated in the next study (Vannini et al. 2013) where seedlings of *Eruca sativa* were treated with different concentrations of AgNPs and $AgNO_3$, to understand the morphological and proteomics changes induced by Ag and Ag in form of nanoparticles. In fact only few studies have been performed on food crops and the interaction with plants and the molecular mechanisms are not fully elucidated. The results show as AgNPs and $AgNO_3$ cause different plant response (increase in root elongation, induction of proteins of the jacalin lecitin family, induction of two key enzymes involved in cysteine biosynthesis, activation of pathways of ROS detoxification), while both treatments cause changes in proteins involved in the sulfur metabolism and in redox regulation, only AgNPs cause an improvement in plant growth and an alteration of proteins in the endoplasmic reticulum and vacuole, indicating these compartments as targets of NPs action. In conclusion, AgNPs cause oxidative stress and alter specific cellular functions and although the macroscopic cell response of the two Ag treatments is similar, the effects only partially overlap at molecular level, showing that the effects are not due only to the release of Ag^+ but to its dimension (Table 7.2).

Oukarroum et al. (2013) investigated the toxicity effect of AgNPs on the aquatic plant *Lemna gibba*, exposed to different concentrations of NPs, to understand whether the behavior is the same as the other plants. In this case a growth inhibition was demonstrated as a function of AgNP concentration. After 7 days of treatment a reduction in plant cellular viability was detected, effect that is correlated with the production of intracellular ROS. The increased ROS formation and the induced oxidative stress within *L. gibba* L. plant cells was proportional with the exposure to AgNPs in the medium. It seems that this stress was due to the release of free Ag inside the plant cells. This study demonstrated the different effects of NPs on different plants and that the accumulation of AgNPs in the aquatic environment can be a potential source of toxicity for duckweeds. Studies on silver nanotoxicity in plants have been controversial centered on whether the cause of toxicity is either the nanosize and shape of the particles or their release as ionic Ag^+. Among metal pollutants, silver ions are one of the most toxic forms for a wide range of microorganisms and, combined with its low toxicity to humans, lead to the development of a wealth of silver-based products in many bactericidal applications (Boenigk et al. 2014). All studies, however, agree that silver nanotoxicity is positively concentration-dependent and negatively size-dependent (Geisler-Lee et al. 2014).

7.1.3 Titanium Dioxide NPs

Titanium dioxide NPs are manufactured worldwide for cosmetics, in particular are used as sunblock to protect the skin from UV light, increased UV- B radiation causes direct and indirect effects including degradation and conformational

Table 7.2 Effects of AgNPs on plants

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	ROS	Formulation used	References
AgNPs	Commercial products as bactericidal		Improvement of silver heavy metal in various plant organs, it is important to monitor the amount of silver heavy metal in plants and define which is the concentration limit for human body that consumes these plants	Increasing the concentration of NPs, the silver heavy metal, the growth index, and the plant morphological characteristics were improved. No significant effect on fruit pH, and on the percentage of fruit dry matter but only on dry roots and leaves	Cucumber fruit performances were significantly enhanced (plant height and the number of leaves); the number, weight, and length of fruit per plant were significantly increased as the TSS. Useful to control plant pathogens		Suspension of NPs sprayed on cucumber plant	Shams et al. (2013)
AgNPs		Alteration of proteins in the endoplasmic reticulum and vacuole		AgNPs and $AgNO_3$ cause different plant response increase in root elongation, induction of proteins of the jacalin lecitin family, induction of two key enzymes involved in cysteine biosynthesis, activation of pathways of ROS detoxification, changes in proteins involved in the sulfur metabolism and in redox regulation	AgNPs cause an improvement in plant growth	Cause oxidative stress and alter specific cellular functions	Seedlings of *Eruca sativa* treated with AgNPs and $AgNO_3$	Vannini et al. (2013)

(continued)

Table 7.2 (continued)

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	ROS	Formulation used	References
AgNPs		Release of free Ag inside the plant cells	Accumulation of AgNPs in the aquatic environment can be a potential source of toxicity for duckweeds	Growth inhibition was demonstrated to depend on AgNPs concentration		Production of intracellular reactive oxygen species, responsible of a reduction in plant cellular viability and induced oxidative stress	Aquatic plant *Lemna gibba*, exposed to different concentrations of NPs	Oukarroum et al. (2013)
AgNPs		NPs were absorbed into plant stems, fruits, and leaves	Phytotoxicity	Decrease of root elongation, significant decrease of biomass, higher superoxide dismutase activity, lower chlorophyll content and fruit productivity			Greenhouse experiments of mature plants of tomato	Song et al. (2013a, b)

changes in DNA, proteins, and alterations in photosynthesis and growth and morphology of plants, furthermore these particles are used in antibacterial products and in wastewater to decompose organic matter. Several studies show the effects in animals (cytotoxicity, neurotoxicity, DNA damage) with controversial results and only few studies analyze the effects of nano TiO_2 on plants with opposite results (Ruffini et al. 2011; Zheng et al. 2005).

In fact nano TiO_2 has excellent biological and optical properties, but, at the same time, can exert toxic effects. To understand its behavior Gao et al. (2013) investigated the effects of nanoanatase-TiO_2 solutions, at different concentrations, on the seedlings of *Ulmus elongata*. This plant was chosen because the seedlings grow fast and are resistant to drought, pest, and diseases, furthermore this is the first study of TiO_2 NPs in a not edible plant and outside the laboratory. Biological effects of nano TiO_2 depend on its structure and plant species. For the study, 2-year-old *U. elongata* seedlings were used to understand the effect of NPs on the photosynthetic characteristics and the environmental factors that determine the effects of NPs on plant growth in the field. These NPs enhanced the synthesis of carbohydrates and lipids, as a toxicity mechanism and, after foliar applications, decreased the net photosynthetic rate via nonstomatal regulation. Furthermore, nanoanatase TiO_2 disrupted the growth of seedlings, increased leaf chlorosis, and caused defoliation. The leaf total carbon and nitrogen content was not affected by the treatment neither the leaf Mg, K, and Mn contents of the sprayed seedlings. Nanoanatase has also positive effects due to a large surface area, high thermal conductivity, and high photocatalytic ability that can inhibit the growth of bacteria. While, high light intensity can increase NPs toxicity reducing the capacity of the plant to fight against strong light. This is the first study assessing as the radiation factor can determine the effects of NPs on plant growth and is the first report on the important roles of environment to determine interaction between NPs and plants.

To avoid confusing results on phytotoxicity or positive results of TiO_2 NPs, Song et al. (2013b) studied the effects on the germination and root elongation of seeds, seedlings, and the uptake and physiological responses of mature plants. A hydroponic system was used to overcome NPs precipitation and to reproduce the environmental conditions. In this case NPs absorbed into seed tissues, did not show any toxicity. In fact nano TiO_2 does not have effects on seed germination, on enzyme activities and chlorophyll content even if the plants absorb NPs, furthermore has positive effects on root elongation. It seems due to its properties to agglomerate and form aggregates with bigger hydrodynamic diameters in distilled water and in hydroponic system, leading to precipitates that are not available to plants and does not cause dangerous effects. Therefore, nanosizes would become larger and not affect plants. Plants did not show any significant difference in total antioxidant capacity (TAC), superoxide dismutase (SOD) activity, and physiological differences when exposed to different treatments. In general, SOD is sensitive to heavy metals and in this experiment it does not change, showing no effects on mature plants. Even at high concentrations, nano TiO_2 does not seem toxic, demonstrating that a higher concentration does not always lead to higher absorption. In particular, these results compared to the previous study, show that the toxicity

of nano TiO_2 at high concentrations is species-dependent. Moreover, root elongation in plants exposed to 5,000 mg/L nano TiO_2 was only 25 % less than the control; thus, toxicity would not be fatal. These results demonstrate as nano TiO_2 has positive effects on plants, rather than negative it is not toxic in situ or in vitro to three plant species (*Brassica campestris*, oilseed rape, *Lactuca sativa*, lettuce, and *Phaseolus vulgaris*, kidney bean). In fact nano TiO_2 is difficult to accumulate in plants and even if it penetrates the seed coat and accumulates into its tissues it does not show toxic effects in plants grown in soil and hydroponics system. Overall, based on these results, TiO_2 is not toxic to plants.

Also for this material, a study was carried out (Feizi et al. 2013) to compare nano with bulk TiO_2 in order to understand if their properties are material-dependent and study its phytotoxic or stimulatory effects on seed germination and growth of fennel. Fennel seeds have weak germination (54 %) that give problems for cultivation; this work shows as seeds exposed for 14d to nano TiO_2 with a concentration of 60 ppm enhanced the indices of seed germination of fennel; similar positive effects were observed on shoot dry weight and germination rate, while bulk TiO_2 at a concentration of 40 ppm decreased shoot biomass and seed germination. Thus using the same concentration, with a nanoformulation, an improvement of germination time and an enhancement of indexes as germination value, vigor index, and mean daily germination, were observed, obtaining a considerable response. Germination tests were performed according to the rule of the International Seed Testing Association (ISTA 2009) and with this method problems with seed germination in some plant species could be overcome. Thus this study assessed the efficacy of the treatments with respect to the size of nanoparticles, but question on the differences using TiO_2 NPs with different crystalline structure and particle size remain. This was investigated by Clement et al. (2013).

These studies were carried out on model organisms as daphnies, algae, rotifers, and plants. The results show as TiO_2 NPs with anatase crystal structure is toxic in the tests conducted but at high concentrations promoted growth of roots for their antimicrobial properties, while rutile is lipophylic and form large aggregates in aqueous medium with a lower toxicity than anatase. In fact these different structures have different reactivities due to different surface properties, leading to a greater toxicity for anatase. Phytotoxicity tests with deeds of Flax show inhibition of germination and root biomass production, compared to controls. The *D.* magna showed a very sensitive response in acute toxicity tests depending mainly on nanoparticle sizes than NPs concentration, demonstrating high sensitivity compared with other tested organisms. This study established the chronic and acute toxicity of TiO_2 NPs for different biological organisms of various taxonomic groups and trophic status. These data should be considered to assess the potential effects on aquatic life. Hashemi and Mosavi (2013) evaluated the effect of four concentrations of nanoanatase (10, 20, 30, and 40 mg/ml) on germination parameters of parsley. At the end of the experiment, the results showed that an increase in the concentration of nanoanatase caused a significant increase in the percentage of germination, germination rate index, root and shoot length, fresh weight, vigor index, and chlorophyll content of seedlings. From the previous studies on TiO_2 it was evidenced as different plants, size,

and crystallinity of NPs determine different positive or negative effects on plants and environment but the results are sometimes controversial. For this reason, Song et al. (2013a) analyzed the effects of different NPs (aerosol of TiO_2 and colloidal AgNPs) on the same plants with the same method to directly compare the results to understand the beneficial or toxic effect of these NPs. Tomato (*Lycopersicon esculentum*) has been chosen as a model plant for toxicity studies of different NPs for its economic importance. Previous studies state the phytotoxicity of AgNPs in some cases but the effect on mature crop plants is unknown. Characteristics of NPs as water soluble or aggregation are important and have been considered in the research. Furthermore, mature plants should be tested because they are not so fragile as seedlings. In this work effects on root elongation and germination have been tested by using the filter paper on Petri dish method obtaining a decrease of root elongation at every concentration for nano Ag, due to a smaller hydrodynamic diameter, compared to nano TiO_2, leading to a higher uptake for Ag. No effects on seed germination were observed. Phytotoxicity was demonstrated in greenhouse experiments of mature plants by a significant decrease of biomass, higher superoxide dismutase activity for AgNPs (compared to TiO_2), lower chlorophyll content and fruit productivity, indicating that exposure to AgNPs stresses the plants, while TiO_2 NPs only showed higher SOD values at the highest concentrations on treatment that is unlikely to exist in natural conditions. Nano TiO_2 treatment induced particle agglomeration, interrupting in such way their uptake and decreasing their effects on plants (Table 7.3). Both NPs were absorbed into plant stems, fruits, and leaves, but some papers have reported also positive effects of nano TiO_2 on plant growth, suggesting the need of studies of the ecological effects, on animal and human health and on management strategies in the specific site in which they occur, in order to mitigate their effects and control their diffusion in the environment.

7.1.4 Zinc Oxide NPs

Nanoparticles absorbed by different plant species can produce positive and negative effects, in particular zinc oxide NPs are used in skin care products (toothpastes, sunscreens, soaps and shampoos, pigments and medicines) and cosmetics with a wide diffusion in the environment. In the present study (Lee et al. 2013a), to test the phytotoxicity of ZnO NPs, a medicinal plant was used: *Fagopyrum esculentum*. This plant can be used as a bioindicator of environmental conditions in different habitats because it is easy to grow and can adapt to environmental stress. Furthermore, this plant is a metal hyperaccumulator that can translocate metals from roots to shoots. Tests of toxicity on plants reduce animal sacrifices and the costs of routine tests. So the effects of ZnO nanoparticles were estimated on bioaccumulation, plant growth, and antioxidative activity. These plants were cultivated under hydroponic cultures and the presence of NPs was observed by SEM and TEM analysis in root cells. A significant biomass reduction, stunted growth, and a higher translocation of Zn in plants were observed using NPs

Table 7.3 Effects of TiO_2 NPs on plants

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	Formulation used	References
TiO_2 NPs	Cosmetics as sunblock, antibacterial products and wastewater		High light intensity can increase NPs toxicity reducing the capacity of the plant to fight against strong light	NPs enhanced the synthesis of carbohydrates and lipids, and, after foliar applications, decreased the net photosynthetic rate via nonstomatal regulation; disrupted the growth of seedlings, increased leaf chlorosis and caused defoliation		Effects of NPs solutions on the seedlings of *Ulmus elongata*	Gao et al. (2013)
TiO_2 NPs		In hydroponic system NPs form aggregates that precipitate and arc not available to plants without dangerous effects	NPs did not show toxicity, but it is species-dependent, It is not toxic in situ or in vivo in oilseed rape, lettuce, and kidney bean	Nano TiO_2 does not have effects on seed germination, on enzyme activities and chlorophyll content even if the plants absorb NPs. No difference in TAC, SOD activity, and physiological differences	Root elongation	Hydroponic system	Song et al. (2013a, b)
TiO_2 NPs					Enhanced the indices of seed germination of fennel, positive effects were observed on shoot dry weight and germination rate, improvement of germination time and an enhancement of indexes as germination value, vigor index, and mean daily germination	Seeds exposed to NPs and bulk	Feizi et al. (2013)

(continued)

Table 7.3 (continued)

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	Formulation used	References
TiO_2 NPs			Greater toxicity for anatase, *D. magna* showed a sensitive response in acute toxicity tests depending on the size of NPs. These data are important to assess the effects on aquatic life	Nano anatase at high concentrations promoted growth of roots, while nano rutile forms aggregates. Inhibition of germination and root biomass production		Acute toxicity tests of NPs for different biological organisms (daphnies, algae, rotifers, plants)	Clement et al. (2013)
TiO_2 NPs		Absorbed into plant stems, fruits, and leaves		Higher SOD, Particle agglomeration interrupts their uptake and decreases their effects on plants		The effect of aerosol and colloidal TiO_2 in a greenhouse experiment of tomato mature plants was studied	Song et al. (2013a, b)

instead of microparticles. In fact NPs can pass the cell membrane and form aggregates within the cell that produce the toxic effect. After the treatment the roots were shortened and damaged and a reduced glutathione level and catalase activity was noted with the generation of reactive oxygen species (ROS) that are predictive biomarkers of nanotoxicity. Catalase (CAT) enzyme is important as antioxidant defense system because converts free radicals H_2O_2 in water and oxygen and protects cells against oxidative damage. Reduced glutathione (GSH) is a free thiol of cells and is responsible of detoxification processes, thus its status indicates the health of the cell. ROS disturb the balance between antioxidant defense mechanism and oxidative stress and plants use antioxidants as defense mechanism as catalase (CAT), glutathione reductase, and glutathione-s-transferase and peroxidase. In the present work, at high NPs concentration, the amount of GSH increased because NPs have stimulated the antioxidant defense system, and at the same time, also CAT activity has increased, but joining saturation. In fact at high doses of NPs, too much ROS were produced and CAT loose the scavenging capacity, producing an oxidative damage. This one was the first study of phytotoxicity of ZnO NPs on a medicinal plant that is commonly used as a model plant in phytotoxicity studies, and was revealed a good indicator in environments polluted with ZnO NPs. From this study it was evidenced as this type of nanomaterial seems to have only negative effects on environment, but are there ways to limit the toxic effects? This was analyzed in another study by Zhao et al. (2013), where corn plants (*Zea mays*) were cultivated for 1 month in soil amended with ZnO NPs and sodium alginate, then the plants were grown with solutions of ZnO NPs and alginate and the dynamics of Zn accumulation in plant tissues were determined. Alginic acid is a component of natural organic matter and its sodium salt (sodium alginate) has been used as capping agent to stabilize NPs in suspension. The fate of NPs in soil and plants in the presence of alginate has not been studied yet and could change the fate and toxicity of ZnO NPs to plants. Previous studies showed negative effects of ZnO NPs on soil and plants but it seems to depend on soil condition and plant species. In fact plants treated with a definite concentration of ZnO NPs plus alginate had Zn in their tissues without reduction in biomass production. This could be due to the fact that alginate increases the level of chlorophyll *a*, helping to maintain photosynthesis. At the same time alginate reduced the activity of stress enzymes (peroxidase and catalase), determining a damage in the defense system, and increasing the release of Zn ions to soil solution and promoting the bioaccumulation in corn plant tissues, leading to an excess of Zn in the aerial parts of the plants. This demonstrates that alginate, a natural component of ecosystems, can modulate the fate of ZnO NPs in soil and plants, limiting its toxic effect on plants.

7.1.5 Cupric Oxide NPs

NPs are bioreactive because of their greater surface area and smaller size than microparticles and it is important to understand their impact on ecosystems. Lee

et al. (2013b) compared the phytotoxic and genotoxic effects of two types of ZnO and CuO NPs on buckwheat (*F. esculentum*) seedlings. The aim of this study was to determine the genotoxic potential of these NPs in the roots of seedlings by using the RAPD assay and estimate the toxic effects of ions released from NPs on biomass and inhibition of root growth. ZnO NPs are used in chemotherapeutic drugs, personal care products (toothpastes, shampoo, sunscreen, soap) and coating catalysts for environmental remediation and can pass into the environment and can be absorbed by plants. In particular, buckwheat seedling growth was affected by these NPs and significant changes in root morphology as inhibition of root length, decrease of root growth, and localization of NPs inside the root epidermis cells were observed. Furthermore, the genotoxic effects of ZnO and CuO NPs on the early growth of buckwheat, an edible plant, was demonstrated showing as high doses might interfere with the cellular homeostatic balance and alter intracellular signaling pathways leading to a cascade of genotoxic effects. The results suggested that high concentrations of ZnO and CuO NPs have damaging effects on genomic DNA, alter gene expression levels and this alteration in DNA structure can be an index to evaluate the phytotoxicity of NPs on buckwheat. Different studies have demonstrated the phytotoxicity of CuO and ZnO NPs for different plants and the translocation of Zn to plant shoots compared to Cu. Dimkpa et al. (2013) evaluated the behaviors of CuO NPs in the environment and in particular in plants of wheat grown in sand treated with these NPs. CuO NPs demonstrated to be phytotoxic to wheat root causing a reduction to their length. In fact when the sand was amended with these NPs their presence was found in root surface and then translocated into plant aerial portions to form complexes with different ligands. In this way, basic information were obtained to understand the fate of NPs from soil to plants confirming the phytotoxicity of CuO NPs. Their fate is similar to that of AgNPs in a sand matrix but these results can vary in different plant species, soil, and NPs sizes and type.

7.1.6 Fullerene

Recently there has been an interest to apply nanomaterials to different fields, as for example fullerene, a popular carbon material used in biomedicine, energy science, and electronics. In particular carboxyfullerene, a water soluble compound had been largely used in biomedical applications with an increasing diffusion also in the environment, for this reason it is important to evaluate its effect on plant. These effects of carbon nanomaterials functionalized and not on plants were illustrated by Husen and Siddiqi (2014). It was found as they influence fruit and crop production in edible plants and vegetables, and increase the water retaining capacity, biomass, and fruit yield in plants up to ~118 %. The internalization and accumulation of these compounds in plant roots and seedlings and their phytotoxicity have been investigated by Liu et al. (2013). The modifications induced by carboxyfullerenes to the cell wall of tobacco plants such as the mechanism of interaction

and survival under repression were analyzed. The adsorption of this component led to the disruption of the cell wall and membrane with the consequent inhibition of cell growth. From AFM measurements and confocal imaging a dose-dependent increase of glycosyl residue on cell wall was observed, with increase of ROS in treated cells. The enhanced intracellular ROS was found in cell membrane but also in all parts of cells. The disruption of cell membrane, thus, can be due to enhanced ROS or these species can be responsible to activate the plant defense system and produce an alteration of cell composition to fight the stress induced by caboxyfullerenes. This is the first study on the changes of plant cell wall subjected to the repression of fullerene, showing the phytotoxicity of this material (Table 7.4).

7.1.7 Gold NPs

Gold NPs (GNPs) are among the most synthesized NPs and are used principally for medical purposes since sixteenth century, but reports, documenting their effect on plant physiology, development and metabolism, are very limited. Different synthesis methods exist and in particular an environmental friendly method using biological routes as plant extracts and microbes. This method, as illustrated by Kumar et al. (2013), can be cost effective for different agricultural applications. In detail AuNPs were synthesized by *Syzygium cumini* leaf extract and exposure studies were carried out on *Arabidopsis thaliana*. *A. thaliana* seeds were exposed to two different concentrations of AuNPs, and germination pattern, antioxidant enzymes, expression of micro RNAs were studied. It was found that exposure to AuNPs enhances the total seed yield of *A. thaliana* three times compared to the control and improves seed germination rate, free radical scavenging activity, and vegetative growth. A correlation was found between expression of regulatory molecules, microRNAs, seed germination, antioxidant potential, growth of *A. thaliana*, and exposure to gold NPs, revealing this to be a good tool to enhance seed yield of plants. Seed germination rate, fresh biomass, and relative water content of *A. thaliana* were significantly increased upon GNP exposure compared to control together with the free radical scavenging potential. This seems due to an increase in water uptake capacity of seeds and thereby, increased seed germination rate and fresh biomass. The activity of enzymes as ascorbate peroxidase (APx), CAT, glutathione reductase (GR), and SOD, that are known to be important for the defense system against reactive oxygen species in plants, were enhanced in seedling exposed to GNPs in a dose-dependent way, acting in antioxidant defense of plants. GNP exposure was found to enhance the root and shoot length compared to control and to modify levels of microRNAs (miR) expression that are involved in the regulation of different morphological, physiological, and metabolic processes in plants. These physiological and molecular modulations might be responsible of the improved seed yield in treated plants with 10 μg/ml of GNPs (24 nm) while 80 μg/ml of GNPs are recommended for better vegetative crop productivity.

Table 7.4 Effects of ZnO NPs, CuO NPs, and carboxyfullerenes on plants

Name	Applications	Localization	Toxicity	Variation observed	ROS	Formulation used	References
ZnO NPs	Skin care products and cosmetics	NPs can pass the cell membrane and form aggregates within the cell that produce the toxic effect	Phytotoxic	Significant biomass reduction, stunted growth, and a higher translocation of Zn in plants, the roots were shortened and damaged	A reduced glutathione level and catalase activity was noted with the generation of ROS	Hydroponic culture of *Fagopyrum esculentum*	Lee et al. (2013a)
ZnO NPs				Plants treated with ZnO NPs plus alginate had Zn in their tissues without reduction in biomass production, Alginate increases the level of chlorophyll a, helping to maintain photosynthesis, alginate reduced the activity of stress enzymes (peroxidase and catalase), determining a damage in the defense system, and increasing the release of Zn ions to soil solution and promoting the bioaccumulation in corn plant tissues, leading to an excess of Zn in the aerial parts of the plants		*Zea mays* was amended in soil with ZnO NPs and sodium alginate, then the plants were grown with s olutions of ZnO NPs and alginate	Zhao et al. (2013)
ZnO NPs	Chemotherapeutic drugs, personal care products, and coating catalysts for environmental remediation	Translocation of Zn to plant shoots				NPs on buckwheat (*F. esculentum*) seedlings	Lee et al. (2013b)

(continued)

Table 7.4 (continued)

Name	Applications	Localization	Toxicity	Variation observed	ROS	Formulation used	References
CuO NPs			Alteration in DNA structure can be an index to evaluate the phytotoxicity	In root morphology as inhibition of root length, decrease of root growth, and localization of NPs inside the root epidermis cells. High doses might interfere with the cellular homeostatic balance and alter intracellular signaling pathways, damaging effects on genomic DNA, alter gene expression levels	Leading to a cascade of genotoxic effects	NPs on buckwheat (*F. esculentum*) seedlings	Lee et al. (2013b)
CuO NPs		Root surface and than translocate into plant aerial portions	Phytotoxic to wheat root causing a reduction to their length			Plants of wheat were grown in sand treated with CuO NPs	Dimkpa et al. (2013)
Carboxyfullerene	Biomedicine, energy science, and electronics		Disruption of the cell wall and membrane with the consequent inhibition of cell growth, phytotoxicity	Increased glycosyl residue on cell wall, disruption of cell membrane, activated plant defense system and produced an alteration of cell composition	Increased ROS in treated cells, enhanced intracellular ROS was found in cell membrane and in all parts of cells	Exposure of fullerene to tobacco plant	Liu et al. (2013)

7.1.8 Multiwalled Carbon Nanotubes (MWCNTs)

Carbon nanotubes (CNTs) are interesting for their physicochemical characteristics, strength, and nanosize. They have attracted attention as potential molecular transporters based on their ability to cross the cell membrane of mammalian cells acting as drug delivery vehicles, in this way they can be released in the environment. In a recent work (Lahiani et al. 2013), CNTs were deposited by air spray on the seed surface or added in the growth medium of plants and it was evidenced as they can penetrate the seed coats and activate germination. In particular MWCNTs were added to agar medium and seed germination and activation of growth was observed in three crops: barley, soybean, and corn. Seed germination was activated when MWCNTs were deposited on seed surfaces and their ability to penetrate was proven by Raman spectroscopy and transmission electron microscopy (TEM). In treated seeds, the expression of genes encoding different water channel proteins (aquaporins) was increased, as evidenced by reverse transcription polymerase chain reaction (RT-PCR), these proteins play a main role in the process of seed germination. These results prove the positive effects of carbon nanotubes on seed germination and growth in different crop species, showing its potential as regulators of germination and plant growth. In fact barley seeds germinated on the second day after planting while 46 % of treated seeds were able to germinate during the first day posttreatment. After 6 days, all treated seeds reached a rate of 100 % germination compared to the control seeds which only reached 63 %. In the present experiment and doses, MWCNTs in the tested doses did not show any toxic effects for crop plants in early stages of development or any negative effects on the development of plants. No significant differences were observed in shoot length, number of leaves, and number of fruits between plants originated from untreated and treated seeds of different crops.

A further study (Serag et al. 2013) evidenced the ability of functionalized carbon nanotubes to penetrate the plant cell wall and induce changes in specific organelles. Their properties to penetrate the cell membrane of tobacco and seed coats, stimulate germination and promote growth in tobacco and tomato plants were studied. CNTs can enter the plant cell wall in two ways, depending on their diameters: if the average diameter of CNTs is below the diameter of plant cell wall, they can enter in the apoplast but chemical methods as ultrasonic assisted chemical oxidative cutting can help the movement of the tubes through the structure of cell wall; if the carbon nanotube diameter exceeds the 5 nm pores of plant cells wall, it must be removed prior to interfacing. Another recent strategy for the penetration consists to immobilize cellulase molecules on the walls and tips of CNTs that help their penetration. Thus the subcellular localization of CNTs depends on their length and on the nature of the tag adsorbed onto their surface. This is important for the delivery of substances to specific subcellular organelles. So they can be used to reinforce fibers as wood materials or as gene delivery vectors to plant cells. In this way, they can lead to advantages in agricultural biology and plant biotechnology at the level of the plant products, for the entire plant and to the plant cells and molecules. Different reports on CNTs toxicity and safety were carried out and it was demonstrated as a functionalization reduces toxic effects while pristine CNTs induce different phytotoxic effects but further investigations are needed.

7.1.9 Mesoporous Silica NPs

Mesoporous silica is actually largely used in catalysis, drug delivery, and imaging and thus released in the environment. Recently, Nair et al. (2011) demonstrated that uptake of nonporous silica nanoparticles (25 nm) labeled with fluorescein isothiocyanate (FITC) had no effect on seed germination at concentrations up to 50 mg L^{-1}. In the present study (Hussain et al. 2013) mesoporous silica nanoparticles functionalized with amine cross-linked fluorescein isothiocyanate were absorbed by lupin, wheat, and *Arabidopsis*. These NPs have a dimension of around 20 nm with interconnected pores of around 2 nm. The uptake and distribution were examined during seed germination, in roots and leaves of plants. It was found that these NPs did not affect seed germination in lupin and did not show phytotoxicity. After germination of lupin and wheat in solution with NPs, they were found within cells, in cell wall of roots, and in the xylem and other cells for the transport of elements. In *Arabidopsis* the NPs were found in leaf, in the intracellular spaces of mesophyll and in the root system (Table 7.5). Although penetration into the cells showed no signs of toxicity they could be used to deliver small NPs in plants, as new biological pesticides with a slow release formulation, or to release fertilizers in soil.

7.1.10 Nickel Oxide NPs

Nickel oxide NPs are used in a number of applications as antibacterial. Faisal et al. (2013) studied the phytotoxicity induced by NiONPs in tomato seedling roots and its translocation in root cells and changes in cell organelles. Furthermore, the potential release of Ni ions, ROS generation, oxidative stress levels, and apoptosis were analyzed. No systematic studies on phytotoxicity were carried out so far on these NPs and in this experiment it was found that NiO NPs induced apoptosis in tomato root cells, trigger the release of caspase-3 proteases from mitochondria, leading to mitochondria dysfunction. Furthermore, they induced imbalance in antioxidant enzymes, induced oxidative stress, apoptosis, and necrosis in tomato seedling roots, and finally damage DNA. This analysis shows its potential to induce cell death through the apoptotic and/or necrotic pathway. In conclusion, these NPs need a particular attention for the potential environmental hazard to plants.

7.1.11 Nanoscale Zero Valent Iron

Nanoscale zero valent iron (nZVI) remains the most predominant way of nanoremediation for the treatment of various environmental pollutants in contaminated water and soil. In this way it has been introduced in the environment in sites containing chlorinated compounds, and heavy metals as As, thus there is the need to

Table 7.5 Effects of AuNPs, CNTs, mesoporous SiO_2 NPs on plants

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	ROS	Formulation used	References
AuNPs	Medical purposes			Enhancement of the total seed yield of *Arabidopsis thaliana* by three times and improvement of seed germination rate, free radical scavenging activity and vegetative growth. Enhancement of the root and shoot length compared to control and changes of the levels of microRNAs (miR) expression that are involved in the regulation of different morphological, physiological, and metabolic processes in plants	Seed germination rate, fresh biomass, and relative water content of *A. thaliana* were significantly increased upon GNP exposure with the free radical scavenging potential. This seems due to an increase in water uptake capacity of seeds	The activity of (APx), CAT, (GR), and SOD, important against ROS in plants, were enhanced In seedling exposed to GNPs in a dose-dependent way, acting in antioxidant defense of plants	*A. thaliana* seeds were exposed to NPs	Kumar et al. (2013)
MWCNTs	Drug delivery vehicles	They can penetrate the seed coats and activate germination	Did not show any toxic effects for crop plants in early stages of development or any negative effects on the development of plants	The expression of genes encoding different water channel proteins (aquaporins) was increased playing a main role in the process of seed germination. No significant differences were observed in shoot length, number of leaves, and number of fruits between plants originated from untreated and treated seeds of different crops	Positive effects of carbon nanotubes on seed germination and growth in different crop species, showing its potential as regulators of germination and plant growth		CNTs were air sprayed on the seed surface or added in the growth medium of plants of barley, soybean, and corn	Lahiani et al. (2013)

(continued)

Table 7.5 (continued)

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	ROS	Formulation used	References
Functionalized CNTs	Reinforcements of fibers or gene delivery vectors to plant cells	Penetrate the plant cell wall and induce changes in specific organelles	Functionalization reduces toxic effects while pristine CNTs induce different phytotoxic effects but further investigations are needed					Serag et al. (2013)
Mesoporous SiO_2 NPs	Catalysis, drug delivery, and imaging	In lupin and wheat NPs were found within cells, in cell wall of roots, and in the xylem and other cells for the transport of elements. In Arabidopsis the NPs were found in leaf, in the intracellular spaces of mesophyll and in the root system	No signs of toxicity were observed	NPs did not affect seed germination and did not show phytotoxicity			Functionalized NPs were absorbed by lupin, wheat, and Arabidopsis	Hussain et al. (2013)

evaluate the potential ecotoxicological impact of nZVI and investigate the fate, impact, accumulation, and toxicity of this nanomaterial on different plants. In a study of Ma et al. (2013) plant seedlings of cattail and hybrid poplars were grown in a greenhouse with hydroponic solutions added for 4 weeks with different concentrations of nZVI (0–1,000 mg/L). At high concentration nZVI exhibited strong toxic effects, reduced the transpiration and growth of hybrid poplars while at low concentrations enhanced plant growth. TEM and STEM show as NPs coated plant roots surface and penetrated into epidermal cells. For both plants the transport to shoots was minimal. The formation of black coating on root surface that can block the root membrane pores and interfere with the water and nutrient uptake process was observed. This can result from the direct deposition of nZVI on the root surface and can be a combination of ferric iron oxides and zero valent iron. In fact nZVI can move into the root cells of poplar plants and can pass through the cell wall and membrane of poplar roots. When these materials are used it is important to consider the remedial benefits but also the potential fate and toxicity of nZVI. From these results nZVI at the concentrations used in field conditions could lead to phytotoxic effects on plants depending upon plant species. This study used bare nZVI and not modified nZVI, the result indicated that large scale introduction of nZVI in the environment can lead to serious consequences thus it needs further attention.

7.1.12 Graphene Oxide Sheet

Graphene is the thinnest and strongest material and has unique properties. In particular a derivative, graphene oxide, is water soluble and has been used as precursor of nanocomposites. In fact it can be used as adsorbent of heavy metals, natural dyes, and pesticides in contaminated environments and can enter in the animal food chain with subsequent transfer to humans. Anjum et al. (2013) evaluated the tolerance of a common food crop, faba bean, to different concentrations of graphene oxide and the glutathione redox system. In fact plants can act as media for the transfer of adsorbed NPs and phytotoxicity studies can show the impact of NPs on environment and health. Previous studies of graphene on plants have been conflicting or speculative with unsubstantiated results on higher plants but it was shown as graphene oxide enhanced the release of different reactive oxygen species leading also to cell death. The results showed in this study report an increase of *Vicia faba* sensitivity under three graphene oxide concentrations (100, 200, and 1,600 mg/L measured as tolerance to graphene oxide) and two promising results with improvements in *V. faba* health status. In fact in the first case the higher sensitivity was due to the occurrence of high-oxidized glutatione, decreased pool of reduced glutathione and the reduced ratio glutathione/glutathione oxidized that did not allow the maintenance of coordination between regeneration and utilization of glutathione. While, *V. faba* seedlings exposed to graphene oxide at concentrations of 400 and 800 mg/L led to a fine tuning between glutathione regeneration and

Table 7.6 Effects of NiO NPs, nZVI, graphene oxide sheet on plants

Name	Applications	Localization	Toxicity	Variation observed	Positive effects	ROS	Formulation used	References
NiO NPs	Antibacterial		They induced imbalance in antioxidant enzymes, induced oxidative stress, apoptosis, and necrosis in tomato seedling roots, and finally damage DNA, Phytotoxic	NiO NPs induced apoptosis in tomato root cells, trigger the release of caspase-3 proteases from mitochondria, leading to mitochondria dysfunction			NPs in tomato seedling roots	Faisal et al. (2013)
nZVI	Nanoremediation	NPs coated plant roots surface and penetrated into epidermal cells, the transport to shoots was minimal. It can move into the root cells of poplar plants and can pass through the cell wall and membrane of poplar roots	Phytotoxic effects on plants depending upon plant species	At high concentrations demonstrated strong toxic effects, reduced the transpiration and growth of hybrid poplars while at low concentrations enhanced plant growth. Black coating on root surface that interfere with the water and nutrient uptake process			Plant seedlings of cattail and hybrid poplars were grown in a greenhouse with hydroponic solutions added for 4 weeks with nZVI	Ma et al. (2013)
Graphene oxide sheet	Precursor of nanocomposites and adsorbent		Graphene oxide enhanced the release of different reactive oxygen species leading also to cell death	Glutathione redox system emerged as the main cause of the different sensitivity of *V. faba* toward graphene oxide concentrations	Enhanced		Faba bean grown with graphene oxide	Anjum et al. (2013)

utilization. So glutathione redox system emerged as the main cause of the different sensitivity of *V. faba* toward graphene oxide concentrations. Furthermore, a tuned coordination between regeneration and utilization of glutathione significantly modulated its pool and its redox couple that controlled the tolerance of *V. faba* seedlings to the graphene oxide concentrations (Table 7.6). So also in this case further studies on risks and benefits evaluation of graphene oxides are needed.

7.2 Conclusion

In this chapter, the last studies of phytotoxicity due to nanomaterials were analyzed and it was observed that their physicochemical properties determine their interaction with living organisms. Cells of plants, and in particular cell walls, are the first site for interaction with nanomaterials and constitute a barrier for the entrance of NPs, for this reason, depending on plant structure, the same NPs can be toxic or not in different organisms. Once entered inside cells, nanomaterials might provoke alterations of cell structures, membranes, and molecules, as physical restraints, solubilization of toxic NP compounds, or production of ROS. Also, positive effects on germination of plant seeds, on the growth of seedlings, on fertilizing effects and on nitrogen assimilation were obtained if the seeds were treated with specific NPs with specific dimension, concentration, and material. In fact their function and use depend on their composition and structure. For this reason, the actual interest is to control their size and shape to manipulate their unique optoelectronic, magnetic, catalytic, and mechanical properties to determine their best interaction with living organisms. As reported in this chapter, different nanomaterials such as CeO_2, Ag, TiO_2 Au, CNTs, SiO_2, showed positive effects, without signs of toxicity, and these results can be applied in agriculture to improve plant production. Before the massive introduction of these nanomaterials in the environment, and the assumption of fruits exposed steadily to these substances, the long-term, multigenerational impact on plants has to be considered. Different behavior of the same nanomaterials was observed, considering different plants (e.g. aquatic plants) or plants in different development periods (seedlings or mature plants). Also, nanomaterials considered phytotoxic (ZnO, CuO, fullerene, NiO, nZVI, graphene) can have positive effects when introduced in low concentrations or the negative effects can be mitigated through measures that consider the presence of organic matter, soil conditions, and limit their absorption by plants and fruits. All these elements need a consideration before the utilization of these nanomaterials in agriculture and a possible danger for plants, environment, and humans.

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Chapter 8
Early Developmental Responses of Plants Exposed to Metals and Oxides Nanomaterials

Lok R. Pokhrel and Brajesh Dubey

Abstract Over the last decade or so, one question about engineered nanomaterials (ENMs) has been constantly asked: Are nanomaterials inherently toxic? It is because characteristics such as "nano" scale size, surface charge, surface plasmon resonance, greater surface area, and propensity to ligand with (in)organic and/or polymeric molecules set ENMs physicochemically apart from their bulk/parent analogs. Related to unique properties, which enable greater functionality in a wide range of consumer applications, is the uncertainty about whether unique risk is posed to the environment, health, and safety (EHS) as ENMs are anthropogenically released into the environment. Recognized as the major sinks, soil, water, and air contamination of ENMs, including their leachable or modified by-products, is inevitable. Understanding of potential impacts on terrestrial plant species has remained unclear as anomalies in morphological, anatomical, and physiological endpoints, which have potential for impairing later development in life, are not routinely screened for, however. In this chapter, we report valuable information synthesized via thorough literature review of the current understanding of potential implications of ENM release and exposure to plants via soil, water, and atmospheric deposition. In particular, we report potential fate, biouptake, site of translocation/associated mechanisms, in vivo transformation, and toxicity (germination rate, growth and development, anatomical and physiological anomalies, and yield) of metal-based ENMs. Additionally, potential mechanisms and factors influencing ENMs' toxicity are explained. Such information is critical to direct future research aimed at uncovering better understanding of nanotoxicology in plants, and to determine whether risk to public health exists from exposure to ENMs through the dietary route.

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Keywords Engineered nanomaterials · Phytotoxicity · Biouptake · Environmental safety · Mechanisms · Developmental effects · Health risk

8.1 Introduction

From doughnuts' icing to world's darkest vantablack (carbon nanotubes-based coating that absorbs 99.96 % of the incident light), from odorless apparels to sunscreen lotion, from energy harvesting devices to environmental remediation (Behar 2013; Owano 2014, www.nanotechproject.org/cpi/): One thing these different products have in common is the applications of engineered nanomaterials (ENMs). ENMs are synthetically engineered materials with size less than 100 nm in one dimension (NNI 2006). When materials exist in near atomic size, they tend to exhibit higher reactivity than their bulk counterparts (Bowker 2002). Due to unique properties, ENMs are touted to bring the next industrial revolution, if not already underway. Realizing ENMs' potential in improving agricultural production, some ENMs may have already made their way into our farms in the form of fertilizer, pesticide, or via contaminated biosolid applications, or remediation technologies (e.g., zero-valent iron to treat subsurface organic plumes) (Pokhrel and Dubey 2013; Gardea-Torresdey et al. 2014). Continual use of ENMs in agriculture, including in various consumer applications, will undoubtedly contaminate the environment (soil, water, and air), potentially impacting the agriculture and food/feed quality, and may pose unknown risk to human health and safety (NNI 2006; Pokhrel et al. 2014a).

Although much research to date focused on animal, mammalian cell lines, microbial, and algal models for understanding nanotoxicology (Navarro et al. 2008; Yu et al. 2009; Pokhrel et al. 2012; Pokhrel and Dubey 2012a, b; Silva et al. 2014), phytotoxicity studies of ENMs are limited for assessing potential risks of this emerging contaminant to human health and the environment (Pokhrel and Dubey 2013). Plants represent basal component (primary producer) in most ecosystems, serve as an important source for trophic transfer of contaminants (heavy metals) including the ENMs, and may represent a pathway for ENM transport in the environment (Gardea-Torresdey et al. 2014). In this review, we assess current understanding of toxicology of ENMs in plants. Specifically, we evaluate the potential fate, biouptake, site of translocation/associated mechanisms, in vivo transformation, and toxicity (germination rate, growth and development, anatomical and physiological anomalies, and yield) of various metal-based (metals and oxides) ENMs. Concurrently, potential factors and mechanisms of ENMs' toxicity are discussed. Figure 8.1 depicts important information as synthesized in this review and also highlights important areas for future research. These information are critical as they direct future research aimed at uncovering better understanding of nanotoxicology in plants, and whether potential risk of ENMs exists in humans through the dietary route.

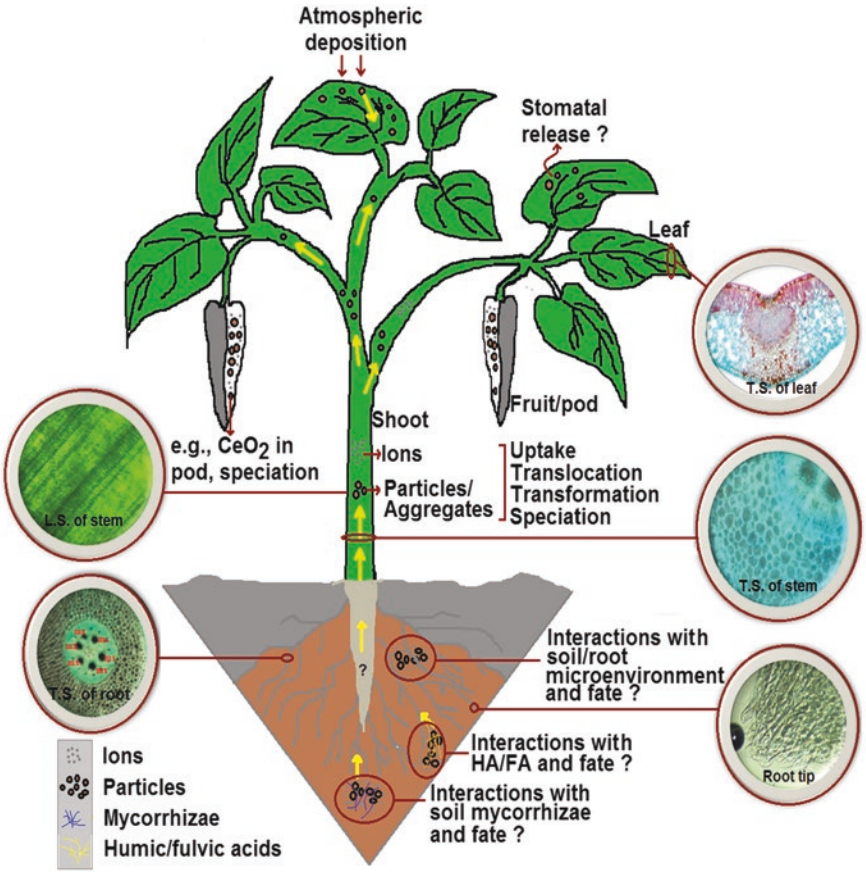

Fig. 8.1 Summary schematic depicting potential for engineered nanomaterial (ENM) interactions with the plant system. Potential fate (e.g., transformation, speciation, etc.) of ENMs within the soil system, uptake by the root system, further translocation, biotransformation, and speciation within the root and shoot systems (stem, leaf, and fruit/pod) are highlighted, with unknowns denoted by "?". Anatomical anomalies upon ENM stress and localization of ENMs can be revealed via microscopic observation (longitudinal [L.S.] or transverse sections [T.S.]) of the respective plant tissues. For additional information, see text. *HA* humic acids; *FA* fulvic acids; *mx* metaxylem; *yellow arrow* showing direction of transport; T.S. of leaf section modified from https://www.flickr.com/photos/blueridgekitties/6475212645/ (BlueRidgeKitties 2011)

8.2 Toxicity of Nanoparticles to Plants

8.2.1 Effects on Seed Germination Rate

Although ZnONPs led to dose-dependent inhibition of seed germination in cabbage, it did not affect germination in maize, however (Pokhrel and Dubey 2013; El-Temsah and Joner 2012). Zn^{+2} ions exposure, on the other hand,

resulted in significantly greater effects in seed germination in both the species tested (Pokhrel and Dubey 2013). Smaller size (6 nm) and more negative (ζ potential $= -49.5$ mV) gum Arabic (GA)-coated AgNPs were found to be relatively more inhibitory than larger size (21 nm) and less negative (ζ potential $= -22.5$ mV) PVP-coated AgNPs, or Ag^+ (used as $AgNO_3$) on germination in American pokeweed (*Phytolacca americana*), soft rush (*Juncus effuses*), or woolgrass (*Scirpus cyperinus*) (Yin et al. 2012). Overall, the effects were greater when assayed in deionized water medium compared to the soil culture experiments, and that plants susceptibility varied by types of species assayed and forms or types of Ag treatments (Yin et al. 2012). Larue et al. (2012) reported no impact of various sized TiO_2NPs on seed germination in wheat (*Triticum aestivum*) despite that the NPs were able to internalize through roots and translocate up to the leaves. Overall, these results suggest that ENM toxicity on seed germination may likely depend on the type of ENMs (material, size, and charge) and the plant species tested as the responses are remarkably variable—ranging from noninhibitory (e.g., TiO_2NP) to inhibitory (e.g., AgNP, ZnONP).

8.2.2 Effects on Plant Growth and Development

Pokhrel and Dubey (2013) investigated early growth and developmental responses of corn (*Zea mays*) and cabbage (*Brassica oleracea* var. *capitata*) exposed to well-characterized nanoparticles of silver (AgNPs) and zinc oxide (ZnONPs) in a week-long experiment. The authors discovered a deep invagination, dubbed *tunneling-like effect*, in the primary root tip in corn exposed to ZnONPs (1,000 mg/L), likely a result of cell dissolution via structural disintegration upon interaction with the NPs. However, microscopic observation of the root surface revealed root hair density being unaffected by NPs or their ionic salts treatments. With AgNPs or ZnONPs, effects on root elongation were minimal, whereas ionic Ag^+ or Zn^{+2} significantly inhibited root development in a dose-dependent fashion in both species (Pokhrel and Dubey 2013). Assaying noncrop species, Yin et al. (2012) observed enhanced biomass growth in root and leaf with PVP-AgNPs treatments in wooly sedge (*Carex lurida*), including a significant root elongation in American pokeweed and switchgrass (*Panicum virgatum*) with PVP-AgNPs or Ag^+ treatments. Yin et al. (2011) documented complete inhibition of root hair development in seedlings of *Lolium multiflorum* with gum Arabic-coated AgNPs (40 mg/L); however, such effect was not observed with similar dose of $AgNO_3$ treatment. Moreover, overall growth effects were more pronounced with 6 nm AgNPs than with 25 nm AgNPs (Yin et al. 2011), suggesting particle size-mediated toxicity of AgNPs. Lee et al. (2012) documented significantly higher toxicity of Ag^+ ions in root and shoot growth in two crop species (*Phaseolus radiates* and *Sorghum bicolor*) compared to AgNPs treatments, and with effects rather greater when assayed in agar medium compared to soil experiments. Pradhan et al. (2013) observed significant enhancement in root and shoot growth, including in plant biomass and rootlet density,

in mung bean (*Vigna radiata*) treated with NPs of manganese (MnNPs) compared to the controls or Mn^{+2} ions (used as $MnSO_4$) treatments. Furthermore, MnNPs promoted O_2 evolution through increased photophosphorylation without eliciting oxidative stress (Pradhan et al. 2013).

Likewise, significant enhancement in fruit yield and biomass were documented in cucumber through foliar applications of higher AgNPs concentrations (500–3,000 mg/L; Shams et al. 2013), which, however, reduced fruit yield and chlorophyll with higher superoxide dismutase activity in tomato at as high as 60 mg/L levels (Song et al. 2013). AuNPs significantly increased vegetative growth and seed production in both noncrop (*Arabidopsis thaliana*) (Kumar et al. 2013) and crop (*Brassica juncea*) species (Arora et al. 2012). Interestingly, in lettuce, foliar applications of AgNPs did not affect leaf biomass and other measured physiological endpoints (Larue et al. 2014). Fe_2O_3NPs enhanced pod and grain biomass by 48 % in soybean (Sheykhbaglou et al. 2010), CeO_2NPs treatments led to increased fruit yield in tomato (Wang et al. 2012), and ZnONPs improved kernel and pod biomass, including shelling percentage, in peanut (Prasad et al. 2012).

On the other hand, several studies have reported no significant implications of various oxide NPs to plants. For example, TiO_2NPs had no effect on biomass in beans or wheat (Jacob et al. 2013), and in fruit yield in tomato (Song et al. 2013). CeO_2NPs did not affect seed production in soybean (Priester et al. 2012) or biomass growth in cilantro (Morales et al. 2013). ZnONPs showed no effect on vegetative growth (Kim et al. 2011; Zhao et al. 2013), photosynthetic pigment content, and gaseous exchange in cucumber (Zhao et al. 2013), except at unusually high concentrations (800 mg/L) of CeO_2NPs which resulted in reduced fruit yield (Zhao et al. 2013).

8.2.3 Effects on Anatomical Structures

Potential modifications which may occur in plant anatomical structures upon exposure to ENMs have not been fully realized as only a few studies have explored anatomical changes with ENM exposure. However, toxicity literature on heavy metal ions suggest that changes in cellular morphology (shape and size) at earlier life stages can severely alter cellular functions related to solute transport and tissue differentiation in plants (Puertas-Mejia et al. 2010; Delmail et al. 2011). In a seminal study, Pokhrel and Dubey (2013) investigated structural changes in primary root cells at the zone of elongation in maize exposed to AgNPs and ZnONPs and compared to responses from respective ionic salt ($AgNO_3$ and $ZnSO_4$) treatments. The authors found consistently elongated cells with each type of NPs treatments, whereas the responses from Ag^+ or Zn^{2+} ions exposure varied: cells showed thinner and irregular morphology with Ag^+ treatments while Zn^{+2} treatment resulted in a relatively shorter but wider cells compared to controls. These novel findings indicate that the mechanism(s) underlying ENM stress is unrelated to that incurred by specific ions during early growth and development

in corn. This is consistent with the results of another study, which independently documented collapsed and highly vacuolated root cortical cells, including disrupted root cap and epidermal cells in *L. multiflorum* when exposed to AgNPs (40 mg/L), whereas $AgNO_3$ exposure did not lead to such anomalies in root cells (Yin et al. 2011). In *Lolium perenne*, considerable effects of ZnONPs in the root epidermis and cortex were observed (Lin and Xing 2008). The evidence suggests that cell structural integrity would likely be compromised when plants are exposed to ENMs of different types, which might have implications on ENM uptake by plants during their early growth and development.

8.3 Biouptake, Localization, and Transformation of Nanoparticles Within Plants

Analysis of biouptake of ENMs are intended to inform about potential risk from consumption of food crops by humans or other organisms higher up in the food chain. Studying multiple types of TiO_2NPs of varied sizes (range: 14–655 nm), Larue et al. (2012) documented low levels of Ti uptake (109.3 μg/g dw.) within wheat root. In another study, Pokhrel and Dubey (2013) reported sevenfold higher Ag uptake with $AgNO_3$ treatment (22 ng Ag/mg dw.) than with Citrate–AgNPs treatment (1.8 ng Ag/mg dw.) in a week old corn seedlings. Geisler-Lee et al. (2013), however, observed higher Ag uptake with AgNPs treatment than $AgNO_3$ treatment. Consistent with the previous study, Yin et al. (2011) reported higher toxicity of gum Arabic-coated AgNPs than the same concentration of Ag^+, with uptake higher with AgNPs compared to Ag^+ treatments, in *L. multiflorum*.

Translocation of AgNPs and CuONPs were reported in sand-grown wheat seedlings (Dimpaka et al. 2013a, b); while Ag speciation occurred in the form of ionic Ag^+ and Ag-GSH, including as NPs themselves, within lettuce leaves with AgNPs treatments (Larue et al. 2014). Transformation of YbO_3NPs into Yb phosphates was recently documented within the root cells in cucumber (Zhang et al. 2012). Mesquite (*Prosopis juliflora* var. *velutina*) did not show characteristic signature of ZnONPs, which the plants were exposed to, within the types of tissues analyzed (e.g., root, stem, and leaf), and that the internalized Zn was identified to be in Zn(II) form, likely bound to unknown organics within the tissues (Hernandez-Viezcas et al. 2011). Likewise, Hernandez-Viezcas et al. (2013) found an absence of ZnONPs in soybean tissues when the plants were treated with high ZnONPs concentration (500 mg/Kg) through soil, and reported that Zn detected in grains, pods, and phloem (see Fig. 5 in Hernandez-Viezcas et al. 2013) resembled to model Zn citrate μ-XANES spectrum, indicating Zn–O bonding. In the same study, the authors located Ce within root nodule including in the root epidermis and pods in soybean. μ-XRF analysis revealed the presence of Ce in CeO_2 form, matching the μ-XRF signature of the applied CeO_2NPs to the plants (see Fig. 2 in Hernandez-Viezcas

et al. 2013). Zhang et al. (2012) found that CeO_2NPs were biotransformed to $CePO_4$, CeO_2 and Ce carboxylates within cucumber root and shoot tissues, and that the transformed NPs demonstrated needle-like, aggregated morphology.

Recently, Wang et al. (2012) reported translocation of CuONPs from root to shoot via xylem vessels and then back from shoot to root via phloem in corn. The authors suggested potential reduction of Cu^{2+} to Cu^{1+} during translocation, and that the NPs were observed in the form of larger aggregates within corn plant. Lin and Xing (2008) reported ZnONPs internalization in endodermal and vascular tissues in *L. perenne*. Wang et al. (2012) observed CuONPs being transported from root to various tissues/organs (xylem, leaf, root) and that root Cu concentration was twofold higher with CuONPs (100 mg Cu/L) than with Cu^{2+} ions treatments in corn. Zhai et al. (2014) observed modulation of particle size distribution as 15 nm or 25 nm AuNPs were taken up and transported to the shoot in poplar, whereas larger particles (50 nm) could retain their size in vivo. The authors could locate AuNPs within the roots in abundance than in leaves. However, plant uptake of Au(III) ions were significantly higher compared to AuNPs treatments. Within the plants, AuNPs were detected in various tissues including in phloem complex, xylem, cell wall, plastids, mitochondria, and more abundantly in plasmodesmata region (Zhai et al. 2014). Observation of TEM images provided an insight into the transport of AuNPs through plasmodesmata-endoplasmic reticulum route where they likely accumulated as the channels narrowed. Presence of AuNPs in the xylem within the leaves indicated that the NPs were transported during nutrients and water uptake (Zhai et al. 2014). Similar observations were previously reported for AgNPs which aggregated within the plasmodesmata and were likely transported via apoplastic pathway (Geisler-Lee et al. 2013).

Direct penetration of NPs through cell wall can be envisioned for smaller size NPs as the pore size on the cell wall (2–20 nm) may limit the passage to the NPs larger than 20 nm (Zhai et al. 2014). Furthermore, cell membrane acts as yet another barrier for extraneous agent to pass through. To test this hypothesis, Sabo-Attwood et al. (2012) investigated uptake and distribution of 3.5 nm or 18 nm sized citrate-coated AuNPs in tobacco (*Nicotiana xanthi*), where the authors observed uptake of Au only from 3.5 nm AuNPs treatments (see Fig. 1 in Sabo-Attwood et al. 2012), unlike with 18 nm sized AuNPs treatments, and that the larger sized particles were found adhered to tobacco root surfaces. Furthermore, exposure to 3.5 nm sized AuNPs resulted in leaf necrosis culminating in plant death, which did not occur with 18 nm AuNPs treatments (Sabo-Attwood et al. 2012). Another study suggested that a size threshold may occur for NPs translocation to the leaves which they reported to be <36 nm; while accumulation of TiO_2NPs in the wheat root could only occur if NPs are <140 nm in diameter, with higher accumulation that occurred when NPs were much smaller (in the size range 14–22 nm) (Larue et al. 2012). Combined these observations from chemically different ENMs bolster the premise that particle size could be an important factor regulating ENM biouptake in plants.

8.4 Potential Factors and Mechanisms of Nanoparticle Toxicity

More recently, measuring the seedling growth in *Phaseolus radiatus* and *S. bicolor*, Lee et al. (2012) reported particle-mediated toxicity of citrate-coated AgNPs (Citrate–AgNPs) in soil, while free Ag^+ toxicity was found to be more pronounced when tested in agar medium. A concentration-dependent inhibition of two different sized (20 nm vs. 100 nm) AgNPs on the biomass growth and frond number, including the greater effects of free Ag^+ compared to AgNPs, were observed in *Lemna minor* (Gubbins et al. 2011). However, the toxicity of gum Arabic-coated AgNPs was higher compared to the same concentration of dissolved Ag^+ in *L. multiflorum*, with greater Ag bioaccumulation observed with AgNPs treatment compared to Ag^+ (Yin et al. 2011).

Negatively charged, anionic carboxylated AuNPs conferred protection to the model lipid membrane against the extreme pH (=12) via shielding effects, whereas positively charged, cationic amino-AuNPs could penetrate and disrupt the model membrane (Tatur et al. 2013). Silva et al. (2014) have experimentally shown interaction between surface charge and particle size to influencing AgNPs toxicity in both the prokaryotic and eukaryotic model organisms, and developed highly precise predicative models based on empirical data to explain nanotoxicity. However, potential surface charge effects and its interaction with particle size in influencing ENM phytotoxicity have not received much attention and therefore what potential effect surface charge density might have remains to be tested in plants.

Amongst the types of ENMs used in commerce, AgNPs are the ones which have undergone high scrutiny as they are widely used in various antimicrobial applications and thus more information is available on potential toxicity mechanisms. Such information would be useful to regulators and industry partners to make informed decision while addressing potential health and safety issues of ENMs. As AgNPs can act as a reservoir for continual ionic Ag^+ release into the environment (Liu and Hurt 2010), it has been argued that AgNPs toxicity could likely be a combined effect of ENMs and the released ions (Pokhrel et al. 2013, 2014b). Direct change in lipid-bilayer structure leading to formation of pits on the cell wall and subsequently altering membrane permeability have been linked to AgNPs exposure (Fabrega et al. 2009). Literature suggests that antimicrobial properties apparently retained in AgNPs might be due to cellular internalization of particulates, thereby causing DNA damage, while potential ionic Ag^+ release within the cell from internalized AgNPs can inhibit ion exchange and cellular respiration (Ratte 1999). Physical interaction of AgNPs with the cell surface has also been implicated as an important factor mediating AgNPs toxicity (El Badawy et al. 2011). Furthermore, AgNPs are known to inhibit β-galactosidase activity leading to cell death (Pokhrel et al. 2012). Reactive oxygen species (ROS) release followed by oxidative stress have also been linked to AgNPs toxicity (Choi et al. 2008).

8.5 The Path Forward

Thorough review of current literature indicates that ENMs, when available in the environment (air, soil, and water), can be taken up by plants including by the food crops, and can potentially transfer from one generation of plants to next (e.g., CeO_2NPs in soybean; Hernandez et al. 2013). Although phytotoxicity of ENMs have been associated with the dissolved ions released from the ENM surfaces, higher toxicity of ENMs themselves have been reported for various types of metal-based ENMs, regardless of whether they tend to release toxic ions (e.g., AgNP, ZnONP) or not (e.g., CeO_2NP and TiO_2NP).

Phytotoxicity investigation using high-purity ENMs is critically important, and to this end, application of ultrapurification techniques such as tangential flow filtration (TFF) coupled with hollow fiber membranes have enabled separation of dissolved ions and impurities form the ENM suspension (El Badawy et al. 2011; Flory et al. 2013; Dorney et al. 2014; Pokhrel et al. 2012, 2014a, b). Use of commercially procured ENMs without further purification (as has been routinely reported in the literature) would complicate the mechanistic understanding of ENM toxicity to biologic receptors, including plants, owing to potential confounding effects of toxic impurities (e.g., heavy metal ions in CNT samples, and dissolved Cd/Se ions in commercial quantum dots samples) already present in the nanosuspensions.

To date, studies have mostly addressed acute toxicity (short-term, high dose) of ENMs at single plant species level and in controlled laboratory setting, providing valuable information on individual species responses to, and basic mechanistic understanding of, ENM stress. However, research investigating ecosystem-level responses, including the field trials, to chronic (long-term, low dose) ENMs exposure has not been fully realized and should be the focus of future research. More research is warranted to assess whether standard USEPA OPPTS 850.4200 bioassay could be adequately used for screening ENMs toxicity (Pokhrel and Dubey 2013), or if molecular endpoints (although less economical) could serve as a more sensitive screening tool. More focused research is needed to elucidate the biological basis of ENM toxicity and how ENMs' and biologic surface characteristics would interact in influencing fate and toxicity of ENMs and of modified by-products both in vivo and ex vivo (Silva et al. 2014). Also, important for nanotechnology to be sustainably used in agriculture, emphasis should be on biouptake, effects on food quality, and crop yield before more nanotechnology-based products (e.g., nano-enabled fertilizer, pesticides, growth stimulant, etc.) find routine applications in our farms.

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Chapter 9
Abiotic Stress Tolerant Transgenic Plants and Nanotechnology

Dhruv Lavania, Amit Kumar Singh, Manzer H. Siddiqui, Mohamed H. Al-Whaibi and Anil Grover

Abstract Crop plants are adversely affected by abiotic stresses. Drought is the most widespread and damaging of all environmental stresses. At the global level, significant proportion of cultivable land masses is affected by high salt levels. Heat and cold stresses profoundly affect agricultural yields of major crops. Also, the level of abiotic stresses is on the rise due to both natural and man-made interventions. The ambient temperature is gradually increasing due to the increased levels of CO_2 and other greenhouse gases. The episodes of drought and flooding stress have become more erratic over the years. The production of transgenic crops that can withstand increased level of abiotic stresses is a silver lining to sustain and increase food production in future. Techniques of producing transgenic crops need to be improvised to achieve high frequency transformation. Current experiments deploying nanotechnology tools for gene delivery are extremely relevant in production of new generation of transgenic plants. With such tools, it would be possible to experimentally produce higher number of transgenic lines and screen out the transgenic lines showing desired phenotype in higher numbers. In the ensuing paragraphs, we delve on the current status of abiotic stress tolerant transgenic crops and also project how nanotechnology tools can help in future endeavors.

Keywords Abiotic stress · Genetic transformation · Nanoparticles · Nanobiotechnology · Transgenics

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9.1 Introduction

9.1.1 Abiotic Stresses and Cultivation of Plants

Crop plants are adversely affected by abiotic stress conditions. Major abiotic stresses which affect plants include heat, cold, salinity, drought, flooding/submergence (anoxia), excess light, and chemical toxicities. Abiotic stresses profoundly affect growth and yield of plants. Drought or water stress is among the most important constraints in obtaining higher crop yields worldwide. Water stress affects plants in several ways. During vegetative stages, water stress reduces leaf expansion, photosynthesis, height of plant, and leaf area. Leaf rolling and leaf tip drying are the primary symptoms resulting from drought stress. Cell enlargement is severely affected by water stress. Decrease in water potential closes stomata and decreases transpiration (Kallarackal et al. 1990). The reproductive processes in plants are particularly sensitive to drought stress. Rice is most sensitive to drought for 10 days before flowering to the end of flowering (Yoshida et al. 1981). The water stress at flowering stage inhibits panicle exertion and spikelet filling. This causes high sterility, leading to decreased yield in rice (Ekanayake et al. 1989). The salt stress (mainly sodium chloride) results in Na^+ toxicity as well as physiological water stress to the plants. Estimates show that more than a third of all the irrigated land in the world is presently affected by salinity (Sahi et al. 2006). This is exclusive of the regions classified as arid and desert lands (which comprise 25 % of the total land of our planet). Salt affected lands occur in all climatic regions from deserts to tropical belts of Africa, Latin America, and Polar regions. The loss of farmable land due to salinization is directly in conflict with the needs of the world population. Excess salt levels affect enzyme activities and photosynthesis. The unfavorable Na^+ and Na^+/K^+ ratios adversely affect the grain yield of crops under salt stress conditions (Kaya et al. 2013). Temperature stress is observed when ambient temperature is below (low temperature stress) or above (high temperature stress) the optimal levels. These temperature extremes are injurious to plant growth and development as plants have evolved to grow in a narrow temperature regime (Singh and Grover 2008). In recent years, there has been a general increase in extreme events including floods, droughts, and heat episodes. The man-made changes in the climate of the earth due to the multifarious activities linked to development have become the focus of scientific attention (Grover et al. 2003). The most imminent of climatic changes of the earth is the increase in the atmospheric temperatures due to increased levels of CO_2 and other greenhouse gases. In certain places, climatic extremes such as droughts, floods, timing of rainfall, and melting of snow have also shown erratic trends. This is adversely affecting agriculture through its direct and indirect effects on crops (Grover et al. 2013).

9.2 Genetic Improvement of Plants Against Abiotic Stresses

From the preceding discussion, it is amply clear that crop production can be appreciably enhanced if proper management practices to save plants from such stressful conditions are devised. In simple terms, the crop production is an outcome of G × E equation where G refers to the genotype; E, the environment (El-Soda et al. 2014). It is relatively difficult, expensive, and hence impractical to change the environmental variables for obtaining optimal growth of crops. On the other hand, it is relatively inexpensive if the G factor (i.e., the genotype) can be suitably altered enabling plants to successfully grow, reproduce, and set seeds under stressful conditions. Crops have been genetically altered and improved for a host of different agronomic traits in the long history of the development of agriculture. Plant breeders have been greatly successful in enhancing the yields of major crops employing the conventional Mendelian tools like selection, hybridization, and progeny analysis. However, the response of crops against abiotic stresses is very complex because it involves multiple genes. The application of conventional plant breeding methods has proven difficult for genetic improvement of plants against abiotic stresses. In recent years, newer breeding tools like 'molecular breeding' have shown promising results in genetic improvement of plants against salt stress, flooding stress etc. (Jenks et al. 2007). The nonconventional tools of genetic improvement like 'transgenic tools' are becoming very significant for genetic improvement of crops against abiotic stresses.

9.3 Births and Growth of Transgenic Technology

Molecular biology science was born essentially in the middle of the twentieth century. The experiments leading to (1) elucidation of DNA structure and proposition of the double helix model of this molecule and (2) the discovery of the mechanisms underlying DNA replication and processes involved in its functioning (i.e., transcription leading to formation of RNA from DNA and translation leading to synthesis of proteins from mRNA) have brought revolutionary changes in our understanding of the living cells. In the beginning of 1980s, the art of producing transgenic plants was crafted (Galun and Breiman 1997). The production of transgenic plants provides a way to modify the genetics of plants so that superior types can be bred in a relatively short term. The gene transfers take place between the sexually compatible individuals in conventional and molecular plant breeding approaches. As against this, gene transfer is practiced across a wider platform of individuals overcoming the barriers of sexual compatibility in the transgenic

approach (Grover et al. 1999). For instance, transgenic plants have been produced for increased insect resistance by incorporating a gene from bacteria and for increased cold resistance incorporating a gene from fish using the methods of plant genetic engineering (Duman and Wisniewski 2014; Ibrahim and Shawer 2014). Since 1980, substantial progress has been made in transgenic plant production science. Transgenic plants with improved resistance to insect infestations using the most celebrated principle of Bt gene technology has been widely practised in a host of different crops (Peferon 1997). Significant success has also been obtained in breeding herbicide and virus resistance in crops by rDNA technology (Chen and Lin 2013). In recent years, transgenic plants have been produced to improve nutritional quality and to change the physiological and developmental aspects of plants (Zhu et al. 2007). To sum up, the approach of transgenic plant production is a new and, highly effective arsenal in the hands of agricultural scientists to enhance crop yields.

9.4 Production of Abiotic Stress Tolerant Transgenic Crops

Coming back to abiotic stresses, question we now address is how to engineer crops against abiotic stresses like salt, drought, and adverse temperatures. While much of the success in transgenic experiments has come for insect resistance and herbicide tolerance as mentioned above, several attempts have also been made to genetically engineer plants against abiotic stresses in the past 25 years. Scientists have explored a host of different transgenes for enhancing resistance of crops against salt, drought, and high and low temperatures. Several genes have enabled production of transgenic rice against drought, salt, flooding, and temperature extremes (Table 9.1). Likewise, host of plant species including model species like *Arabidopsis* and tobacco and crops like tomato and wheat have been genetically transformed for different abiotic stresses. It can be safely argued that results obtained from these experiments are of somewhat mixed nature. On the one hand, there are definite reports showing that plants have been successfully produced with enhanced abiotic stress tolerance by transgenic methods. On the other hand, there is poor success in field application of abiotic stress tolerant transgenic crops in spite of all the intensive efforts. Most of the success reported till this day is from laboratory-based experiments (Grover et al. 2013). It is therefore important to revisit these experiments and address what we possibly lack in our efforts in producing abiotic stress tolerant crops at the level of field cultivation.

The success in production of transgenic plants is principally governed by three critical inputs: (a) the availability of the effective gene, (b) relevant techniques for transferring the transgene inside the genome of the desired trans-host species, and (c) regulated expression of the transgene in the trans-host (Grover et al. 2001). The transfer of genes in genetic engineering experiments is largely a random event. Experiments have shown that the level of expression of the transcripts/proteins from the transgenes in the trans-hosts is highly variable. A great deal of effort is

Table 9.1 Protocol details involved in genetic engineering for increased abiotic stress tolerance in rice

Gene name	Encoding protein	Source species	Promoter	Phenotype of overexpression transgenic plants	References
Signal transduction genes					
SAPK4	Sucrose nonfermenting I-type serine threonine protein kinase	*Oryza sativa*	CaMV35S	Overexpression transgenic plants grew faster under salt stress conditions both at seedling and at mature plant stages	Diédhou et al. (2008)
DSM1	Mitogen activated protein kinase kinase kinase	*O. sativa*	Ubi-1 (*Zea mays* ubiquitin promoter)	Increased dehydration stress tolerance at seedling stage of overexpression transgenic plants	Ning et al. (2010)
OsSIK1	Receptor-like kinase	*O. sativa*	Double CaMV35S	Overexpression transgenic plants more tolerant to salt and drought stresses; enhanced peroxidase, superoxide dismutase, and catalase activities. Reduced stomatal density in overexpression transgenics	Ouyang et al. (2010)
OsCPK21	Calcium-dependent protein kinase	*O. sativa*	Ubi-1	Increased survival of overexpression transgenics under salt stress conditions. Growth inhibition of transgenic seedlings by abscisic acid (ABA) more than that of wild type (WT) seedlings	Asano et al. (2011)
OsITPK2	Inositol 1,3,4-triphosphate 5/6-kinase	*O. sativa*	CaMV35S	Hypersensitivity of overexpression transgenics to drought and salt stresses. Reduced levels of inositol triphosphate and genes related to osmoregulation and reactive oxygen species (ROS) homeostasis	Du et al. (2011)
OsCPK12	Calcium-dependent protein kinase	*O. sativa*	CaMV35S	Overexpression resulted in enhanced salinity tolerance, upregulation of ROS scavengers, greater ABA sensitivity and more susceptibility to blast disease resistance	Asano et al. (2012)
OsSIK2	S-domain receptor-like kinase	*O. sativa*	CaMV35S	Salt and drought stress tolerance resulting from overexpression. Early leaf development and delayed dark-induced senescence in transgenics	Chen et al. (2013)

(continued)

Table 9.1 (continued)

Gene name	Encoding protein	Source species	Promoter	Phenotype of overexpression transgenic plants	References
OsCPK9	Calcium-dependent protein kinase	*O. sativa*	CaMV35S	Increased drought tolerance through enhanced stomatal closure and better osmoregulation in transgenics. Higher pollen viability leading to increased spikelet fertility	Wei et al. (2014)
OsCPK4	Calcium-dependent protein kinase	*O. sativa*	Ubi-1	Salt and drought stress tolerance by prevention of stress-induced membrane lipid peroxidation in the transgenics. Overexpression transgenics exhibited higher water holding capacity and reduced electrolyte leakage under stress	Campo et al. (2014)
Transcription factor genes					
JERF3	Ethylene response factor protein	*O. sativa*	CaMV35S	Increased drought and osmotic stress tolerance; higher accumulation of soluble sugars and proline, upregulation of stress-responsive genes like *WCOR413-like*, *OsEnol* and *OsSPDS2* in overexpression transgenics	Zhang et al. (2010)
OsNAC10	NAC protein (acronym for *NAM* [no apical meristem], *ATAF1*-2, *CUC2*[cup-shaped cotyledon])	*O. sativa*	Constitutive GOS2 promoter and root specific RCc3 promoter	Increased tolerance to drought, salt, and low temperature at vegetative stage. Enlarged roots, greater tolerance, and improved grain yield under field level drought stress	Jeong et al. (2010)
OsWRKY45-1 and OsWRKY45-2	WRKY protein	*O. sativa*	Ubi-1	Against salt stress, OsWRKY45-1 allele overexpression showed no difference, OsWRKY45-2 allele overexpression showed increased tolerance. ABA signaling negatively regulated by OsWRKY45-1 allele and positively regulated by OsWRKY45-2 allele	Tao et al. (2011)

(continued)

Table 9.1 (continued)

Gene name	Encoding protein	Source species	Promoter	Phenotype of overexpression transgenic plants	References
ZmCBF3	C-repeat binding transcription factor	*Z. mays*	Ubi-1	Increased tolerance to drought, salt and cold stresses; no growth retardation and yield penalty under normal conditions	Xu et al. (2011)
OsDREB2A	Dehydration response element binding factor	*O. sativa*	4ABRC(stress-inducible promoter)	Induced overexpression enhanced survival of transgenic plants under severe drought and salt conditions	Cui et al. (2011)
OsNAC9	NAC protein	*O. sativa*	GOS2 and RCc3	Increased grain yield under normal conditions; under drought stress, increased yield in RCc3:OsNAC9 transgenics. Changed root architecture possibly imparting increased drought stress tolerance	Redillas et al. (2012)
OsbZIP46	Basic leucine zipper transcription factor	*O. sativa*	Ubi-1	Overexpression of native gene showed no effect on drought tolerance whereas overexpression of a constitutively active form of OsbZIP46 (named OsbZIP46CA1) enhanced drought and salt stress tolerance	Tang et al. (2012)
OsbZIP16	Basic leucine zipper transcription factor	*O. sativa*	Act-1(rice actin 1 promoter)	Increased tolerance to drought stress at seedling and tillering stages; increased sensitivity to ABA	Chen et al. (2012)
ZFP182	TFIII-A type zinc finger transcription factor	*O. sativa*	CaMV35S	Increased tolerance to salt, cold, and drought stresses; accumulation of proline and soluble sugars in transgenic plants	Huang et al. (2012)
EDT1/HDG11	Homeodomain-leucine zipper transcription factor	*Arabidopsis thaliana*	Act-1	Drought stress tolerance assigned to the extensively developed root system, lesser stomatal density, greater water use efficiency, accumulation of compatible osmolytes and higher antioxidant enzyme activities. Increased seed setting, bigger panicles, more tillers, and enhanced photosynthesis activity leading to higher grain yield of transgenic plants	Yu et al. (2013)

(continued)

Table 9.1 (continued)

Gene name	Encoding protein	Source species	Promoter	Phenotype of overexpression transgenic plants	References
AtDREB1A	Dehydration response element binding factor	*A. thaliana*	rd29-A (stress-inducible promoter)	Higher drought stress tolerance associated with osmolyte accumulation, chlorophyll maintenance, higher relative water content, and reduced ion leakage. Increased spikelet fertility and grain yield under normal and stressful conditions	Ravikumar et al. (2014)
Antioxidant genes					
katE	Catalase	*Escherichia coli*	CaMV35S	Overexpression transgenics were tolerant to salt stress throughout the life cycle. Higher catalase activity was observed in the transgenics	Nagamiya et al. (2007)
glyII	Glyoxylase II	*O. sativa*	CaMV35S	Higher tolerance to toxic levels of methylglyoxal and NaCl in overexpression transgenics with sustained growth under salt stress conditions	Singla-Pareek et al. (2008)
Sod1	Copper Zinc superoxide dismutase	*Avicennia marina*	Ubi-1	Increased tolerance to salt and drought stress and to oxidative stress induced by methyl viologen	Prashanth et al. (2008)
GST and *CAT1*	Glutathione-*S*-transferase (GST) and catalase	*O. sativa*	CaMV35S	Increased tolerance to cadmium alone and a combination of cadmium and heat stress. Lesser oxidative damage under stressful conditions mediated by coexpression of GST and catalase enzymes	Zhao et al. (2009)
AeMDHAR	Monodehydroascorbate reductase	*Acanthus ebracteatus*	Double CaMV358	Salt tolerance at germination and seedling stages. Higher number of tillers and 1,000-grain weight of transgenic plants	Sultana et al. (2012)
OsMIOX	Myo-inositol oxygenase	*O. sativa*	CaMV35S	Increased tolerance to polyethylene glycol induced drought stress. Higher expression of ROS scavenging genes and higher ROS scavenging enzyme activity and proline content in transgenic plants	Duan et al. (2012)

(continued)

Table 9.1 (continued)

Gene name	Encoding protein	Source species	Promoter	Phenotype of overexpression transgenic plants	References
γ-ECS	γ-glutamylcysteine synthetase	*O. sativa*	Rab21 (stress-inducible promoter)	Higher salt tolerance concurrent with efficient redox homeostasis and 1.5-fold higher germination rate; lesser ion leakage and higher chlorophyll-fluorescence upon methyl viologen treatment. Higher grain yield and biomass of field grown transgenic plants	Choe et al. (2013)
Osmotic homeostasis genes					
SAMDC	*S*-adenosylmethionine decarboxylase	*Tritordeum*	ABA-inducible promoter	Higher biomass and increased spermidine and spermine accumulation under salt stress	Roy and Wu (2002)
codA	Choline oxidase	*Arthrobacter globiformis*	CaMV35S	Glycine betaine accumulation; more than 50 % transgenic plants tolerant to salt stress	Mohanty et al. (2002)
otsA and *otsB* fusion	Trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP)	*E. coli*	ABA-inducible promoter and rbcS (Rice rubisco small subunit) promoter	Tolerance to salt, drought, and low-temperature stresses. Greater trehalose accumulation, increase photosynthesis, maintained plant growth, reduced photo-oxidation, and better ion homeostasis under stressful conditions	Garg et al. (2002)
otsA and *otsB* fusion	TPS and TPP	*E. coli*	Ubi-1	High accumulation of trehalose in leaves and seeds of transgenic plants. Greater tolerance to cold, drought, and salt stresses without stunting of growth	Jang et al. (2003)
p5cs	Δ^1-pyrroline-5-carboxylate synthetase	*Vigna aconitifolia*	Act-1 and an ABA-inducible promoter	Higher accumulation of proline both under constitutive and stress-induced production of *p5cs* in transgenic plants. Faster growth under salt and water deficiency stress. Transgenic plants with stress-inducible synthesis of *p5cs* showed greater biomass than constitutively synthesizing transgenic plants	Su and Wu (2004)

(continued)

Table 9.1 (continued)

Gene name	Encoding protein	Source species	Promoter	Phenotype of overexpression transgenic plants	References
adc	Arginine decarboxylase	*Datura stramonium*	Ubi-1	Higher putrescine production in transgenic plants under drought stress leading to greater accumulation of spermidine and spermine and stress tolerance	Capell et al. (2004)
COX	Choline oxidase	*Arthrobacter pascens*	SIP (ABA-inducible promoter) and Ubi-1	Greater salt stress tolerance in SIP transgenics than Ubi-1 transgenics in spite of lower glycine betaine accumulation in the former. Greater biomass production in SIP transgenics grown under salt stress conditions	Su et al. (2006)
codA	Choline oxidase	*A. globiformis*	CaMV35S	Tolerance to water stress and better yield; higher photo system II activity, increased detoxification of ROS. Survival rate and agronomic performance of transgenics is better under prolonged water stress	Kathuria et al. (2009)
OsTPS1	Trehalose-6-phosphate synthase	*O. sativa*	Act-1	Transgenic plants tolerant to cold, salt, and drought stresses; higher trehalose and proline accumulation, upregulation of several stress-responsive genes	Li et al. (2011)
Ion homeostasis genes					
AgNHX1	Vacuolar-type Na^+/H^+ antiporter	*Atriplex gmelini*	CaMV35S plus first intron of catalase from *Ricinus communis* L.	Overexpression in salt sensitive rice cultivar *Kinuhikari* resulted in increased salt stress tolerance. As against WT rice, transgenic plants showed survival under 3 day treatment of 300 mM NaCl	Ohta et al. (2002)
OsNHX1	Vacuolar Na^+/H^+ antiporter	*O. sativa*	CaMV35S	Increased tolerance to salt stress in overexpression transgenics plants	Fukuda et al. (2004)

(continued)

Table 9.1 (continued)

Gene name	Encoding protein	Source species	Promoter	Phenotype of overexpression transgenic plants	References
nhaA	Na^+/H^+ antiporter	*E. coli*	CaMV35S	Enhanced growth of transgenic plants compared to WT under salt stress conditions; higher sodium and proline contents under salt or drought stress	Wu et al. (2005)
SOD2	Plasma membrane Na^+/H^+antiporter	*Saccharomyces pombe*	CaMV35S	Increased salt stress tolerance in transgenic plants associated with higher P-ATPase activity, enhanced photosynthesis and H^+ exchange capacity in roots and reduced ROS generation	Zhao et al. (2006)
PgNHX1	Vacuolar Na^+/H^+ antiporter	*Pennisetum glaucam*	ABA-inducible promoter	Overexpression resulted in enhanced tolerance to salinity with extensive root development as compared to WT plants	Verma et al. (2007)
OsKAT1	Shaker family Potassium channel	*O. sativa*	Ubi-1	Higher salt tolerance and increased cellular K^+ content; lower Na^+ to K^+ ratios in overexpression cells	Obata et al. (2007)
OsHAK5	Potassium transporter	*O. sativa*	CaMV35S	Tolerance to salt stress; increased uptake of K^+ by roots and root to shoot transport of K^+, higher K^+/Na^+ ratio in the shoots	Yang et al. (2014)

therefore needed to select the progenies that optimally express the trans-protein. To do so, it is important that methods of genetic transformation must yield larger populations of the transformed progenies. The progenies need to be adequately screened to select transgenic types with the optimal levels of gene expression. For this input, it is critical that gene transfer technologies are suitably optimized for effective biotechnological applications.

9.5 Methods of Genetic Transformation of Crops

The transfer of genes in destined plant cells is a critical step in rDNA technology. Since 1970s, several techniques have been optimized to deliver the desired genes to plant cells (Anami et al. 2013). Among these, *Agrobacterium tumefaciens* has been the major workhorse for plant scientists for the gene transfer. For a large number of plant species, this is the most-promising method of gene delivery. When *Agrobacterium* infects a wound site, a portion of its DNA (called transfer/T-DNA) is mobilized and gets integrated into the chromosome of the host cells which begin to proliferate, leading to the formation of tumor-like growth. The genes transferred from the bacterium to the plant are carried on the extrachromosomal circular plasmids called tumor-inducing (Ti) plasmids (Tzfira and Citovsky 2006). For the *Agrobacterium* to be an effective vehicle for DNA transfer, the tumor-inducing gene of Ti plasmids is removed through a process called 'disarming' (Simpson et al. 1986). The bulk of the genetic transformations with *Agrobacterium* were initially shown to work with dicot plant species. In recent years, there has been significant success in genetic transformation of monocot species like rice, corn, and wheat by *Agrobacterium* (Ji et al. 2013).

Apart from *Agrobacterium*, several other methods for genetic transformation have been devised. One such method is PEG-mediated DNA uptake where PEG (polyethylene glycol) and $CaCl_2$ are used for stimulation of the DNA uptake process as well as its integration in the isolated protoplasts (Datta et al. 1992). Another method is a related technique of 'electroporation' where the isolated protoplasts are given a shock treatment electrically by giving a short, high voltage pulse, to make transient pores in the cell membranes. This enables the plasmid DNA molecules to pass through the pores and enter from culture medium to inside plant cells (Ji et al. 2013). Microprojectile bombardment or biolistics is another innovative way for DNA transfer (Ji et al. 2013). In this method, a microprojectile gun or DNA particle gun is used to deliver DNA directly into plant cells by shooting it through the cell. Microscopic particles of tungsten or gold are coated with plasmid DNA molecules and then shot into the target cells. In the process, DNA is transferred to the nucleus which then integrates into the plant genome. Among the various methods mentioned above, *Agrobacterium*-based approach stands out as the most favored technique for genetic transformation of crops because of its simplicity, reliability, and wider applicability.

9.6 Nanotechnology in Gene Transfer Experiments

Of late, principles of nanotechnology have been extensively applied in physical, biological, agricultural, and medical sciences in a highly beneficial manner. In textile industry, the use of nanotechnology includes minimizing loss of cellulose during processing of cotton into end product, coming out with 100 nm diameter fibers capable of being good absorbent of chemical input in agriculture and boosting the efficacy of enzymes required in cellulose–ethanol conversion (Lang 2013). The application of nanotechnology to produce nanosilica from rice husk has not only addressed the problem of rice husk disposal but also has come handy in making substances like glass and concrete (Awizar et al. 2013). Delivery systems for pests, nutrients, and plant hormones have been manufactured using nanotechnology tools (Sekhon 2014; Nair et al. 2010). Nanotechnology facilitates controlled release of agrochemicals allowing direct delivery of various macromolecules at the preconceived sites for enhancing plant disease resistance, nutrient utilization, and plant growth (Nair et al. 2010). Nanoencapsulation increases efficiency in terms of usage and safe handling of pesticides (Nair et al. 2010).

Lately, researchers have started applying nanotechnology in plant genetic engineering with noticeable results. Torney et al. (2007) demonstrated that a honeycomb mesoporous silica nanoparticle (MSN) system with 3 nm pores can act as an effective conduit for DNA and chemicals for their onward journey to segregated plant cells and intact leaves. Using MSN particles coated with a plasmid expressing green fluorescent protein (GFP) under CaMV35S promoter, the researchers showed transient expression in tobacco mesophyll protoplasts. Intact plant cells of tobacco cotyledons were also successfully transformed using gene gun after the mesopores of the DNA-coated MSN particles were capped by gold nanoparticles. Efficacy of this system for generating stable transgenic plants was established by raising callus from GFP bombarded immature maize embryos. Subsequently, the researchers generated transgenic tobacco plants harboring GFP gene driven by an inducible promoter which could be chemically triggered by addition of β-oestradiol. Transgenic plantlets were bombarded with β-oestradiol-loaded MSN particles having their pores capped by gold nanoparticles to prevent leaching. Presence of DTT as an uncapping agent in the germination medium allowed for the controlled release of the chemical inducer followed by expression of the GFP transgene. As the gold nanoparticles can prevent the molecules from moving out, the group, utilizing this trait, coated the β-oestradiol containing MSN particles with inducible GFP gene, capped the extremes with gold nanoparticles, and bombarded this system into wild type plants. Undoing the capping in presence of DTT resulted into controlled release of chemicals triggering gene expression in plants. Further diversification like increasing the probe size and multifunctionalization of these MSNs can help achieve target-specific delivery of proteins, nucleotides, and chemicals in plant cells. In addition, several biogenic species can be delivered together and released in a controlled fashion at the target sites by the use of MSN technology. This group proposed that nanotechnology can be applied to plant science to aid further investigation of plant genomics and gene function as well as improvement of crop species.

9.7 Future Generation Stress Tolerant Transgenic Plants

As discussed above, the production of abiotic stress tolerant transgenic plants is a highly desired commercial goal in present-day agriculture. To realize this goal, newer and more effective methods of genetic transformations are needed. The new discovery that using nanotechnology tools, gene transfers can be achieved in a controlled manner opens up new directions. Using this new method, it should be possible to transform wider range of species. Further, it should be possible to apply nanotechnology-based approach for genetically transforming those species which are not amenable to genetic transformation using *Agrobacterium*. By combining biotechnology and nanotechnology sciences, it should be possible to more effectively achieve goals like production of abiotic stress tolerant crops and plants with other desired agronomic traits.

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Chapter 10
Carbon Nanotubes and Modern Nanoagriculture

Maged F. Serag, Noritada Kaji, Manabu Tokeshi and Yoshinobu Baba

Abstract Since their discovery, carbon nanotubes have been prominent members of the nanomaterial family. Owing to their extraordinary physical, chemical, and mechanical properties, carbon nanotubes have been proven to be a useful tool in the field of plant science. They were frequently perceived to bring about valuable biotechnological and agricultural applications that still remain beyond experimental realization. An increasing number of studies have demonstrated the ability of carbon nanotubes to traverse different plant cell barriers. These studies, also, assessed the toxicity and environmental impacts of these nanomaterials. The knowledge provided by these studies is of practical and fundamental importance for diverse applications including intracellular labeling and imaging, genetic transformation, and for enhancing our knowledge of plant cell biology. Although different types of nanoparticles have been found to activate physiological processes in plants, carbon nanotubes received particular interest. Following addition to germination medium, carbon nanotubes enhanced root growth and elongation of some plants such as onion, cucumber and rye-grass. They, also, modulated the expression of some genes that are essential for cell division and plant development. In addition, multi-walled carbon nanotubes were evidenced to penetrate thick seed coats, stimulate germination, and to enhance growth of young tomato seedlings. Multi-walled carbon nanotubes can penetrate deeply into the root system and further distribute into the leaves and the fruits. In recent studies, carbon nanotubes were reported to be chemically entrapped into the structure of plant tracheary

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elements. This should activate studies in the fields of plant defense and wood engineering. Although, all of these effects on plant physiology and plant developmental biology have not been fully understood, the valuable findings promises more research activity in the near future toward complete scientific understanding of the behavior of carbon nanotubes in plants. This chapter focuses on the impact of carbon nanotubes on plants and the potential use of these unique nanomaterials in crop management and plant biotechnology.

Keywords Carbon nanotubes · Modern nanoagriculture · Crop plants

10.1 Introduction

Carbon nanomaterials are experiencing renewed interest as motivated by their interesting size, shape, and structure, as well as attractive, unique physical properties (Chen et al. 2003; Kam et al. 2004; Bianco et al. 2005; Cherukuri et al. 2004). A wide variety of carbon based nanomaterials such as fullerene, fullerene cages, single-walled carbon nanotubes, multi-walled carbon nanotubes, and cup-stacked carbon nanotubes have been engineered with high precision and quality. Carbon nanotubes are rolled up seamless cylinders of graphene sheets (Iijima 1991; Dai 2002; Dresselhaus and Dai 2004; Golberg et al. 2008). Single-walled carbon nanotubes are composed of one rolled sheet of graphene, while multi-walled carbon nanotubes are composed of several sheets of graphenes rolled up into concentric cylinders. Single-walled carbon nanotubes have a diameter close to 1 nm, with a tube length that can reach few micrometers, while the diameter of multi-walled carbon nanotubes can attain several tenths of nanometers. In recent years, efforts have been dedicated to unveil the biological application of carbon nanotubes because of their unique physicochemical properties (Singh et al. 2005; Gao et al. 2006; Kam et al. 2005, 2006). Work in this area is motivated both by the hope that carbon nanotubes will have useful applications in biology and by the concern that they may exert harmful effects on organisms. A considerable volume of the literature has appeared in the last decade on the application of carbon nanotubes in biological and medical sciences. Indeed, many valuable applications have emerged which admirably enriched the bioscience field (Kam et al. 2005; Bianco et al. 2005, 2011; Kostarelos et al. 2007; Pantarotto et al. 2004; Mènard-Moyon et al. 2010; Lacerda et al. 2006, 2012; Ali-Boucetta 2011).

During the past few years, there has been extensive interest in applying nanoparticles to plants for agricultural and crop management (Nanotechnology in Agriculture and Food 2006; Toreney et al. 2007; Khodakaovskaya et al. 2009, 2012; Serag et al. 2011a, 2012a; Husen and Siddiqi 2014). Indeed, the application of carbon nanomaterials in crop management becomes more pressing in the context of the increasing population and depleting resources. Researchers have demonstrated that the exposure of carbon nanotubes to plant seeds can increase the germination percentage and can enhance the growth of seedlings. These findings could result in significant developments of the production of valuable crops such

as maize and tomato, by taking advantage of the enhancement in the biomass of the plants. However, conflicting reports on the safety of carbon nanotubes have been outlined. These controversial findings require clarification to avoid confusion to the public (Liu et al. 2007, 2008; Singh et al. 2006; Lacerda et al. 2008).

In this chapter, we first shed light on the uptake and distribution of carbon nanotubes in plant cells, and then we give specific examples illustrating the application of carbon nanotubes in agricultural biotechnology. Specifically, we discuss the capability of carbon nanotubes to promote crops yield and reduce the pesticides uptake. Finally, we discuss the environmental and toxicity impacts of carbon nanotubes and further shed light on the future applications of carbon nanotubes in agriculture.

10.2 Plant Cell Uptake, Distribution, and Elimination of Carbon Nanotubes

The cell wall—a multilayered structure surrounding the cell—is unique to the plant kingdom, so it is not surprising that plants grow with defined shape, strength, and rigidity. The plant cell wall is a composite material built from a meshwork of hard cellulose fibers imbedded in a matrix of sugar polymers known as glycans. The plant cell wall represents a formidable barrier to parasites, bacteria, and macromolecules (McNeil et al. 1984). The ability to overcome this barrier has turned out to be beneficial for wide variety of biotechnology application including genetic manipulation and germplasm production (Evans 1983). The plant cell wall has narrow pores of average diameter of 5 nm. These pores allow the passage of solutes while limiting the diffusion of larger particles and macromolecules including some enzymes (Meiners et al. 1991). To tackle this barrier, enzymatic digestion was successfully employed. Enzymatic digestion of the cellulose meshwork via cellulose generates fragile protoplasts—plant cells appearing as spheroplasts after removal of their cell wall—that are easily damaged either physically or chemically. Also, the osmolality of the surrounding medium should be adjusted carefully to prevent cellular burst.

Several strategies have been employed to help carbon nanotubes to enter through the plant cell wall and plant cell membrane. These strategies are largely dependent on the diameter of carbon nanotubes with respect to the diameter of the plant cell wall pores:

1. Since the diameter of single-walled carbon nanotubes (Fig. 10.1) is 1–2 nm which is smaller than the diameter of the plant cell wall pores (5 nm), they were perceived to spontaneously leak into the apoplast (the fluidic space sandwiched between the plant cell wall and the plasma membrane). In order for this spontaneous leakage to be successful, the single-walled carbon nanotubes have to be shortened to a comparable size. Chemical methods such as ultrasonic-assisted chemical oxidative cutting are commonly used to break the carbon nanotubes into small pieces and, simultaneously, introduce carboxylic groups at their tips and walls to enhance their water solubility (Nakayama-Ratchford et al. 2007).

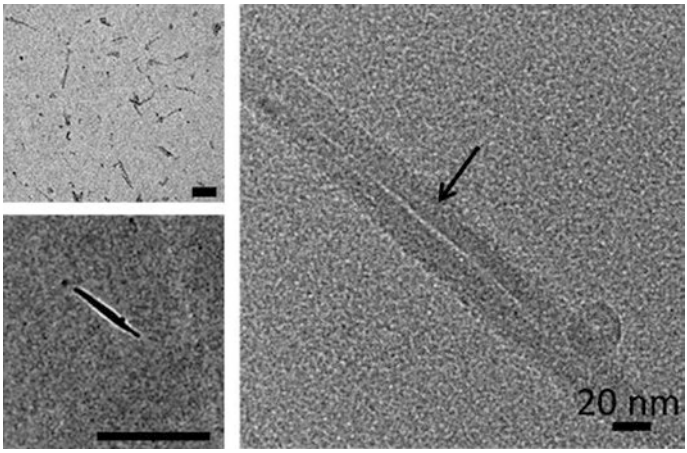

Fig. 10.1 Transmission electron micrograph of single-walled and multi-walled carbon nanotubes. *Top left* Single-walled carbon nanotubes. *Scale bar* 100 nm. *Bottom left* Multi-walled carbon nanotubes. *Scale bar* 500 nm. *Right* Zoomed image of multi-walled carbon nanotubes. The *arrow* indicates the thick wall of the nanotubes. Reprinted and adapted with permission from (Serag et al. 2011a, b). Copyright (2011) American Chemical Society

2. Generation of protoplasts was conceived to tackle the robust barrier of the cell by complete removal of the cell walls. Carbon nanotubes of diameters as wide as tens of nanometers (Fig. 10.1) can be made to penetrate the cell via simple interfacing after cell wall removal. Multi-walled carbon nanotubes were observed to penetrate the cell membrane of *Catharanthus roseus* protoplasts as nanoneedles similar to what have been previously shown for mammalian cells (Pantarotto et al. 2004; Lacerda et al. 2012).
3. Wide-diameter carbon nanotubes have been introduced into walled plant cells via local hydrolysis of the cellulosic cell wall. Cellulose molecules immobilized on the tips and walls of cup-stacked carbon nanotubes generated local lesions in the cell wall, through which the uptake of carbon nanotubes was facilitated.

10.2.1 Cellular Uptake, Distribution, and Exocytosis of Single-Walled Carbon Nanotubes

Single-walled carbon nanotubes have shown a unique ability to leak through the cell wall pores of both *Nicotiana tobacum* and *C. roseus* cells (Serag et al. 2011a, 2013). The first evidence on the internalization of single-walled carbon has been shown for *N. tobacum* in 2009 (Liu et al. 2009). *Nicotiana tobacum* showed temperature-dependent uptake of single-walled carbon nanotubes which suggested that the cell membrane internalization occurred via endocytosis (Fig. 10.2).

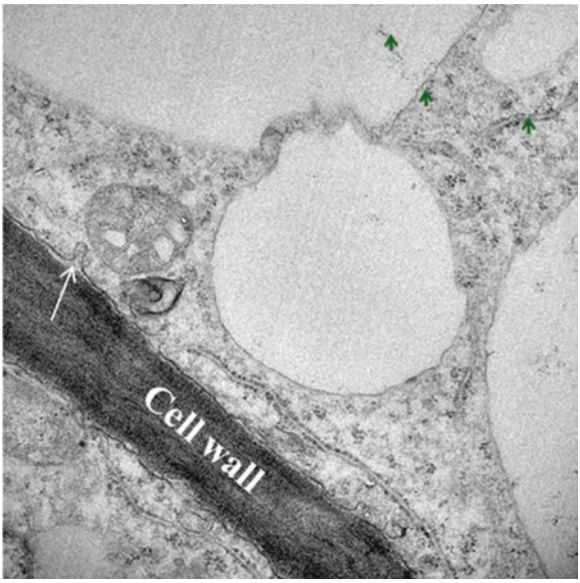

Fig. 10.2 Transmission electron micrograph showing a cytoplasmic view of *Catharanthus roseus* cell. The *white arrow* indicates invagination of plasmalemma leading to formation of small endocytosis organelle. *Scale bar* 500 nm. Reprinted and adapted with permission from (Serag et al. 2011a). Copyright (2011) American Chemical Society

Moreover, wortmanin—an endocytosis inhibitor—hampered the cell uptake and this provided conclusive evidence on the endocytosis-mediated internalization of single-walled carbon nanotubes. The capacity of *N. tobacum* cells to internalize single-walled carbon nanotubes was used to effectively co-transport molecular cargoes into the cellular compartments. Fluorescein isothiocyanate either free or conjugated to single-stranded DNA were successfully introduced into *N. tobacum* cells using single-walled carbon nanotubes. Interestingly, surface modification of single-walled carbon nanotubes by these molecular constructs has directed them to different cellular compartments. Single-walled carbon nanotubes with free fluorescein isothiocyanate were mainly internalized into the cell vacuole, while nanotubes wrapped with fluorescein isothiocyanate linked to single-stranded DNA were sequestered into the cytoplasm (Liu et al. 2009). The mechanism by which different payloads target different cellular compartments is still unclear. Nevertheless, it has been confirmed that single-walled carbon nanotubes follow the same mechanism by which free fluorescein isothiocyanates is translocated from the cytoplasm to the vacuole. After internalization, free fluorescein isothiocyanate anions were evidenced to move from the cytoplasm to the vacuole via protein carriers distributed through the tonoplast (the vacuolar membrane) (Oparka 1991). This carrier mediated transport is inhibited by the uricosuric drug probenecid, causing fluorescein isothiocyanate to exclusively accumulate in the cytoplasm. Single-walled carbon nanotubes conjugated with fluorescein isothiocyanate showed the same translocation/accumulation mechanisms after plant cell interfacing. Furthermore, washing-out probenecid caused the conjugates to redistribute from the cytoplasm to the vacuolar compartment. This indicated that single-walled

carbon nanotubes had no effect on the distribution mechanism of fluorescein isothiocyanate in the plant cell (Khodakovskaya et al. 2012). This observation is not surprising because the drastic sonication-assisted oxidation of single-walled carbon nanotubes usually downsizes the carbon nanotubes to a comparable size to the fluorescein isothiocyanate molecule. Therefore, the small size of fluorescein isothiocyanate conjugates and the high loading density on the surface of carbon nanotubes compared with the loading density of the single-stranded DNA-fluorescein isothiocyanate conjugate was hypothesized to favor the delivery of the former conjugate into the vacuoles via the protein carriers and to inhibit the vacuolar delivery of the later conjugates. These observations might explain the organelles-targeted delivery of carbon nanotubes.

The exclusive cytoplasmic accumulation of carbon nanotubes with stacked fluorescein isothiocyanate molecules in presence of probenecid increased their chances to enter the cell nucleus. Fluorescence recovery after photobleaching[1] revealed that single-walled carbon nanotubes conjugated with fluorescein isothiocyanate accumulated inside the nucleus of *C. roseus* cells. The positively charged nuclear proteins assisted the liberation of fluorescein isothiocyanate molecules in the nucleoplasm where they further accumulated inside the nucleolus.

Using raster scan image correlation spectroscopy,[2] it was further revealed that single-walled carbon nanotubes induce autophagy in *C. roseus* cells. Raster scan image correlation spectroscopy was used to generate quantitative spatial maps of single-walled carbon nanotubes dynamics inside different cell compartments. Raster scan image correlation spectroscopy revealed that the diffusion coefficient of single-walled carbon nanotubes conjugated with fluorescein isothiocyanate

[1] FRAP (Fluorescence recovery after photobleaching) is an optical technique used to quantify the lateral mobility of molecules inside the cells. The experimental setup comprises a microscope, a laser light source and a fluorescent probe (e.g. fluorescein isothiocyanate molecule) coupled to the molecule of interest (single-walled carbon nanotubes). Several images of the fluorescent probe were acquired to determine its initial fluorescence intensity, and then a bright laser illumination are focused for a short time on the region of interest to photobleach the fluorescence of the probe. Finally, another series of images are acquired using low level of illumination to track the gradual recovery of the fluorescence in the bleached area. This should give information about the lateral diffusional mobility of the molecules of interest.

[2] Raster scan image correlation spectroscopy (RICS) is an optical technique to measure the spatiotemporal distribution of fluorescent probes inside the cell. RICS is considered a merge of the conventional fluorescence correlation spectroscopy (FCS) and image correlation spectroscopy (ICS) technologies. FCS is an optical method that monitors the spontaneous fluctuations of fluorescence intensity collected from fluorophores in a small, open excitation laser beam volume. FCS can measure fast diffusion with only temporal information. ICS monitors temporal fluctuations at every point in a stack of 2-D images. ICS can measure very slow diffusion with spatial information. RICS is used to measure molecular dynamics from fluorescence confocal images such as binding and diffusion. It can be used to efficiently measure a wide range of diffusion coefficients ranging between 0.1 and 1,000 $\mu m^2 s^{-1}$.

inside *C. roseus* cell vacuoles was close to the diffusion coefficient in the cytoplasm. This indicated that parts of the cytoplasm leaked inside the vacuoles through autophagy (Autophagy ('self-eating'), is an ubiquitous stress response in eukaryotic organisms that targets damaged organelles for vacuolar degradation) (Serag et al. 2013; Minibayeva et al. 2012). High-resolution transmission electron microscopy further revealed that Golgi apparatus was involved in this stress response (Fig. 10.3) (Serag et al. 2012c).

The plant cell has a unique ability to limit the cytoplasmic localization of single-walled carbon nanotubes to minimize their toxicity. Laser scanning confocal microscopy showed that the plant cell rapidly distributes the internalized single-walled carbon nanotubes into the cell vacuole. Following this vacuolar redistribution, single-walled carbon nanotubes migrate toward the cell membrane via a vesicle-mediated transport pathway. The vesicles, filled with the nanotubes, shuttle between the tonoplast (the vacuolar membrane) and the plasma membrane. These shuttling vesicles, then, fuse with the plasma membrane expelling the nanotubes outside the cell (Fig. 10.4) (Serag et al. 2011a). Adding inhibitors of the vesicle-mediated transport to the cell medium (e.g., Exo1) resulted in trapping of the nanotubes inside the vesicles. This suggested the involvement of the vesicle-mediated transport in the exocytosis process of single-walled carbon nanotubes (Serag et al. 2011a).

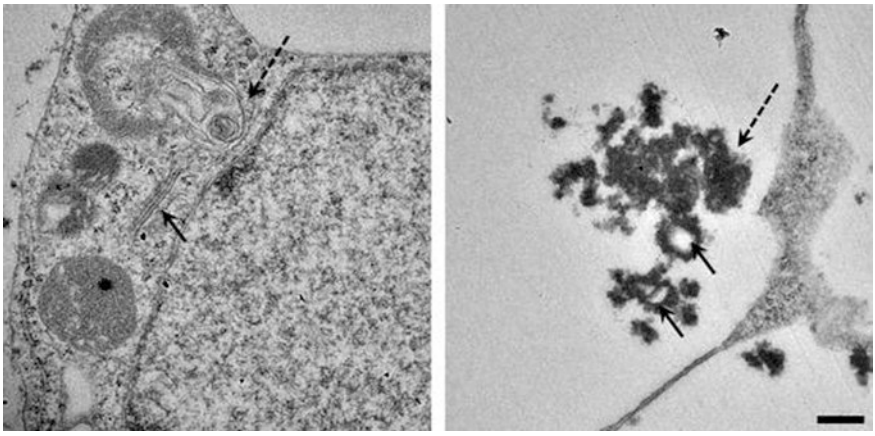

Fig. 10.3 High-resolution transmission electron microscopy imaging of the autophagy phenomenon. *Left* Cytoplasmic view of *C. roseus* cell incubated with single-walled carbon nanotubes. The *solid arrow* indicates an intact Golgi body, while the *dashed arrow* indicates a damaged one. *Right* Vacuolar view of *C. roseus* cell incubated with single-walled carbon nanotubes. The *arrows* indicate damaged Golgi-associated vesicles. The *dashed arrow* indicates extensive aggregates of single-walled carbon nanotubes due to the high salt concentration inside the cell vacuole. Reprinted and adapted with permission from (Serag et al. 2012b). Copyright (2012) American Chemical Society

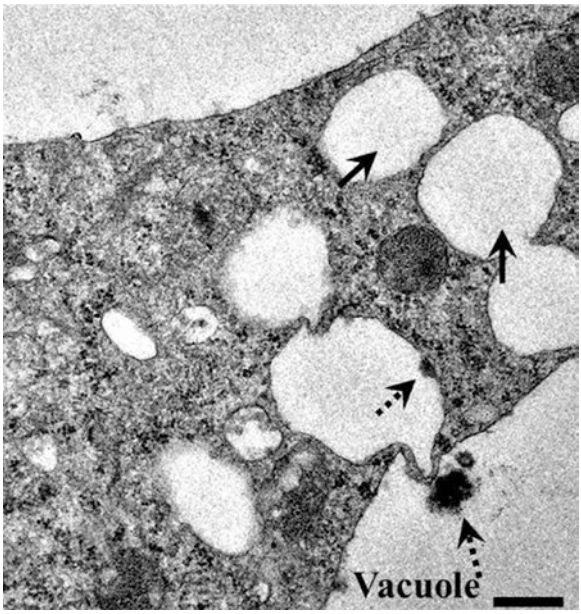

Fig. 10.4 Transmission electron micrograph showing a cytoplasmic view of *C. roseus* cell. The *black dashed arrows* indicate aggregates of single-walled carbon nanotubes associated with a vesicular membrane or inside vacuoles during vesicle-mediated transport. The *solid arrows* indicate large trafficking vesicles. *Scale bar* 500 nm. Reprinted and adapted with permission from (Serag et al. 2011b). Copyright (2011) American Chemical Society

10.2.2 Cellular Uptake, Distribution, and Exocytosis of Multi-walled Carbon Nanotubes

Unlike single-walled carbon nanotubes, the multi-walled nanotubes physically penetrated the cell membrane of *C. roseus* protoplasts through a nonendosomal route. Using high-resolution transmission electron microscopy, multi-walled carbon nanotubes were observed to penetrate the subcellular membranes as nanoneedles. Electron microscopy further revealed that the uptake of multi-walled carbon nanotubes are poorly associated with the endosomal organelles. Although the uptake of multiwalled carbon nanotubes occurs through an energy-independent route, increasing the concentration of nanotubes in the cell medium resulted in a decrease in the rate of the normal endocytosis function of the cell. This phenomenon was attributed to a carbon nanotubes-dependent increase in the medium tonicity which resulted in temporary inhibition of the endosomal cycle. This inhibition was proposed to promote the direct penetration of multi-walled carbon nanotubes into the cell rather than the endosomal route (Serag et al. 2011a).

The direct penetration of multi-walled carbon nanotubes assisted their translocation to most of the cell organelles. High-resolution transmission electron microscopy identified vacuoles, plastids and nucleus as the primary locations of nanotubes accumulations (Figs. 10.5 and 10.6). Following the uptake, multi-walled carbon nanotubes penetrated deeply into cell nucleus and localized in the peri-nuclear region. The average lengths of multi-walled carbon nanotubes distributed into different cell organelles were around 100 nm. Multi-walled carbon

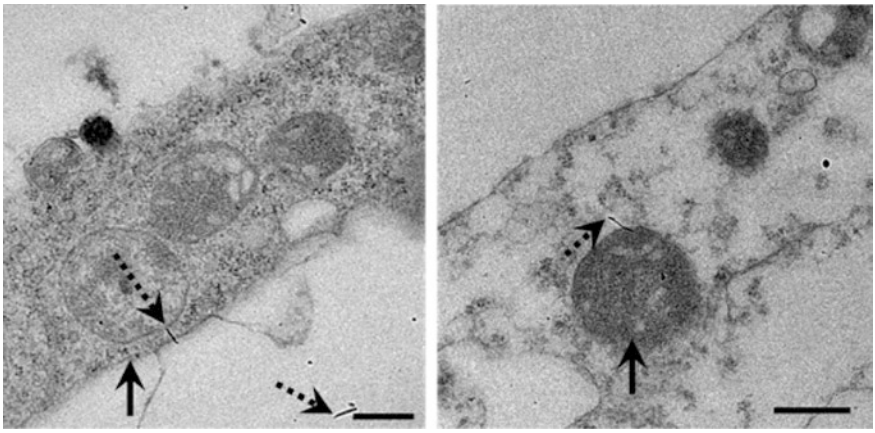

Fig. 10.5 Transmission electron micrograph showing cytoplasmic views of *C. roseus* cell. *Left* The micrograph shows a multi-walled carbon nanotubes penetrating the vacuolar membrane. *Dashed arrows* indicate multi-walled carbon nanotubes. The *solid arrow* indicates the vacuolar membrane (tonoplast). *Right* The *dashed arrow* indicates a multi-walled carbon nanotube penetrating the mitochondrial membrane. The *solid arrow* indicates a mitochondrion. *Scale bars* 500 nm. Reprinted and adapted with permission from (Serag et al. 2011b). Copyright (2011) American Chemical Society

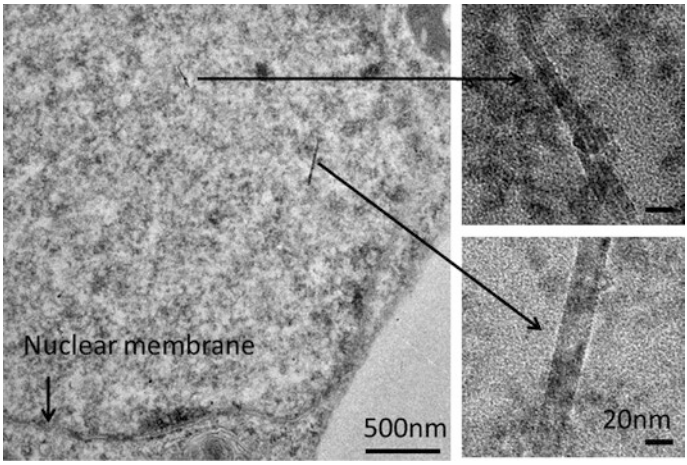

Fig. 10.6 Transmission electron micrographs showing carbon nanotubes accumulation inside the nucleus of *C. roseus*. *Left* The micrograph shows multi-walled carbon nanotubes inside the nuclear matrix. *Right* The corresponding zoomed-in images of the nucleus. Reprinted and adapted with permission from (Serag et al. 2011b). Copyright (2011) American Chemical Society

nanotubes longer than 100 nm were accumulated in most of the organelles of *C. roseus* cells, while short tubes (30–100 nm) accumulated inside vacuoles, plastids and the nucleus. Endoplasmic reticulum and mitochondria were resistant to the penetration of short multi-walled carbon nanotubes (Fig. 10.5). This suggested a

size-dependent distribution of multi-walled carbon nanotubes in *C. roseus* cells. This size-dependent distribution was proposed to offer a strategy of organelles-targeting via multi-walled carbon nanotubes.

10.2.3 Cellular Uptake and Fate of Cup-Stacked Carbon Nanotubes

Cup-stacked carbon nanotubes consist of closely-packed truncated graphene buckets. This unique structure differentiates them from the seamless cylinders structure characteristic of both single and multi-walled carbon nanotubes (Endo et al. 2002; Kim et al. 2004; Hasobe et al. 2006; Saito et al. 2006). This truncated conical structure provides wide room of reactive edges in the outer as well as inner surfaces and hence permits efficient functionalization. The average diameter of these nano-buckets range between 60 and 100 nm (Serag et al. 2012a).

A simple strategy has been proposed to deliver the cup-stacked carbon nanotubes into the walled plant cells. Cellulase enzyme that has the ability to hydrolyze the cellulosic wall surrounding the plant cell was immobilized on the tips of the cup-stacked carbon nanotubes. Cellulase subunits are tadpole-like protein structures with 21.5 nm size (Pilz et al. 1990). Therefore, the wide diameter cup-stacked carbon nanotubes are suited to carry this large protein structure. To introduce the cup-stacked carbon nanotubes into the plant cell, cellulose enzyme was covalently attached to the tips and walls of the cup-stacked carbon nanotubes. The immobilized cellulose induced tiny openings in the plant cell wall, through which the cup-stacked carbon nanotubes penetrated the cell. Indeed, this strategy bypassed the complete removal of the cell wall that negatively affects cell viability and plant regeneration (Serag et al. 2011a).

The cellular fate of cup-stacked carbon nanotubes has been elucidated using *Arabidopsis thaliana* cells. Cup-stacked carbon nanotubes were observed to participate in the cell transdifferentiation process into tracheary cells.[3] The presence of cup-stacked carbon nanotubes inside *A. Thaliana* cells has promoted their attachment to the cell microtubules. This was followed by oxidative cross-linking of monolignol (precursors of lignin biosynthesis) to the surface of the nanotubes leading to their deposition in the structure of the tracheary cells' walls (Serag et al. 2012a).

[3] Tracheary elements are lignified structures associated with xylem tissues in the plants. They are highly specialized for moving water and solutes from the root to the shoot system. In cell culture, the cells of some plants transdifferentiate into tracheary cells by the effect of phytohormones present in the cell medium. The transdifferentiation process begins with the deposition of secondary cell wall consisting of cellulose, lignin and hemicellulose. The transdifferentiation ends with a process called "programmed cell death" where all of the cells contents are removed leaving the cell empty.

10.3 Carbon Nanotechnology for Crops Biotechnology

The development of nanotechnology-based applications in agriculture is essential to tackle many difficulties that farming and food industry face across the world. The challenge has always been how to increase the agricultural output, detect plant diseases at appropriate timing, and efficiently advance the treatment. The process of maximizing the agricultural output requires improving the ability of plants to absorb nutrients from the soil. In this section, several examples are presented to illustrate the capability of carbon nanotubes to improve the agriculture output of edible plants.

10.3.1 Tomato

Compared with the plant cell wall and cell membrane, the thick seed coat is expected to hamper the penetration of carbon nanotubes into the embryo. However, researchers elucidated that multi-walled carbon nanotubes have the ability to penetrate the thick seed coat of tomato plant and increase seed germination and plant growth (Khodakaovskaya et al. 2009). Adding multi-walled carbon nanotubes at concentrations 10, 20, and 40 μg/ml to the nutrient medium significantly shortened the period of germination compared to the control sample lacking the multi-walled carbon nanotubes. The tomato plantlets germinated in presence of the multi-walled carbon nanotubes showed an increase in the biomass and, also, possessed well-developed stems compared with the control. The length of the rootlets appeared the same in both cases. Overall, tomato plants were shown to grow up to two times faster than the control. This was attributed to the increased number of pores in the seed coat that the multi-walled carbon nanotubes generate upon their penetration. These pores were hypothesized to provide better water and nutrient permeation (Khodakaovskaya et al. 2009, 2013).

Researchers also demonstrated the effect of multi-walled carbon nanotubes on the tomato plant in all stages of plant developments (Nair et al. 2012). In addition to the double height attained after treatment with multi-walled carbon nanotubes, the number of flowers was also doubled compared with the control. The mechanisms of all of these effects remain to be investigated.

The effect of single-walled carbon nanotubes on root elongation has been studied (Canas et al. 2008). Pristine single-walled carbon nanotubes negatively affected root elongation in tomato at 24 and 48 h incubation times. This inhibitory effect was attributed to an enhanced accumulation of the single-walled carbon nanotubes around the base of the apical meristem of tomato roots. Since the apical meristem is located in the zone of cell division, root elongation was inhibited.

10.3.2 Rice

The effect of carbon nanomaterials on the germination of rice seeds has been demonstrated (Nair et al. 2012). Particularly significant, carbon nanotubes were found to increase the water contents of the seeds during germination. The carbon nanotubes-treated seeds showed healthy and well-developed roots and shoots compared with control seedlings.

Experiments done on cell suspension of rice cells with multi-walled carbon nanotubes, however, showed various toxic effects. Primarily, multi-walled carbon nanotubes elicited the release of reactive oxygen species with consequent increase in the dead cells. Multi-walled carbon nanotubes also suppressed the activity of superoxide dismutase and decreased chlorophyll contents. These changes have been accounted for the natural self-defense of the plant to avoid affecting other cells (Tan et al. 2009).

Second generation rice plants grown from plant seeds initially germinated in nutrient media containing single-walled carbon nanotubes and multi-walled carbon nanotubes and, then, harvested after 6 months showed residual carbon nanotubes in their tissues. The accumulated carbon nanotubes in the seed resulted in 1 month delay in the germination of the seeds. The seed had more residual carbon nanotubes followed by root, stem, and then leaves. This indicated that carbon nanotubes were transmitted to the next generation of the plant and resulted in adverse effects (Tan et al. 2009).

10.3.3 Maize

Maize seedlings grown in agar nutrient media and treated with different concentration of multi-walled carbon nanotubes had dramatic effect on the growth. Pristine multi-walled carbon nanotubes increased the growth of plant rootlets. The water imbibition rate was found to be higher in the presence of the multi-walled carbon nanotubes. Furthermore, ionic nutrient transport showed dramatic enhancement that could be facilitated by water inflow. Higher concentrations of multi-walled carbon nanotubes, however, had less dramatic effect. These findings provided a clue for the utilization of multi-walled carbon nanotubes for optimizing water transport and increasing the biomass yield of maize (Tiwari et al. 2013).

10.3.4 Mustard

The rate of germination and growth of *Brassica juncea* has been evaluated in presence of pristine multi-walled carbon nanotubes and oxidized multi-walled carbon nanotubes. Researchers found that oxidized multi-walled carbon nanotubes

increased the moisture contents of seeds and enhanced the water absorption machinery of root tissues. The also had a positive effect on the seed germination rate. These effects were observed at very low concentration of the nanotubes (2.3×10^{-3} mg/mL). The enhancement of the growth rate could be obtained by soaking the seed in a solution of the oxidized multi-walled carbon nanotubes instead of adding them to the germination and growth media (Mondal et al. 2011). The mechanism of water influx into the seed in presence of carbon nanotubes is still under intensive research. It was shown that immersing the plant tissues in a solution of carbon nanomaterials increased its electrical conductance (Zheng et al. 2005). This effect was suggested to affect the plant aquaporins (specialized channels that regulate water and salts influx by selectively allow water molecules to flow into the plant tissues to maintain equilibrium). Furthermore, it has been reported that multi-walled carbon nanotubes activates the expression of the aquaporin gene (*LeAqp2*) (Zheng et al. 2005).

High concentration of the oxidized multi-walled carbon nanotubes (exceeding 46×10^{-3} mg/mL) had harmful effect to the plant growth and the biomass production. It was concluded that the rate of growth was concentration dependent. Researchers, also, found that the nanotubes migrate through the plant vascular tissues through the plant. These significant effects were proposed to be useful in horticulture, agriculture, and biofuel production (Mondal et al. 2011).

10.3.5 Onobrychis

Multi-walled carbon nanotubes have been shown to enhance the growth of *Onobrychis arenaria* plant and stimulate the peroxidase activity as well. The translocation of multi-walled carbon nanotubes to leaves and roots was also reported for *Onobrychis*. The increase in the peroxidase activity was associated with the oxidative stress, a characteristic effect of carbon nanomaterials on plant cells. Researchers have shown that multi-walled carbon nanotubes accumulated at the root surface and penetrated the epidermal cells. This penetration resulted in an injury associated elevation of peroxidase activity. Transmission electron microscopy images have supported the accumulation of multi-walled carbon nanotubes in different plant tissues (Smirnova et al. 2012).

10.3.6 Wheat

Wheat germination exhibited significant enhancement in presence of multi-walled carbon nanotubes. After treating wheat with Fe_3O_4-functionalized multi-walled carbon nanotubes, they were detected in the epidermis of the root. With the aid of Raman spectroscopy, it was elucidated that the functionalized carbon nanotubes adsorbed onto the root surface without affecting the plant development or

root-tissue morphology. Researchers detected functional groups such as carboxylic acids and isothiocyanates at the sites of carbon nanotubes adsorption. This might be a consequence of the chemical binding of Fe_3O_4-functionalized multi-walled carbon nanotubes to the plant tissues (Miralles et al. 2012).

In the same study, (Miralles et al. 2012) the researchers have compared the effect of multiwalled carbon nanotubes on the germination rate of wheat and alfalfa plants. Multi-walled carbon nanotubes enhanced wheat germination rate compared with that of alfalfa. This has been accounted for the ease of nanotubes penetrability in the case of Wheat. Wheat seed is relatively softer that than of alfalfa, therefore, the uptake of carbon nanotubes was conceived to be much more facilitated.

10.3.7 Cucumber, Onion, Lettuce, Carrot, and Cabbage

In a pilot study, the effect of single-walled carbon nanotubes on root elongation of cucumber, onion, lettuce, carrot, and cabbage was explored (Canas et al. 2008). Interestingly, different crops showed different responses to single-walled carbon nanotubes. Cucumber and onion were the only crops that responded positively to single-walled carbon nanotubes where root elongation was enhanced at concentrations more than 100 mg/L. Root elongation of cabbage and carrot was unaffected by single-walled carbon nanotubes while the root elongation in only one species, lettuce, was inhibited after 48 h. Alteration of surface chemistry of the root was hypothesized to be a major factor that contribute to these effects. For example, single-walled carbon nanotubes may affect microbial–root interaction in the rhizosphere and may cause microbial toxicity.

Cucumber was chosen as a model plant to explore the root uptake. After exposure to single-walled carbon nanotubes for 48 h, the predominant mechanism of the uptake was energy-independent passive uptake where no sign of active uptake has been detected.

10.4 Carbon Nanotubes and Pesticides

The effect of multi-walled carbon nanotubes on the residues of pesticides in a number of plants was studied (De La Torre-Roche et al. 2013). It was evidenced that pesticide residues in zucchini, corn, tomato and soybean was reduced in presence of multi-walled carbon nanotubes. For example, chlordane and DDT accumulation in different parts of the above plants has been reduced in a dose-dependent fashion by the presence of multi-walled carbon nanotubes. Although this effect has been demonstrated for different species, the underlying mechanisms and implications for food safety should receive rigorous investigations.

10.5 Environmental and Toxicity Impact of Carbon Nanotubes

An increasing number of studies outlined the environmental impacts and safety profile of carbon nanomaterials (Lin and Xing 2007; Schwab et al. 2011; Khodakovskaya et al. 2011; Oleszczuk et al. 2011; Wei et al. 2010; Shen et al. 2010; Stampoulis et al. 2009). It was revealed that carbon nanotubes functionalization dramatically reduces their toxic effects (Sayes et al. 2006; Kostarelos et al. 2009). In several studies, functionalized carbon nanotubes have not shown toxic effects on plant cell (Serag et al. 2011a, 2012b). However, in other studies pristine carbon nanotubes were reported to induce various toxic effects. For example, single-walled carbon nanotubes induced toxic effects in rice and *Arabidopsis* including cell aggregation, chromatin condensation, plasma membrane deposition, and H_2O_2 accumulation. Twenty-five mg/mL of single-walled carbon nanotubes was sufficient to cause death to 25 % of cultured protoplasts within 6 h. This toxic effect was due to oxidative stress that led to apoptosis (Shen et al. 2010). With a similar mechanism, pristine multi-walled carbon nanotubes were reported to reduce the biomass of *Cucurbita Pepo* plants in hydroponic cultures indicating a high degree of toxicity.

10.6 Future of Nanoagriculture Technologies Based on Carbon Nanotubes

The emerging science of nanoagriculture continues to rapidly advance and promises valuable technologies for enhancing the agricultural output. In a recent study (Giraldo et al. 2014), it has been shown that single-walled carbon nanotubes passively transport and target the lipid envelope of extracted plant chloroplasts. Upon their accumulation, they enhance the electron transport rate and the photosynthetic activity over three times compared with that of the control. The semiconducting single-walled carbon nanotubes were shown to increase chloroplast carbon capture by promoting chloroplast solar energy utilization. Furthermore, single-walled carbon nanotubes were shown to enable near-infrared fluorescence monitoring of nitric oxide. This property could open new avenue toward development of plants as photonic chemical sensors. For example, single-walled carbon nanotubes real-time sensing of nitric oxide in leaves could be extended to detect pesticides, herbicides, and environmental pollutants.

Several studies have focused on the interface of nanotechnology and plant biology (Serag et al. 2011a, 2012b). Carbon nanotubes were able to traffic through the plant cell wall and most of the subcellular organelles. The subcellular distribution of carbon nanotubes was strongly dependent on their lengths and the nature of the functional tag adsorbed or covalently linked to their surfaces. The demonstrated ability of preferential accumulation is of particular importance for plant biotechnology and agriculture where biomolecules could be co-delivered to specific subcellular organelles.

The incorporation of carbon nanotubes in the structure of tracheary elements (Serag et al. 2012b) could prove useful for wood bioengineering. It was conceived that plant woods can be reinforced with carbon nanotubes fibers to impart novel characteristics to the wood such as more strength (Serag et al. 2013).

Carbon nanotubes could be regarded as a potential nano-vector to transfect plant cells with genes of interest. Single-stranded DNA molecules wrapped around SWCNTs were able to target the cytoplasm of walled plant cells. This property could be used to introduce RNA pieces into the nucleus to activate or silence the genes of interest. Similarly, protoplast could be a target for delivering larger DNA molecules such as the delivery of plasmids into the plant cell genome.

In conclusion, carbon nanotechnology has the potential to enable advanced application in agriculture. Starting from increased crops yield passing by organelles-targeted gene delivery and ending with wood and chloroplasts engineering, in the following years carbon nanotubes are expected to show more opportunities in the agricultural field that remained beyond technical realization.

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Chapter 11
Phytosynthesis of Nanoparticles

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Abstract Increasing pressure to develop green and eco-friendly methods for synthesizing nanoparticles has provoked researchers to shift to microorganisms and biological systems. Plants can be used in large-scale production of nanoparticles, and in order to improve their potential in nanoparticle synthesis, it is necessary to investigate the biochemical and molecular mechanisms of nanoparticle formation. This chapter reviews phytosynthesis of nanoparticles, the influences of reaction conditions, and mechanistic aspects.

Keywords Green chemistry · Green synthesis · Nanoparticles · Nanoparticle synthesis · Phytosynthesis · Plants

11.1 Introduction

Development of reliable and environmentally friendly process for synthesis of metallic nanoparticles (NPs) is an important step in the field of application of nanobiotechnology and nanoscience. There are various methods for the synthesis

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Table 11.1 Photosynthesis of metal and metal oxide NPs

Plants	Nanoparticle	Size (nm)	Morphology	References
Acalypha indica	Silver	20–30	Spherical	Krishnaraj et al. (2010)
Allium cepa	Silver	~100	Spherical, cubic	Parida et al. (2011)
Allium sativum	Silver	3.7 ± 0.9 (1.0 mL extract), 3.8 ± 1.3 (1.5 mL extract), 4.1 ± 1.5 (2.0 mL extract)	Spherical	White II et al. (2012
A. sativum	Gold	1.8 ± 0.85–23.2 ± 4.1	Spherical, crystalline	Rastogi and Arunachalam (2012)
A. sativum	Gold	9–15	Spherical	Coman et al. (2013)
Aloe vera	Gold and silver	–	Spherical, triangular	Chandran et al. (2006)
A. vera	Indium oxide	5–50	Spherical	Maensiri et al. (2008)
Alternanthera dentate	Silver	50–100	Spherical	Kumar et al. (2014)
Annona squamosa	Silver	35 ± 5 nm	Spherical	Kumar et al. (2012)
A. squamosa	Titanium dioxide	23 ± 2 nm	Spherical	Roopan et al. (2012b)
Apiin extracted from henna leaves	Silver	39	Spherical, triangular	Kasthuri et al. (2009b)
Apiin extracted from henna leaves	Gold	7.5–65	Quasi-spherical	Kasthuri et al. (2009b)
Arbutus unedo	Silver	9–15	Spherical	Naik et al. (2013)
Artemisia nilagirica (Astcraccae)	Silver	70–90	Spherical, triangular, hexagonal	Vijayakumar et al. (2013)
Avena sativa	Gold	5–20 (pH 3 and 4), 25–85 (pH 2)	Rod-shaped	Armendariz et al. (2004a)
Azadirachta indica	Gold, silver and silver–gold alloys	5–35 and 50–100	Spherical, triangular, hexagonal	Shankar et al. (2004a)
Black tea leaf extracts	Gold and silver	20	Spherical, prism	Begum et al. (2009)

(continued)

Table 11.1 (continued)

Plants	Nanoparticle	Size (nm)	Morphology	References
Boswellia serrata	Silver	7.5 ± 3.8	Spherical	Kora et al. (2012)
Brassica juncea	Silver	2–35	Spherical	Haverkamp and Marshall (2009)
Bryophyllum sp.	Silver	2–5	Fee unit cell structure	Jha et al. (2009b)
Cacumen platycladi	Gold–palladium biomettalic	7.4 ± 0.8 nm	Spherical	Zhan et al. (2011)
Cajanus cajan	Silver	5–60	Spherical	Nagati et al. (2012)
Camellia sinensis	Gold	40	Spherical, triangular, irregular	Vilchis-Nestor et al. (2008)
C. sinensis	Gold	2.94–45.58	Spherical, triangular, hexagonal	Boruah et al. (2012)
Carica papaya	Silver	60–80	Spherical	Mude et al. (2009)
Chenopodium album	Gold and silver	10–30	Quasi-spherical	Dwivedi and Gopal (2010)
Cinnamomum camphora	Gold and silver	55–80	Triangular, spherical (Au) and quasi-spherical (Ag)	Huang et al. (2007)
C. camphora	Palladium	3.2–6		Yang et al. (2010)
Cinnamon zeylanicum	Silver	31–40	Cubic, hexagonal	Sathishkumar et al. (2009b)
C. zeylanicum	Palladium	15–20	Crystalline	Sathishkumar et al. (2009a)
Citrus limon	Silver	<50	Spherical, spheroidal	Prathna et al. (2011)
Cochlospermum gossypium	Silver	3	Spherical	Kora et al. (2010)
Cocos nucifera	Silver	23 ± 2	Spherical	Roopan et al. (2013)
C. nucifera	Silver	22	Spherical	Mariselvam et al. (2014)
Coriandrum sativum	Gold	6.75–57.91	Spherical, triangular, truncated triangular, decahedral	Badri Narayanan and Sakthivel (2008)

(continued)

Table 11.1 (continued)

Plants	Nanoparticle	Size (nm)	Morphology	References
Cuminum cyminum	Silver	6–15	Spherical	Kudle et al. (2012)
Cymbopogon flexuosus	Gold	200–500	Spherical, triangular	Shankar et al. (2004b)
Cyperus sp.	Silver	2–5	Fee unit cell structure	Jha et al. (2009b)
Cycas sp.	Silver	2–6	Spherical	Jha and Prasad (2010)
Datura metel	Silver	16–40	Spherical, ellipsoidal	Kesharwani et al. (2009)
Delonix elata	Silver	35–45	Spherical	Sathiya and Akilandeswari (2014)
Delphinium denudatum	Silver	<85	Spherical	Suresh et al. (2014)
Desmodium triflorum	Silver	5–20	Spherical	Ahmad et al. (2011)
Diospyros kaki	Bimetallic Gold/silver	50–500	Cubic	Song and Kim (2008)
Eclipta sp.	Silver	2–6	Spherical	Jha et al. (2009a)
Emblica Officinalis	Gold and silver	(10–20) and (15–25)	–	Ankamwar et al. (2005b)
Enhydra fluctuans	Silver	100–400	Spherical	Roy and Barik (2010)
Eucalyptus camaldulensis	Gold	1.25–17.5	Crystalline, spherical	Ramezani et al. (2008)
Eucalyptus citriodora	Silver	~20	Spherical	Ravindra et al. (2010)
Eucalyptus hybrida	Silver	50–150	Crystalline, spherical	Dubey et al. (2009)
Euphorbia hirta	Silver	40–50	Spherical	Elumalai et al. (2010)
Ficus bengalensis	Silver	~20	Spherical	Ravindra et al. (2010)
Garcinia mangos tana	Silver	35	Spherical	Veerasamy et al. (2011)
Gardenia jasminoides E.	Palladium	3–5	–	Jia et al. (2009)
Gliricidia sepium	Silver	10–50	Spherical	Rajesh et al. (2009)
Hibiscus rosa sinensis	Gold and silver	14	Spherical, prism	Philip (2010)
Hydrilla sp.	Silver	2–5	Fee unit cell structure	Jha et al. (2009b)

(continued)

Table 11.1 (continued)

Plants	Nanoparticle	Size (nm)	Morphology	References
Ipomoea aquatica	Silver	100–400	Spherical and cubic	Roy and Barik (2010)
Ipomoea indica	Silver	10–50	Spherical	Pavani et al. (2013)
Jatropha curcas	Silver	10–20	Fee unit cell structure	Bar et al. (2009c)
J. curcas	Silver	15–50	Spherical	Bar et al. (2009b)
J. curcas	Lead	5–17.5	Spherical	Joglekar et al. (2011)
Lantana camara	Silver	39.6	Irregular, spherical	Thirumurugan et al. (2011)
Lawsonia inermis	Copper	27–45	Spherical	Cheirmadurai et al. (2014)
Ludwigia adscendens	Silver	100–400	Spherical	Roy and Barik (2010)
Malus domes tic a	Silver	50–300	Flower-like	Umoren et al. (2014)
Medicago sativa	Titanium–nickel alloys	1–4	FCC-likc geometry for smallest dusters and complex arrays for biggest ones	Schabes-Retchkiman et al. (2006)
M. sativa	Gold	2–40	Irregular, tetrahedral, hexagonal platelet, decahedral, icosahedral	Gardea-Torresdey et al. (2002a, b, 2003)
M. sativa	Iron oxide	2–10	Crystalline	Herrcra-Bccerra et al. (2008)
Mentha piperita	Silver	5–30	Spherical	Parashar et al. (2009a, b)
M. piperita	Silver	90	Spherical	Ali et al. (2011)
M. piperita	Gold	150	Spherical	Ali et al. (2011)
Millingtonia hortensis	Silver	10–40	Spherical	Gnanajobitha et al. (2013)
Moringa oleifera	Silver	57	Spherical	Prasad and Elumalai (2011)
Murraya koenigii	Silver	10	Crystalline, spherical	Philip et al. (2010)
Murraya koenigii	Gold	20	Spherical, triangular	Philip et al. (2010)
Musa paradisiaca	Silver	–	Crystalline, irregular	Bankar et al. (2010)
Musa sapientum L.	Silver	96.74–156.94	Spherical	Dineshkumar et al. (2012)

(continued)

Table 11.1 (continued)

Plants	Nanoparticle	Size (nm)	Morphology	References
Nelumbo nucifera	Silver	25–80	Spherical, triangular, truncated triangular, decahedral	Santhoshkumar et al. (2010)
Nigella sativa	Silver	1.5–4	Spherical	Ranjan et al. (2013)
Ocimum sanctum	Silver	10 ± 2 and 5 ± 1.5 nm	Spherical	Ahmad et al. (2010)
O. sanctum	Gold	30	Crystalline, hexagonal, triangular	Philip and Unni (2010)
O. sanctum	Silver	10–20	Spherical	Philip and Unni (2010)
Ocimum tenuiflorum	Silver	7–15	Spherical, ovoid crystalline	Vignesh et al. (2013)
Origanum vulgare	Silver	136 ± 10.09	Spherical	Sankar et al. (2013)
Parthenium hysterophorus	Silver	~50	Irregular	Parashar et al. (2009a, b)
Pear fruit extract	Gold	200–500	Triangular, hexagonal	Ghodake et al. (2010)
Pelargonium graveolens	Gold	20–40	Decahedral, icosahedral	Shankar et al. (2003b)
P. graveolens	Silver	16–40	Crystalline	Shankar et al. (2003a)
Pelargonium roseum	Gold	2.5–27.5	Crystalline	Ramezani et al. (2008)
Phoma glomerata	Silver	2–100	Spherical	Gade et al. (2014)
Physalis alkekengi	Zinc oxide	72.5	Crystalline	Qu et al. (2011b)
Pinus eldarica	Silver	10–40	Spherical	Iravani and Zolfaghari (2013)
Piper betle	Palladium	4 ± 1	Spherical, crystalline	Mallikarjuna et al. (2013)
Plectranthus amboinicus	Silver	~18	Spherical	Ajitha et al. (2014)
Polyalthia longifolia	Silver	~50 (10^{-3} M, 25 °C), ~20 (10^{-4} M, 25 °C), ~35 (10^{-3} M, 60 °C), ~15 (10^{-4} M, 60 °C)	Spherical	Kaviya et al. (2011)
Prosopis juliflora	Silver	11–19 (XRD pattern)/35–60 (SEM images)	Triangular, tetragonal, pentagonal and hexagonal	Raja et al. (2012)

(continued)

Table 11.1 (continued)

Plants	Nanoparticle	Size (nm)	Morphology	References
Psidium guajava	Gold	25–30	Mostly spherical	Raghunandan et al. (2009)
Punica granatum (seed cxtract)	Silver	30	Spherical	Chauhan et al. (2011)
Rhizophora apiculata	Silver	19–42	Spherical	Antony et al. (2011)
Rumex hymenosepalus	Silver	2–40	Fee, hexagonal, spherical	Rodríguez-León et al. (2013)
Salicornia brachiata	Gold	22–35	Spherical	Ahmed et al. (2014)
Saraca indica	Gold	15–23	Spherical, triangular, tetragonal, pentagonal, hexagonal	Dash et al. (2014)
Scutellaria barbata D. Don *(Barbated skullcup)*	Gold	5–30	Spherical, triangular	Wang et al. (2009)
Securinega leucopyrus	Silver	11–20	Spherical	Donda et al. (2013)
Sedum alfredii Hancc	Zinc oxide	53.7	Hexagonal wurtzite and pseudospherical	Qu et al. (2011a)
Sesbania drummondii	gold	6–20	Spherical	Sharma et al. (2007)
Sesbania grandiflora	Silver	10–25	Spherical	Das et al. (2013)
Sesuvium portulacastrum L.	Silver	5–20	Spherical	Nabikhan et al. (2010)
Solanum lycopersicums	Silver	14–33	Spherical	Bindhu and Umadevi (2014)
S. lycopersicums	Gold	5–19	Spherical, triangular	Bindhu and Umadevi (2014)
Solanum torvum	Silver, gold	5–50	Agglomerated, spherical	Ramamurthy et al. (2013)
Sorbus aucuparia	Silver, gold	16–18	Spherical, triangular, hexagonal	Dubey et al. (2010a)
Sorghum bicolor (L.) Mocnch	Silver	10	Fee, crystalline	Njagi et al. (2011)
Sorghum bicolor (L.) Mocnch	Iron	50	Amorphous	Njagi et al. (2011)

(continued)

Table 11.1 (continued)

Plants	Nanoparticle	Size (nm)	Morphology	References
Sphearenthus amarenthoides	Gold	39–47	Spherical	Nellore et al. (2012)
Stigmaphyllon littorale	Silver	5–25	Spherical	Kudle et al. (2013)
Syzygium aromaticum	Gold	5–100	Crystalline, irregular, spherical, elliptical	Deshpande et al. (2010)
Syzygium cumini	Silver	29–92	Spherical	Kumar et al. (2010)
Tamarindus indica	Gold	20–40	Triangular	Ankamwar et al. (2005a)
Tanacetum vulgare	Gold and silver	11, 16	Triangular, spherical	Dubcy et al. (2010b)
Terminalia catappa	Gold	10–35	Spherical	Ankamwar (2010)
Terminalia chebula	Silver	Less than 100	Pentagonal, triangular, spherical	Kumar et al. (2012)
Trichoderma koningii	Gold	30–40	Triangular	Maliszewska et al. (2009)

of NPs, but most of them are environmentally unfriendly and expensive (Senapati 2005; Klaus-Joerger et al. 2001). Consequently, there is an ever-growing need to develop nontoxic and eco-friendly procedures for synthesis and assembly of NPs with desired morphologies and sizes, fast, and clean. One of the options to achieve this objective is to use natural processes such as use of microorganisms and biological systems. One approach that shows immense potential is based on the phytosynthesis of NPs using plants (Table 11.1) (Iravani 2011; Iravani et al. 2014a, b; Korbekandi et al. 2009; Iravani and Zolfaghari 2013). The use of plants in this area is rapidly developing due to their growing success and ease of formation of NPs. The objectives of recent studies tend to provide a controlled and upscalable process for synthesis of monodispersed and highly stable NPs (Iravani 2011; Korbekandi et al. 2009; Iravani et al. 2014a). In this chapter, most of the plants used in nanoparticle synthesis are mentioned, and detailed examination of numerous syntheses examples and case studies is presented.

11.2 Phytosynthesis of Metal NPs

11.2.1 Silver and Gold NPs

Gardea-Torresdey et al. (2002a) reported phytosynthesis of gold (Au) NPs within live *Medicago sativa* (alfalfa) plants by gold ion uptake from solid media. The alfalfa plants were grown in an $AuCl_4^-$ rich environment (Gardea-Torresdy et al. 2002b, 2003). Moreover, colloidal silver (Ag) NPs were synthesized by reacting aqueous silver nitrate with *M. sativa* seed exudates under nonphotomediated conditions (Lukman et al. 2011). Upon contact, rapid reduction of Ag^+ ions was observed in <1 min with silver nanoparticle formation reaching 90 % completion in <50 min. It was observed that largely spherical NPs (~5–51 nm) were produced at $[Ag^+] = 0.01$ M and 30 °C, while flower-like particle clusters (~104 nm) were observed on treatment at higher silver concentrations. Predilution of the exudate induced the formation of single-crystalline silver nanoplates, forming hexagonal particles and nanotriangles with edge lengths of about 86–108 nm, while pH adjustment to 11 resulted in monodisperse silver NPs (~12 nm). It was mentioned that repeated centrifugation and redispersion enhanced the percentage of nanoplates from 10 to 75 % in solution (Lukman et al. 2011).

Armendariz et al. (2004b) reported the formation of rod-shaped gold NPs by biomaterials. They characterized the gold NPs formed by wheat biomass exposed to a 0.3 mM potassium tetrachloroaurate solution at pH values of 2–6 at room temperature. It was concluded that wheat biomass was able to reduce Au(III)–Au(0) forming face-centered cubic (FCC) tetrahedral, hexagonal, decahedral, icosahedral multitwinned, irregular shape, and rod shape NPs. In another study, pH-dependent synthesis of rod-shaped gold NPs using *Avena sativa* has shown that biomass might carry more positive functional groups such as positive amino groups, sulfhydryl groups, and carboxylic groups which allowed the gold ions to get more

closure to binding sites and approved the reduction of Au(III)–Au(0) (Gardea-Torresdey et al. 2002a; Armendariz et al. 2004a). A 0.1 mM solution of Au(III) was reacted with powdered oat biomass at pH values of 2–6 for 1 h. As in the case of wheat, oat biomass produced FCC tetrahedral, hexagonal, decahedral, icosahedral multitwinned, irregular , and rod shaped NPs. It was reported that most of the NPs synthesized by using alfalfa, wheat, and oat at pH 2 had an irregular shape. However, it seems that pH has a major impact on the size of the synthesized NPs rather than on the shape of them.

Sastry and coworkers have explored the formation mechanism of triangular gold nanoprisms by *Cymbopogon flexuosus* extracts, the nanotriangles seemed to grow by a process involving rapid bioreduction, assembly, and room temperature sintering of spherical gold NPs (Shankar et al. 2004b). Also rapid synthesis of stable gold nanotriangles using *Tamarindus indica* leaf extract as a reducing agent could be achieved (Ankamwar et al. 2005a). The shape of metal NPs considerably changed their optical and electronic properties (Kelly et al. 2003). They have demonstrated synthesis of gold NPs with variety of shapes (spherical and triangular) and sizes using *Aloe vera* plant extracts, as well (Chandran et al. 2006). It was explained that only biomolecules of molecular weights less than 3 kDa caused reduction of chloroaurate ions, leading to the formation of gold nanotriangles.

The aqueous solution of gold ions when exposed to *Coriandrum sativum* leaf extract was reduced and resulted in the extracellular biosynthesis of gold NPs with spherical, triangle, truncated triangles, and decahedral morphologies ranging from 6.75 to 57.91 nm. These NPs were stable in solution over a period of 1 month at room temperature (Badri Narayanan and Sakthivel 2008). Gold NPs synthesized using *Azadirachta indica* leaf broth appeared to have a propensity to form thin, planar structures rather than just spherical particles. The planar particles formed were predominantly triangular with a very small percentage of hexagonal-shaped particles (Shankar et al. 2004a). Ghosh et al. (2012a) reported the synthesis of gold NPs using *Gnidia glauca* flower extract. The concentration of chloroauric acid and temperature was optimized to be 0.7 mM and 50 °C, respectively. The NPs varied in morphology from nanotriangles to nanohexagons majority being spherical. Spherical particles (~10 nm) were found in majority. However, particles of larger dimensions were in range between 50 and 150 nm. The gold NPs exhibited remarkable catalytic properties in a reduction reaction of 4-nitrophenol to 4-aminophenol by $NaBH_4$ in aqueous phase (Ghosh et al. 2012a).

Magnolia kobus and *Diospyros kaki* were capable of eco-friendly extracellular synthesis of gold NPs (~5–300 nm) with different triangular, pentagonal, hexagonal, and spherical shapes within a few minutes (for up to 90 % conversion at a reaction temperature of 95 °C) (Song et al. 2009). It was suggested that the rate of synthesis of the NPs was related to the reaction and incubation temperature, and increased temperature levels allowed nanoparticle growth at a faster rate. Moreover, by increasing the temperatures and leaf broth concentrations, size of NPs became smaller. Fourier Transformed Infrared Spectroscopy (FTIR) analysis has shown that gold NPs produced by *M. kobus* extract were surrounded by proteins and metabolites (such as terpenoids having functional groups of amines,

aldehydes, carboxylic acid, and alcohols). It was also reported that the use of low concentration of phyllanthin extract reacting with $HAuCl_4$ led to synthesis of hexagonal or triangular gold NPs, but spherical NPs could be formed by addition of higher concentration of the extract (Song et al. 2009).

Triangular and hexagonal gold NPs (~200–500 nm) were synthesized using pear fruit extract (Ghodake et al. 2010). Pear extract contains essential phytochemicals consisting of organic acids, peptides, proteins, and amino acids. In addition, it contains saccharides which provide synergetic reduction power for the bioreduction of chloroaurate ions into gold NPs. The pear fruit extract when exposed to chloroaurate ions in an alkaline condition resulted in gold NPs with plate-like morphologies in a highly productive state. The gold NPs formed under normal conditions also exhibited plate-like morphologies with a low productivity. In addition, the synthesis of gold NPs (~11 nm) with spherical and triangular shapes by fruit extract of *Tanacetum vulgare* was reported (Dubey et al. 2010b). Carbonyl group was involved in synthesis of these NPs. In another study, Singh et al. (2013) reported the green synthesis of silver NPs (~40–100 nm) using *Dillenia indica* fruit extract. It was reported that the stability of the colloidal silver NPs for more than 6 days (166 h) might be attributed to the citrate component of the *D. indica* fruit juice (Singh et al. 2013).

Green synthesis of biocompatible gold NPs from chloroauric acid using water extract of *Eclipta alba* leaves at room temperature was reported. The gold NPs were produced in very short time, even in less than 10 min. The in vitro stability of as-synthesized gold NPs was studied in different buffer solutions. Mukherjee et al. (2012) designed and developed a gold NPs-based drug delivery system containing doxorubicin, an FDA-approved anticancer drug. Consequently, administration of this to breast cancer cells (MCF-7 and MDA-MB-231) showed significant inhibition of breast cancer cell proliferation compared to pristine doxorubicin. They suggested this method for large-scale synthesis of biocompatible gold NPs which can be used as a delivery vehicle for the treatment of cancer diseases (Mukherjee et al. 2012).

Silver and gold NPs were synthesized using an aqueous extract of the seaweed *Turbinaria conoides*. Spherical and triangular nanostructures of the silver and gold NPs were observed between the size ranges of 2–17 and 2–19 nm, respectively. The synthesized silver NPs were efficient in controlling the bacterial biofilm formation, but, gold NPs did not show any remarkable anti-biofilm activity. The maximum zone of inhibition was recorded against *Escherichia coli* (17.6 ± 0.42 mm), followed by *Salmonella* sp., *Serratia liquefaciens*, and *Aeromonas hydrophila* (Vijayan et al. 2014). Moreover, silver NPs were synthesized from aqueous silver nitrate through a simple green route using the leaf extract of *Coccinia grandis* (as the reducing and capping agents) (Arunachalam et al. 2012). The synthesized NPs were in range of 20–30 nm and were crystallized in face-centered cubic symmetry. The thermal stability of NPs was studied using Thermo Gravimetric Analyzer (TGA) which showed that the NPs synthesized by plant extracts began to degrade at around 300 °C. Moreover, there was a steady weight loss until 800 °C. The total weight loss up to 800 °C for the synthesized silver NPs was about 36.26 %.

The observed behavior was most likely as a consequence of the surface desorption of bio-organic compounds present in nanoparticle powder. Therefore, plant leaf extract-stabilized silver NPs were expected to be made up of molecules responsible for the reduction of metal ion and stabilizing particles in the solution. Further, the thermal stability of NPs was studied using differential scanning calorimeter (DSC). As a result, the synthesized silver NPs showed an endothermic peak at 65 °C. Photocatalytic property of the silver NPs were investigated by degradation of Coomassie Brilliant Blue G-250 under UV light. The results showed that silver NPs have suitable activity for the degradation of Coomassie Brilliant Blue G-250. FTIR spectrum was examined to identify the possible biomolecules responsible for capping and efficient stabilization of the silver NPs synthesized by plant leaf extract. The peaks observed for silver NPs formed through reduction by *C. grandis*, at 1,228 cm^{-1} (ether linkages), 1,376 cm^{-1} (–O–H bending), 1,050 cm^{-1} (ether linkages), 1,484 cm^{-1} (=NH), and 1,632 cm^{-1} (amide I) suggested the presence of alkaloids and terpenoids adsorbed on the surface of silver NPs (Arunachalam et al. 2012).

Silver NPs were synthesized by reaction of the biomass of *Cinnamomum camphora* leaf with aqueous silver precursors at ambient temperature (Huang et al. 2007). Size dispersity of quasi-spherical silver NPs could be facilely controlled by simple variation of the amount of biomass reacting with aqueous solution of silver nitrate. The polyol components and the water-soluble heterocyclic components were mainly found to be responsible for the reduction of silver ions and the stabilization of the NPs, respectively. Furthermore, Huang et al. (2008) investigated biological formation of silver NPs by lixivium of sun-dried *C. camphora* leaf in continuous-flow tubular microreactors. They introduced polyols in the lixivium as possible reducing agents. In another study, silver and gold NPs with a particle size of 10–20 nm, using *Zingiber officinale* root extract as a reducing and capping agent were synthesized (Velmurugan et al. 2014). Chloroauric acid and silver nitrate were mixed with *Z. officinale* root extract for the synthesis of silver and gold NPs. As a result, optimum nanoparticle formation was achieved at pH 8 and 9, 1 mM metal ion, a reaction temperature 50 °C, and reaction time of 150–180 min for silver NPs (~10–20 nm) and gold NPs (~5–20 nm), respectively. FTIR spectroscopy analysis showed the respective peaks for the potential biomolecules in the ginger rhizome extract, which were responsible for the bioreduction in metal ions and synthesized silver and gold NPs. Further, it was observed that the synthesized silver NPs showed a moderate antibacterial activity against bacterial food pathogens (Velmurugan et al. 2014).

Spherical and ovoid crystalline silver NPs (~7–15 nm) were synthesized by phytoreduction of silver nitrate using aqueous leaf extract of *Ocimum tenuiflorum* (Vignesh et al. 2013). FTIR spectrum was analyzed to identify the feasible biological functional groups responsible for the reduction of silver ions and stabilizing the synthesized NPs. A glycosidic linkage present in the FTIR spectrum of silver NPs suggested that the plant-based polysaccharides might contribute for the reduction of silver ion into silver NPs. The colloidal solution of silver NPs showed significant antimicrobial activity against variety of human and fish

pathogens in solid medium. The implications of colloidal silver NPs on hematological and biochemical functions of fresh water fish *Labeo rohita* were tested. The experiments revealed that the amount of hemoglobin and total count of blood cells were considerably increased. The activity of functional enzymes such as glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), anti-protease, and myeloperoxidase were radically improved due to the treatment of fish with colloidal silver NPs. The authors of this study mentioned that the colloidal silver NPs having prominent immunomodulatory effects in fresh water fish, thus these NPs can be used as an immunomodulator in aquaculture sectors instead of synthetic growth factors (Vignesh et al. 2013).

Brassica juncea and *M. sativa* can be used for phytosynthesis of silver NPs. Harris and Bali (2008) have investigated the limits (substrate metal concentration and time exposure) of uptake of metallic silver by two common metallophytes, *B. juncea* and *M. sativa*. *B. juncea*, when exposed to an aqueous substrate containing 1,000 ppm silver nitrate for 72 h, accumulated up to 12.4 wt% silver. *M. sativa* accumulated up to 13.6 wt% silver when exposed to an aqueous substrate containing 10,000 ppm silver nitrate for 24 h. In the case of *M. sativa*, an increase in metal uptake was observed with a corresponding increase in the exposure time and substrate concentration. In both cases, Transmission Electron Microscopy (TEM) analysis showed the presence of roughly spherical silver NPs, with a mean size of 50 nm. In addition, the in vivo formation of silver NPs was observed in *B. juncea*, *Festuca rubra*, and *M. sativa* (Marchiol et al. 2014). Marchiol et al. (2014) reported that the mentioned plants were grown in Hoagland's solution for 30 days and then exposed for 24 h to a solution of 1,000 ppm $AgNO_3$. Despite the short exposure time, the silver uptake and translocation to plant leaves was very high, reaching 6,156 and 2,459 mg kg^{-1} in *B. juncea* and *F. rubra*, respectively. TEM images of plant fractions showed the in vivo formation of silver NPs in the roots, stems, and leaves of the plants. In the roots, silver NPs were present in the cortical parenchymal cells, on the cell wall of the xylem vessels and in regions corresponding to the pits. In leaf tissues, silver NPs of different sizes and shapes were located close to the cell wall, as well as in the cytoplasm and within chloroplasts. The NPs were not observed in the phloem of the three plant species. The contents of reducing sugars and antioxidant compounds, proposed as being involved in the green synthesis of silver NPs. Ascorbic acid has been proposed as the reducing agent responsible for this process. In contrast, *F. rubra* had a level of reducing sugars much higher than *B. juncea* and *M. sativa*. The authors of this study mentioned that nanoparticle synthesis started in healthy cells, which then rapidly undergo a progressive alteration until they were completely disrupted due to silver toxicity. Thus, nanoparticle synthesis was initiated within the chloroplasts in a healthy cell and ends in the cytoplasm of the same cell, which has been damaged (Marchiol et al. 2014). Further, in another study, after a 9-week growth in gold-, silver-, and copper-enriched soil, seeds of *B. juncea* grow into a plant containing Au–Ag–Cu alloy NPs (Haverkamp et al. 2007). Starnes et al. (2010) detected the formation of gold NPs in *M. sativa* and other species as early as 6 h after the start of exposure to $KAuCl_4$. It was also verified that plant growth conditions (e.g., variations in

temperature, pH, and photosynthetically active radiation) influenced the size and shape of growing gold NPs (Starnes et al. 2010). Beattie and Haverkamp (2011) demonstrated that in *B. juncea*, the sites of the most abundant reduction of metal salts to NPs were the chloroplasts, in which high reducing sugars (i.e., glucose and fructose) may be responsible for the metal reduction (Beattie and Haverkamp 2011).

Rodríguez-León et al. (2013) reported the synthesis of silver NPs (~2–40 nm) from silver nitrate solutions using extracts of *Rumex hymenosepalus*. High-resolution transmission electron microscopy and fast Fourier transform analysis show that two kinds of crystal structures are obtained: FCC and hexagonal. They observed that the FCC NPs displayed two size populations: one with a small average diameter (~10 nm) and a second one with a larger diameter (~28 nm). On the other hand, the hexagonal NPs have only one size population and larger diameters (~38 nm). The polyphenols contained in the *R. hymenosepalus* extracts acted effectively as the reducing agents for the Ag^+ ions due to their antioxidant activity.

Shankar et al. (2004a) reported the synthesis of silver NPs by the reduction of aqueous Ag^+ ions and also the synthesis of bimetallic core-shell NPs of silver by simultaneous reduction of aqueous Ag^+ ions with the broth of *A. indica* leaves. They observed that the metal particles were stable in solution even 4 weeks after their synthesis. Moreover, stabilization of NPs was possibly facilitated by reducing sugars and/or terpenoids present in *A. indica* leaf broth. The synthesized silver NPs were predominantly spherical and polydisperse with diameters in the range 5–35 nm. Furthermore, *Pelargonium graveolens* (geranium) leaf broth, when exposed to aqueous silver nitrate solution, resulted in enzymatic synthesis of stable crystalline silver NPs, extracellularly (Shankar et al. 2003a). The bioreduction of the metal ions was fairly rapid, occurred readily in solution, and resulted in a high density of stable silver NPs in the size range 16–40 nm. The synthesized NPs appeared to be assembled into open, quasilinear superstructures and were predominantly spherical in shape. It was believed that proteins, terpenoids, and other bio-organic compounds in the geranium leaf broth participated in the bioreduction of silver ions and in the stabilization of the NPs thus formed by surface capping. Shankar et al. (2003b) reported the possibility of terpenoids from geranium leaf in the silver nanoparticle synthesis. Polyols such as terpenoids, polysaccharides, and flavones in the *C. camphora* leaf were believed to be the main cause of the reduction of silver and chloroaurate ions (Huang et al. 2007). Moreover, green synthesis of silver NPs using methanol extract of *Eucalyptus hybrida* leaf was reported. Flavonoid and terpenoid constituents present in *E. hybrid* leaf extract are responsible for the stabilization of produced silver NPs (~50–150 nm) (Dubey et al. 2009). *Cinnamon zeylanicum* bark extract could be used in biosynthesis of cubic and hexagonal silver nanocrystals (~31–40 nm) (Sathishkumar et al. 2009b). The particle size distribution varied with variation in the dosage of *C. zeylanicum* bark extract. The number of particles increased with increasing dosage due to the variation in the amount of reductive biomolecules. Small NPs were formed at high pH. The shape of silver NPs at high pH was more spherical in nature rather than ellipsoidal. Moreover, bactericidal effect of produced nanocrystalline silver particles

was tested against *E. coli* strain. As a result, the various tested concentrations of 2, 5, 10, 25, and 50 mg/L produced inhibition of 10.9, 32.4, 55.8, 82, and 98.8 %, respectively. The minimum inhibitory concentration was found to be 50 mg/L. *C. zeylanicum* bark is rich in terpenoids (linalool, methyl chavicol, and eugenol) and in chemicals (such as cinnamaldehyde, ethyl cinnamate, and β-caryophyllene) which contribute to its special aroma. Furthermore, proteins are also present in the bark. It was believed that water-soluble organics present in *C. zeylanicum* bark were the reasons of the bioreduction of silver ions to silver NPs. Moreover, proteins from *C. zeylanicum* bark capped the synthesized NPs either through free amine groups or cysteine residues, and thus stabilized them (Sathishkumar et al. 2009b).

The reaction of aqueous silver ions with *Desmodium trifolium* extract resulted in synthesis of silver NPs at room temperature (Ahmad et al. 2011). The authors of this article believed that H^+ ions produced along with NAD during glycolysis were responsible for the formation of nanosilver particles as well as water-soluble antioxidative agents (e.g., ascorbic acids). These antioxidative agents were especially participating in reduction of silver ions. Jha and Prasad (2010) demonstrated that cycas leaf extract could be used in order to produce stable silver NPs. X-ray data indicated that silver NPs had FCC unit cell structure. Phytochemicals such as polyphenols, glutathiones, metallothioneins, and ascorbates probably were responsible for formation of the NPs.

High density of extremely stable silver NPs (~16–40 nm) were rapidly synthesized by challenging silver ions with *Datura metel* leaf extract (Kesharwani et al. 2009). Leaf extracts of this plant contains biomolecules such as alkaloids, proteins/enzymes, amino acid, alcoholic compound, and polysaccharides which could be used as reluctant to react with silver ions and scaffolds to direct the formation of silver NPs in solution. Quinol and chlorophyll pigment were responsible for the reduction of silver ions and stabilization of produced NPs. In another study, green synthesis of silver NPs (~20–30 nm) from silver nitrate (1 mM) solution through the extract of *D. metel* flower (as the reducing and capping agents) was reported. In addition, the synthesized NPs showed antimicrobial activity (Nethradevi et al. 2012).

Geraniol as a volatile compound obtained from *P. graveolens* was used for biosynthesis of silver NPs (~1–10 nm). The cytotoxicity of the synthesized silver NPs was evaluated in vitro against Fibrosarcoma Wehi 164 cell line at different concentrations (1–5 μg/mL) (Safaepour et al. 2009). The presence of silver NPs (5 μg/mL) significantly inhibited the cell line's growth (up to 60 %). Therefore, it seems that silver NPs have inhibitory effects against the proliferation of cancer cells.

Song and Kim (2008) have elucidated that *Pinus desiflora*, *D. kaki*, *Ginko biloba*, *M. kobus*, and *Platanus orientalis* leaves broth synthesized stable silver NPs with average particle size ranging from 15 to 500 nm, extracellularly. In the case of *M. kobus* and *D. kaki* leaves broth, synthesis rate and final conversion to silver NPs became faster, when reaction temperature increased. But the average particle sizes produced by *D. kaki* leaf broth decreased from 50 to 16 nm, when temperature increased from 25 to 95 °C. They also illustrated that only 11 min was

required for more than 90 % conversion at the reaction temperature of 95 °C using *M. kobus* leaf broth (Song and Kim 2008). Moreover, phytosynthesis of silver NPs (~44–64 nm) using *Iresine herbstii* and evaluation of their antibacterial, antioxidant, and cytotoxic activities were reported (Dipankar and Murugan 2012). The synthesized NPs were cubic and face-centered cubic in shape. The synthesized silver NPs showed potent antibacterial activity against human pathogenic bacteria. These NPs exhibited strong antioxidant activity as well as cytotoxicity against HeLa cervical cell lines. Furthermore, Suman et al. (2013) reported the phytosynthesis of spherical silver NPs (~30–55 nm) from the root of *Morinda citrifolia*. FTIR showed that the NPs were capped with plant compounds. The X-ray diffraction spectrum XRD pattern clearly indicated that the silver NPs formed in the present synthesis were crystalline in nature. The synthesized NPs were also proved to exhibit excellent cytotoxic effect on HeLa cell (Suman et al. 2013). Logeswari et al. (2013) reported the green synthesis of silver NPs from silver nitrate solution by commercially available plant powders, such as *Solanum tricobatum*, *Syzygium cumini*, *Centella asiatica,* and *Citrus sinensis*. Atomic Force Microscopy (AFM) showed the irregular shapes of silver NPs and the formation of them were found to be 53, 41, 52, and 42 nm, corresponding to *S. cumini*, *C. sinensis*, *S. tricobatum,* and *C. asiatica*, respectively. FTIR spectroscopy confirmed the presence of protein as the stabilizing agent surrounding the silver NPs. Antimicrobial activity of the silver bionanoparticles was performed by a well diffusion method. The highest antimicrobial activity of silver NPs synthesized by *C. sinensis* and *C. asiatica* was found against *Pseudomonas aeruginosa* (Logeswari et al. 2013).

Vilchis-Nestor et al. (2008) used *Camellia sinensis* (green tea) extract to produce gold NPs and silver nanostructures in aqueous solution at ambient conditions. They also investigated the control of size, morphology, and optical properties of the nanostructures and reported initial concentrations of metal ions and tea extract as the controlling factors. It was investigated that when the amount of *C. sinensis* extract was increased, the resulted NPs were slightly bigger and more spherical. Authors of this study believed that phenolic acid-type biomolecules present in *C. sinensis* extract were responsible for synthesis and stabilization of silver and gold NPs. Caffeine and theophylline present in tea extracts might be responsible for catalysis and synthesis of NPs (Vilchis-Nestor et al. 2008). In another study, green synthesis of spherical gold NPs (~2.94–45.58 nm) was done by using fresh young leaves and leaf buds of *C. sinensis* (Boruah et al. 2012). Phytoreduction of $HAuCl_4$ by polyphenols present in young leaves and leaf buds of tea extract at room temperature produced gold NPs. The kinetics of the reaction suggested that the reaction was fast and completed in 28 min. In addition, black tea leaf extracts were used in synthesis of gold and silver NPs (Begum et al. 2009). The NPs are stable and have different shapes, such as spheres, trapezoids, prisms, and rods. Findings of this study demonstrated that polyphenols and flavonoids were responsible for synthesis of silver and gold NPs. Sun et al. (2014) reported a simple and cost-effective method for synthesis of silver NPs using tea leaf extract. The synthesized NPs were nearly spherical, with the sizes ranging from 20 to 90 nm. In addition, the antibacterial activity of silver NPs was determined by

monitoring the growth curve and also by the Kirby-Bauer disk diffusion method. But, the silver NPs produced by tea extract showed low antibacterial activity against *E. coli* (Sun et al. 2014).

Mude et al. (2009) reported synthesis of spherical silver NPs (~60–80 nm) using callus extract of *Carica papaya*. Proteins and other ligands seemed to be responsible for the synthesis and stabilization of silver NPs. Furthermore, FCC silver NPs (~10–20 nm) were synthesized by using the latex of *Jatropha curcas* as reducing and capping agent (Bar et al. 2009c). It was demonstrated that leaf extracts from the aquatic medicinal plant, *Nelumbo nucifera* (Nymphaeaceae), could be able to reduce silver ions and produce silver NPs (~45 nm) in different shapes (Santhoshkumar et al. 2010). Biosynthesized silver NPs showed larvicidal activity against malaria (*Anopheles subpictus*) and filariasis (*Culex quinquefasciatus*) vectors. Silver NPs were synthesized biologically by using *Sorbus aucuparia* leaf extract within 15 min. The synthesized NPs were found to be stable for more than 3 months. The authors of this study believed that sorbate ion in the leave extract of *S. aucuparia* encapsulated the NPs and this matter was responsible for maintenance of the stability (Dubey et al. 2010a). The effect of leaf extract quantity, substrate concentration, temperature, and contact time were also evaluated to optimize the process of producing NPs. With increase in the concentration of metal ions from 10^{-4} to 10^{-2} M, increase in particle size was also found (Dubey et al. 2010a).

The aqueous extract of *Alternanthera sessilis* L. (Amaranthaceae) was used for synthesizing silver NPs from silver nitrate aqueous (Niraimathi et al. 2013). Phytochemical analysis of the extract revealed the presence of alkaloid, tannins, ascorbic acid, carbohydrates, and proteins and they served as effective reducing and capping agents for converting silver nitrate into NPs. FTIR spectrum showed the silver NPs having a coating of proteins indicating a dual role of biomolecules responsible for capping and efficient stabilization of the silver NPs (Niraimathi et al. 2013). Moreover, polyethylene glycol-stabilized colloidal silver NPs (~7–14 nm) were synthesized using the reductive potency of the aqueous extract of *Thuja occidentalis* leaves under ambient conditions (Barua et al. 2013). MTT assay revealed the dose-dependent cytocompatibility and toxicity of the NPs with the L929 normal cell line. On the other hand, the antiproliferative action of the NPs was evaluated on HeLa cell line. Fluorescence and phase contrast microscopic imaging indicated the appearance of multinucleate stages with aggregation and nuclear membrane disruption of the HeLa cells post treatment with the NPs. The interaction at the prokaryotic level was also assessed via differential antibacterial efficacy against *Staphylococcus aureus* and *E. coli* (Barua et al. 2013).

Silver NPs were prepared by using *Bryophyllum* sp., *Cyperus* sp., and *Hydrilla* sp. plant extracts (Jha et al. 2009b). X-ray analysis demonstrated that silver NPs (~2–5 nm) have FCC unit cell structure. The reduction of silver ions was due to water-soluble phytochemicals such as flavones, quinones, and organic acids (e.g., oxalic, malic, tartaric, and protocatechuic acid) present in plant tissues. It was demonstrated that silver NPs might have resulted due to different aforementioned metabolites or fluxes and other oxidoreductively labile metabolites such as ascorbates or catechol/protocatechuic acid (Jha et al. 2009b). In the case of

Chenopodium album, organic acids were also responsible for nanoparticle biosynthesis. The plant leaf has high level of oxalic acid as well as lignin which can act as reducing agents. *C. album* leaf extract was used for the single-pot bioinspired synthesis of spherical silver and gold NPs (Dwivedi and Gopal 2010). Quasi-spherical shapes were observed for produced NPs within range of about 10–30 nm.

Silver NPs (~20–40 nm) were successfully synthesized using switchgrass (*Panicum virgatum*) extract used at room temperature. Phytosynthesis of silver NPs through this process was fairly rapid, with 90 % of silver ion reduction completed within 90 min. XRD analysis indicated the formation of phase pure silver NPs with FCC symmetry (Mason et al. 2012). In another study, spherical silver NPs (~16–28 nm) were synthesized using plant fruit bodies (*Tribulus terrestris* L.) (Gopinath et al. 2012). The active phytochemicals present in the plant were responsible for the quick reduction of silver ions to metallic silver NPs. The antibacterial property of synthesized NPs was observed by Kirby-Bauer method with clinically isolated multidrug resistant bacteria such as *Streptococcus pyogens*, *P. aeruginosa*, *E. coli*, *Bacillus subtilis* and *S. aureus* (Gopinath et al. 2012). Sathiya and Akilandeswari (2014) reported the phytoreduction of silver nitrate into silver NPs using the leaf extract of *Delonix elata*. SEM analysis revealed the spherical shape of the NPs with sizes in the range of 35–45 nm and Energy Dispersive Spectroscopy (EDS) spectrum confirmed the presence of silver along with other elements in the plant metabolite. The X-ray diffraction analysis showed that the synthesized NPs were crystalline in nature and had FCC structure. FTIR analysis revealed the presence of phenolic compounds along with the silver NPs, and flavonoids present in the leaf broth of *D. elata* were responsible for the bioreduction and stabilization of silver NPs (Sathiya and Akilandeswari 2014).

Santhosh kumar et al. (2012) reported the efficacies of antiparasitic activities of biosynthesized silver NPs using stem aqueous extract of *Cissus quadrangularis* against the adult of hematophagous fly, *Hippobosca maculata*, and the larvae of cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*. Contact toxicity method was followed to determine the potential of parasitic activity. Twelve milliliters of stem aqueous extract of *C. quadrangularis* was treated with 88 mL of 1 mM silver nitrate (as the substrate) solution at room temperature for 30 min. FTIR analysis confirmed that the bioreduction of silver ions to silver NPs were due to the reduction by capping material of plant extract. The synthesized silver NPs (~42.46 nm) were spherical and oval in shape. The mortality obtained by the synthesized silver NPs from the *C. quadrangularis* was more effective than the aqueous extract of *C. quadrangularis* and silver nitrate solution (1 mM). The adulticidal activity was observed in the aqueous extract, silver nitrate solution, and produced silver NPs against the adult of *H. maculata* with LC_{50} values of 37.08, 40.35, and 6.30 mg/L; LC_{90} values of 175.46, 192.17, and 18.14 mg/L and r^2 values of 0.970, 0.992, and 0.969, respectively. The maximum efficacy showed in the aqueous extract, silver nitrate solution, and produced silver NPs against the larvae of *R. (B.) microplus* with LC_{50} values of 50.00, 21.72, and 7.61 mg/L; LC_{90} values of 205.12, 82.99, and 22.68 mg/L and r^2 values of 0.968, 0.945, and 0.994,

respectively (Santhoshkumar et al. 2012). In another study, the antiparasitic activities of hexane, chloroform, ethyl acetate, acetone, methanol, and the aqueous leaf extracts of *Euphorbia prostrata* Ait. (Euphorbiaceae) and synthesized silver NPs using aqueous leaf extract against the adult cattle tick *Haemaphysalis bispinosa* Neumann (Acarina: Ixodidae) and the haematophagous fly *H. maculata* Leach (Diptera: Hippoboscidae) were determined (Zahir and Rahuman 2012). Parasites were exposed to varying concentrations of plant extracts and produced silver NPs for 24 h. All extracts showed the maximum toxic effect on parasites; however, the highest mortality was found in the hexane, chloroform, ethyl acetate, acetone, methanol, and aqueous leaf extracts of *E. prostrate* and biosynthesized silver NPs against the adult of *H. bispinosa* (LC_{50} = 45.24, 40.07, 21.91, 25.32, 19.30, 10.16, and 2.30 ppm; LC_{90} = 86.95, 88.66, 70.92, 83.22, 48.28, 70.27, and 8.28 ppm) and against *H. maculata* (LC_{50} = 39.37, 41.98, 19.92, 27. 93, 21.97, 9.79, and 2.55 ppm; LC_{90} = 89.44, 98.52, 76.59, 90.18, 55.07, 54.35, and 9.03 ppm), respectively. Mortality of 100 % was found in biosynthesized silver NPs at a concentration of 10 mgL^{-1}. It was observed that the synthesized silver NPs (~25–80 nm) were rod shaped. The chemical composition of aqueous leaf extract was analyzed by gas chromatography–mass spectrometry (GC–MS). The major chemical constituent was identified as 2-phenylethanol. In addition, toxicity tests were conducted to analyze the toxicological effects of particle size on *Daphnia magna* and *Ceriodaphnia dubia*, and the animal model test was evaluated against *Bos indicus* for 24-h treatment. No toxicity on daphnids and no adverse effects were noted on animals after exposure to solvent extracts and produced silver NPs (Zahir and Rahuman 2012). In addition, silver NPs were synthesized by employing an aqueous peel extract of *Annona squamosa* in silver nitrate (Kumar et al. 2012). The synthesized NPs were irregular spherical in shape and the average particle size was about 35 ± 5 nm. The authors of this study reported that reducing sugars (aldoses) and terpenoids are proposed to play a key role for the phytoreduction of silver ions and the formation of corresponding NPs, while ketones/aldehydes bind to emerging spherical NPs to form large nanotriangles and hexagons. Moreover, to identify the capping reagents and the molecules responsible for the reduction of silver ions in *A. squamosa* aqueous peel extract GC–MS has been evaluated. GC–MS chromatogram of aqueous extract of *A. squamosa* revealed that a major constituent contains –OH/–CHO as functional group in the moiety. Further, ^1H NMR spectrum of *A. squamosa* L. aqueous peel extract revealed that strong signal observed at δ = 4.76–4.81 ppm is due to =C–H. Signal at δ = 5.35 ppm could be due to aliphatic –OH group, whereas the signals appearing between δ = 1.26–1.32 ppm are related to aliphatic C–CH_2–C groups. Further, water-soluble hydroxy functional group containing compounds were reported to be responsible for the reduction of Ag^+ ions. Consequently, the hydroxyl compounds bind to the NPs and enhance the stability as well as it will forms a layer (capping material) over silver NPs which provide stability to NPs (Kumar et al. 2012).

Jagtap and Bapat (2013) reported the phytosynthesis of silver NPs (with an average size 10.78 nm) from aqueous solution of silver nitrate using *Artocarpus heterophyllus* Lam. seed powder extract, as a reducing agent. The reaction of

A. heterophyllus Lam. seed powder extract and silver nitrate was carried out in an autoclave at 15 psi, 121 °C for 5 min. The synthesized NPs were generally found to be irregular in shapes. The FTIR spectra indicated the role of amino acids, amides group I in the synthetic process. The seed contains Jacalin, a lectin which is a single major protein representing more than 50 % of the proteins from the jackfruit crude seed extract having several biological activities. The synthesized NPs showed highly potent antibacterial activity toward *B. cereus*, *B. subtilis*, *S. aureus*, and *P. aeruginosa* (Jagtap and Bapat 2013). In another study, the extract of *Benincasa hispida* seeds (as the reducing and capping agents) was used for the biosynthesis of stable and nearly spherical gold NPs (~10–30 nm). The particle size could be easily tuned by the reaction conditions including quantity of extract, temperature, and pH (Aswathy Aromal and Philip 2012a). It was found that the NPs were more stable at pH 6. Moreover, from FTIR spectrum, it was suggested that the possible reducing agent was polyols and the capping material responsible for stabilization was proteins present in the extract. Carboxylic group (COOH) present in the extract becomes COO–, and this carboxylate group present in proteins can act as a surfactant to attach on the surface of gold NPs and it stabilizes gold NPs through electrostatic stabilization (Aswathy Aromal and Philip 2012a).

11.2.2 Palladium NPs

Sathishkumar et al. (2009a) have investigated phytocrystallization of palladium (Pd) through reduction process using *Cinnamomum zeylanicum* bark extract. As a result, nanocrystalline palladium particles (~15–20 nm) were successfully synthesized. It was demonstrated that reaction conditions such as pH, temperature, and biomaterial dosage had no major effects on the shape and size of produced NPs. In another study, nanocrystalline palladium particles (10–15 nm) have been synthesized using *Curcuma longa* tuber extract as the biomaterial. Temperature and pH had no major effect on size and shape of the NPs. It was found that the zeta potential of formed palladium NPs was negative and it increased with increase in pH. It has been also reported that palladium NPs could be synthesized by using coffee and tea extract. The synthesized NPs were in the size range of about 20–60 nm and crystallized in face-centered cubic symmetry (Nadagouda and Varma 2008). It was reported that *Gardenia jasminoides* E.′ water crude extract could be used for bioreduction of palladium chloride. Identified antioxidants, including geniposide, chlorogenic acid, crocins, and crocetin were reducing and stabilizing agents for synthesizing palladium NPs (~3–5 nm) in water crude extract. The particle size and disparity of NPs were temperature dependent, and the best dispersity was revealed at 70 °C (Jia et al. 2009). Roopan et al. (2012a) reported the green synthesis of spherical shape palladium NPs (~80 ± 5 nm) using *A. squamosa* L. (Annonaceae), commonly known as custard apple. The report revealed the presence of secondary metabolites contained −OH group which was responsible for the reduction of Pd(II)–Pd(0) (Roopan et al. 2012a). In another study, a facile and

environmentally friendly method was reported for the synthesis of palladium NPs using an aqueous solution of *Pulicaria glutinosa*, a plant widely found in a large region of Saudi Arabia, as a bioreductant (Khan et al. 2014). The hydroxyl groups of the plant extract molecules were found mainly responsible for the reduction and growth of palladium NPs. Results from FTIR analysis confirmed the dual role of the *P. glutinosa* extract, both as a bioreductant as well as a capping ligand, which stabilized the surface of the NPs. It was demonstrated that the synthesized NPs were crystalline NPs, and the results of XRD analysis confirmed the formation of a face-centered cubic structure. These palladium NPs demonstrated excellent catalytic activity toward the Suzuki coupling reaction under aqueous and aerobic conditions. Moreover, kinetic studies of the catalytic reaction monitored using GC confirmed that the reaction completes in less than 5 min (Khan et al. 2014).

Palladium NPs were synthesized by using *Piper betle* leave extract. The FTIR spectrum indicated the influence of flavonoids, terpenoids, and proteins in palladium NPs synthesis and stabilization in the aqueous medium. The peaks at 1,600 and 1,406 cm^{-1} showed the presence of flavonoids and terpenoids. It was demonstrated that the water-soluble flavonoids present in the *P. betle* extract could have been adsorbed on the surface of the NPs and same might have induced the reduction of Pd^{+2} to stable Pd^{0} NPs with interaction of carbonyl groups. Further, it was reported that the protein molecules in the *P. betle* extract might also participate in the process of capping and stabilization of NPs (Mallikarjuna et al. 2013).

11.2.3 Platinum NPs

Platinum (Pt) NPs (~2–12 nm) could be synthesized using the leaf extract of *D. kaki.* The leaf extract of *D. kaki* was reacted with an aqueous $H_2PtCl_6 \cdot 6H_2O$ solution at various temperatures between 25 and 95 °C. It was reported that more than 90 % of the platinum ions were converted into NPs with a 10 % leaf biomass concentration at 95 °C (Song et al. 2010). The authors proved that the size platinum NPs could be controlled by the temperature or the composition of the reaction mixture. The average size of the NPs decreased from 12 nm at 25 °C to 5 nm at 95 °C, and decreased from 11 nm at 5 % to 3 nm at 50 % the lead broth concentration. The size of platinum NPs increased from 3 to 9 nm as $PtCl_6$ ion concentration increased from 0.1 to 2 mM (Song et al. 2010). The leaf extract of *Ocimum sanctum* was used as a reducing agent for the synthesis of platinum NPs (~23 nm). Fourier transform infrared spectroscopy revealed that the compounds such as ascorbic acid, gallic acid, terpenoids, certain proteins and amino acids act as reducing agents for platinum ions reduction. It was reported that the reduced platinum showed similar hydrogen evolution potential and catalytic activity like pure platinum using linear scan voltammetry (Soundarrajan et al. 2012). In another study, one-pot synthesis of platinum and palladium NPs using lignin isolated from red pine (*Pinus resinosa*) was reported. In the TEM images, spherical NPs of diameters in the range of 16–20 nm were observed, in the case of

palladium, and smaller ones of not so well-defined shapes for platinum. These NPs showed good catalytic activity in the reduction reaction (Coccia et al. 2012).

11.2.4 Copper NPs

Copper (Cu) NPs were biosynthesized using Magnolia leaf extract. When aqueous solutions of $CuSO_4 \cdot 5H_2O$ treated with the leaf extract, stable copper NPs (~40–100 nm) were formed. Foams coated with biologically synthesized copper NPs showed higher antibacterial activity against *E. coli* cells (Lee et al. 2011). In another study, extracellular synthesis of copper NPs was carried out using stem latex of a medicinally important plant, *Euphorbia nivulia*. The synthesized NPs were stabilized and subsequently capped by peptides and terpenoids present within the latex. The copper NPs are toxic to adenocarcinomic human alveolar basal epithelial cells (A549 cells) in a dose-dependent manner. It was concluded that the nontoxic aqueous formulation of latex capped copper NPs could be directly used for administration/in vivo delivery of NPs for cancer therapy (Valodkar et al. 2011). Moreover, an eco-friendly method for synthesis of copper NPs (~15 ± 1.7 nm) was reported (Harne et al. 2012). Cysteine proteases present in the latex of *Calotropis procera* L. were used to fabricate copper NPs from copper acetate. FTIR was performed to confirm capping behavior of the latex proteins that contributed to long term stability of copper NPs (6 months) in aqueous medium. The FTIR spectrum results suggested that antioxidant enzymes (AOEs), cysteine protease, and tryptophan with functional groups of amines, alcohols, ketones, aldehydes, and carboxylic acids might be adsorbed on the surface of the synthesized copper NPs using *C. procera* L. latex. Further, cytotoxicity studies of latex-stabilized copper NPs were carried out on HeLa, A549, and BHK21 cell lines by MTT dye conversion assay. HeLa, A549, and BHK21 cells showed excellent viability even at 120 μM concentration of copper NPs (Harne et al. 2012).

Terminalia arjuna bark extract was used to produce copper NPs (~23 nm) under microwave irradiation (Yallappa et al. 2013). FTIR result indicated the presence of flavonones and terpenoids which might be responsible for the reduction and stabilization process. A solid-state ^{13}C NMR spectrum also indicated the presence of biomolecules on copper NPs. As a result, the prominent peaks at 211 and 200 ppm were attributed to ketones and aldehydes (flavonones, terpenoid aldehydes) from the plant extract on copper NPs. The peak at 187 ppm indicated the presence of amides while the peaks at 155, 145, 132, and 77 ppm indicated unsaturated compounds, reducing sugar, aromatic benzenes, and carbonyl groups, respectively (Yallappa et al. 2013). In another study, extract of leaves of henna (*Lawsonia inermis*) was used for synthesizing spherical copper NPs (~27–45 nm). Results from FTIR spectrum showed that the synthesized copper NPs were coated with lawsone and oxide layers. The authors fabricated a conducting nanobiocomposite films using the prepared copper NPs and collagen fibers discarded from leather industry. They demonstrated that the synthesized copper NPs and

nanobiocomposite films had potential for various electronic device applications (Cheirmadurai et al. 2014).

11.2.5 Lead NPs

Rapid synthesis of lead (Pb) NPs (~10–12.5 nm) by using 0.5 % aqueous extract of *J. curcas* L. latex was reported. Lead NPs were characterized initially by UV-Vis spectroscopy and shown distinct peak at 218 nm. This peak was highly specific for lead NPs. FTIR was performed to find the role of cyclic peptides namely curcacycline A (an octapeptide) and curcacycline B (a nonapeptide) as possible reducing and capping agents present in the latex of *J. curcas* L. (Joglekar et al. 2011).

11.2.6 Gold–Palladium Bimetallic NPs

Gold–palladium (Au–Pd) bimetallic NPs (~7.4 ± 0.8 nm) were produced based on simultaneous bioreduction of Au(III) and Pd(II) precursors (aqueous $HAuCl_4/PdCl_2$ mixture) with *Cacumen platycladi* leaf extract in aqueous environment. TEM micrograph demonstrated that the gold–palladium bimetallic NPs were well-defined spherical in shape. FTIR showed that the C=O and C–O groups in the plant extract played a critical role in capping the NPs. It was reported that some water-soluble polyhydroxy biomolecules in the *C. platycladi* leaf extract such as flavonoid and reducing sugar were responsible for the phytoreduction of the NPs (Zhan et al. 2011).

11.2.7 Zero-Valent Iron NPs

Generally, several methods can be used for the synthesis of zero-valent iron NPs including vacuum sputtering, decomposition of iron pentacarbonyl ($Fe(CO)_5$) in organic solvents, and the reaction of iron(II) or iron(III) salts with sodium borohydride (Kuhn et al. 2002; Karlsson et al. 2005; Wang and Zhang 1997). Further, green methods for synthesis of zero-valent iron NPs have been developed. Nadagouda et al. (2010) compared the zero-valent iron NPs produced using the green and borohydride methods and observed that some of the green zero-valent iron NPs were found to be nontoxic when compared with control samples prepared using the conventional borohydride reduction protocols. In this study, tea polyphenols were used for the rapid generation of nanoscale zero-valent iron particles. The zero-valent iron NPs were subsequently examined for in vitro biocompatibility using the human keratinocyte cell (HaCaT) line as a representative

skin exposure model. The cells were exposed to zero-valent iron NPs for time periods of 24 and 48 h. Biocompatibility was assessed using the methyl tetrazolium, or MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)) and lactate dehydrogenase (LDH) assays to determine in vitro cytotoxicity. The evaluation of mitochondrial function (MTS) and membrane integrity (LDH) in human keratinocytes showed that these green synthesized zero-valent iron NPs were nontoxic in the human keratinocytes exposed when compared with control samples synthesized using a borohydride protocol (Nadagouda et al. 2010). It was demonstrated that these NPs could have an adverse effect on pure cultures of bacteria, such as *E. coli*, *Pseudomonas fluorescens*, and *B. subtilis* var. *niger* (Auffan et al. 2008; Lee et al. 2008; Diao and Yao 2009).

Machado et al. (2013) evaluated the viability of the utilization of several plant leaves to produce extracts which were capable of reducing iron(III) in aqueous solution to form zero-valent iron NPs (~10–20 nm). The extracts could be classified into three categories according to their antioxidant capacity (expressed as Fe(II) concentration): >40 mmol L^{-1}; 20–40 mmol L^{-1}; and 2–10 mmol L^{-1}; with oak, pomegranate, and green tea leaves producing the richest extracts. It was concluded that the best results were obtained when the extraction is performed at 80 °C, while the best contact time and leaf mass: solvent volume ratio depended on the type of leaf. The authors of this chapter believed that polyphenols were responsible for synthesis of zero-valent iron NPs (Machado et al. 2013).

11.3 Phytosynthesis of Metal Oxide NPs

11.3.1 Iron Oxide NPs

It was reported that iron oxide NPs could be synthesized by using alfalfa biomass (Herrera-Becerra et al. 2008). Results showed that pH 10 yielded smaller particles with greater proportion of the Fe_2O_3, and the size could be controlled in the range of 1–4 nm. When pH decreased (pH 5), larger NPs were produced. In another study, Au–Fe_3O_4 composite NPs were prepared with a combined chemical and biological reducing process ("semi-biosynthesis method"). Magnetic cores were primarily produced using a fabrication method consisting of co-precipitation of Fe^{2+} and Fe^{3+}. An ethanol extract of *Eucalyptus camaldulensis* was used for the reduction of Au^{+3} on the surface of the magnetite NPs and for the functionalization of the Au–Fe_3O_4 nanocomposite particles (Haratifar et al. 2009). Iron oxide NPs (Fe_3O_4) were rapidly synthesized by reduction of ferric chloride solution with *Tridax procumbens* leaf extract containing carbohydrates as a major component which act as reducing agent (Senthil and Ramesh 2012). The results indicated that water-soluble carbohydrates which have aldehyde groups may cause the formation of iron oxide NPs. The purification process of the Fe_3O_4 product did not require any expensive method, since solid product was obtained from the reaction in liquid

phase. The antibacterial effect of iron oxide NPs against *P. aeruginosa* was also investigated. Bacteriological tests were performed in Potato Dextrose Agar (PDA) medium on solid agar plates and in liquid systems supplemented with different concentrations of iron oxide NPs. These particles were shown to be an effective bactericide (Senthil and Ramesh 2012).

11.3.2 Indium Oxide NPs

It was reported that indium oxide (In_2O_3) NPs were biologically synthesized using *Aloe barbadensis* Miller (*A. vera*) plant extracted solution. In_2O_3 NPs (~5–50 nm) were produced by using indium acetylacetonate and *A. vera* plant extracted solution (Maensiri et al. 2008). The NPs were formed after calcinations the dried precursor of indium oxide in air at 400–600 °C. The morphologies and sizes of indium oxide materials were affected by the temperature of calcinations.

11.3.3 Copper Oxide NPs

Copper oxide NPs were synthesized using aqueous extract of *Acalypha indica* leaf. The synthesized NPs were highly stable, spherical, and particle size was in the range of 26–30 nm. The antimicrobial activity of *A. indica*-mediated copper oxide NPs was tested against selected pathogens. These NPs showed efficient antibacterial and antifungal effect against *E. coli*, *P. fluorescens*, and *Candida albicans*. The cytotoxic activity of *A. indica*-mediated copper oxide NPs was evaluated by MTT assay against MCF-7 breast cancer cell lines and confirmed that these NPs have cytotoxicity activity (Sivaraj et al. 2014). Moreover, Gunalan et al. (2012a) reported that *A. barbadensis*-mediated copper oxide NPs were spherical in nature and sizes ranging from 15 to 30 nm. Results from FTIR indicated the existence of some phenolic compounds, terpenoids, or proteins which were bound to the surface of copper oxide NPs that remained despite repeated washing. The stability of copper oxide NPs might be due to the free amino and carboxylic groups which had interacted with the copper surface. Moreover, the proteins in the medium prevented agglomeration and stabilized the NPs (Gunalan et al. 2012a).

11.3.4 Chromium Oxide NPs

Chromium oxide (Cr_2O_3) NPs were rapidly synthesized by reduction of potassium dichromate solution with *Arachis hypogaea* leaf extract containing reducing sugars which act as reducing agent (Ramesh et al. 2012). The results indicated that the aldehyde groups present in the plant extract played an important role in the

formation of chromium oxide NPs. The antibacterial effect of chromium oxide NPs against *E. coli* was investigated. Bacteriological tests were performed in PDA medium on solid agar plates and in liquid systems supplemented with different concentrations of chromium oxide NPs. These particles were shown to be an effective bactericide. The shapes of the chromium oxide NPs appeared like hexagonal and cubic with rough surfaces (Ramesh et al. 2012). In another study, chromium oxide NPs (~65 nm) were phytosynthesized by the reduction of potassium dichromate solution with *Mukia maderaspatana* plant extract. For this, 20 mL of potassium dichromate solution was mixed with 20 mL of plant extract in a beaker and stirred for 10–15 min. The color of the solution changed from orange to green indicating the formation of chromium(III) oxide NPs. The solution was kept at room temperature for evaporation of aqueous phase. The green solid product was dried in hot air oven at 65–70 °C for an hour. The resulting solid was calcined at 650–700 °C for 3 h. The addition of potassium dichromate solution to the plant extract containing mild reducing agents caused the reduction of orange dichromate(VI) ions to green chromium(III) ions. The enhancing influence of chromium oxide NPs as a catalyst for the decomposition of $KMnO_4$ was studied. The antibacterial effect of chromium oxide NPs against *E. coli* was investigated. These particles were shown to have an effective bactericide (Rakesh et al. 2013).

11.3.5 Zinc Oxide NPs

It was reported that crystalline zinc oxide (ZnO) NPs (~72.5 nm) were synthesized using *Physalis alkekengi* L. The TEM micrographs demonstrated that the synthesized NPs were polydispersed and not uniformly distributed (Qu et al. 2011b). Furthermore, the branches (stems) of *Euphorbia tirucalli* was used for synthesizing spherical zinc oxide NPs (~20 nm) (Hiremath et al. 2013). The synthesis of hexagonal wurtzite zinc oxide NPs from Zn-hyperaccumulator (*Sedum alfredii* Hance) plants was reported, as well. The formed NPs were agglomerated, and single zinc oxide NPs were pseudospherical in shape with a mean size of 53.7 nm (Qu et al. 2011a). In another study, the exploit of aqueous leaf extract of *Corriandrum sativum* as an eco-friendly agent for the pattern of zinc oxide nanoparticle using zinc acetate and sodium hydroxide as a surrogate for chemical method was reported. FTIR studies of aqueous *Corriandrum* leaf extract revealed the presence of phytoconstituents such as alcohol, aldehyde, and amine which were the surface active molecules stabilized the NPs and this phytochemicals have interacted with the zinc surface and stabilized zinc oxide NPs (Gnanasangeetha and SaralaThambavani 2013b). Moreover, the antimicrobial efficacy of green and chemical synthesized zinc oxide NPs against various bacterial and fungal pathogens was determined. Zinc oxide NPs are known to be one of the multifunctional inorganic NPs with effective antimicrobial activities. Actually, metal oxide powders (e.g., ZnO, MgO, and CaO) showed antimicrobial activities against *S. aureus*, *E. coli*, and fungi. It is considered that the detected active oxygen

species generated by these metal oxide particles could be the main mechanism of their antibacterial activity. Various microbiological tests were performed using varying concentrations of green and chemical zinc oxide NPs with sizes 40 and 25 nm, respectively. It was reported that green zinc oxide NPs showed more enhanced biocidal activity against various pathogens when compared to chemical synthesized zinc oxide NPs. Also effectiveness of NPs were increased with increasing particle dose, treatment time, and synthesis method. This study demonstrated that the particle size variation and surface area to volume ratio of green zinc oxide nanoparticle were responsible for significant higher antimicrobial activity (Gunalan et al. 2012b). Aqueous leaf extract of *A. indica* were used to green synthesis of zinc oxide NPs (~100–200 nm). This green method showed that the environmentally benign and renewable aqueous leaf extract of *A. indica* could be used as a stabilizing agent for the synthesis of zinc oxide NPs. XRD pattern illustrated that the synthesized NPs were in wurtzite nature. The flavonoid and terpenoid constituents present in *A. indica* leaf extract were the surface dynamic molecules stabilized the NPs (Gnanasangeetha and SaralaThambavani 2013a). Moreover, green synthesis of spherical zinc oxide NPs (~30–35 nm) by zinc nitrate and utilizing the biocomponents of leaves extract of *Calotropis gigantea* was reported. The X-ray patterns showed hexagonal crystal type for zinc oxide (Vidya et al. 2013).

Nagajyothi et al. (2013) reported the green synthesis of zinc oxide NPs (~8.48–32.51 nm) using trifoliate orange (*Poncirus trifoliata*). Scanning electron microscope (SEM) image showed that the morphology of zinc oxide NPs was nearly spherical shape. The utility of phytosynthesized zinc oxide NPs as catalyst in Claisen Schmidt Condensation reaction was described. Accordingly, this ecofriendly, inexpensive and chemically stable catalyst was used for the condensation of 3,4-dimethyl benzaldehyde and acetophenone (Nagajyothi et al. 2013). Furthermore, zinc oxide nanocrystals were synthesized by employing *Nephelium lappaceum* L., peel extract as a natural ligation agent. Green synthesis of zinc oxide nanocrystals was carried out via zinc–ellagate complex formation using rambutan peel wastes. It was reported that zinc oxide nanocrystals-coated cotton showed good antibacterial activity toward *E. coli* and *S. aureus* (Yuvakkumar et al. 2014).

Zinc oxide NPs were synthesized by using *Borassus flabellifer* fruit extract (~50–60 nm) (Vimala et al. 2014). The UV/Vis spectrum showed an absorption peak at 368 nm that reflects surface plasmon resonance zinc oxide NPs. The synthesized NPs were porous in nature and rod-like structure. The possible mechanism for the green synthesis of zinc oxide NPs involved reduction of zinc nitrate ions that can form intermediate complexes with phenolic OH groups present in hydrolyzable tannins, which subsequently undergo oxidation to quinine forms with consequent reduction of zinc to zinc oxide NPs. Results from analysis proved the existence of some phenolic compounds, terpenoids or proteins which were bound to the surface of zinc oxide NPs. Moreover, it was suggested that the proteins prevented agglomeration and stabilized the NPs by forming a coat, covering the NPs. The authors examined the synthesized doxorubicin-zinc oxide NPs

exhibited a dose-dependent cytotoxicity against MCF-7 and HT-29. The inhibitory concentration (IC_{50}) was found to be 0.125 μg mL^{-1} for MCF-7 and HT-29 cells. An induction of apoptosis was evidenced by nuclear stain Hoechst 33,258. In vivo toxicity assessment showed that doxorubicin-zinc oxide NPs had low systemic toxicity in murine model system. The results proved that the synthesized zinc oxide NPs can be considered as a promising candidate for a drug delivery system (Vimala et al. 2014).

11.3.6 Titanium Dioxide NPs

An eco-friendly approach for synthesis of titanium dioxide (TiO_2) NPs from titanium isopropoxide solution using *Nyctanthes arbor-tristis* (night-flowering jasmine) leaves extract has been reported. SEM analysis demonstrated that the average size was from 100 to 150 nm, and the shapes were uniformed spherical (Sundrarajan and Gowri 2011). In another study, titanium dioxide NPs were synthesized using *A. squamosa* peel extract and their catalytic applications were studied on the 2,3-disubstituted dihydroquinazolin-4(^{1}H)-one synthesis. Synthesized compounds were confirmed using FTIR, ^{1}H NMR, ^{13}C NMR and GC–MS analyses (Bharathi et al. 2014). Moreover, Roopan et al. (2012b) reported the phytosynthesis of rutile titanium dioxide NPs using fruit peel *A. squamosa* aqueous extract. The UV-Vis spectrophotometer results were promising and showed a rapid synthesis of titanium dioxide NPs with a surface plasmon resonance occurring at 284 nm. The TEM images showed polydisperse NPs with spherical shapes and size 23 ± 2 nm ranges. The authors of this study used GC–MS to find the possible mechanisms for the formation of titanium dioxide NPs. Consequently, results from GC–MS analysis showed that the aqueous extract of *A. squamosa* fruit peel contained compounds having hydroxyl group as a functional group in the structure. Titanyl hydroxide could be dehydrated to give titanium dioxide NPs by heating it with an *A. squamosa* aqueous peel extract at about 60 °C. Here, the plant extract served as catalysts due to the present of compounds having hydroxyl group as a functional group in the structures. Further, water-soluble compounds containing hydroxyl functional group were reported to be responsible for the stabilization of titanium dioxide NPs (Roopan et al. 2012b).

Eclipta prostrata leaf extract was used for synthesizing of titanium dioxide NPs (Rajakumar et al. 2012). FTIR peak implicated the role of carboxyl group O–H stretching amine N–H stretch in the formation of titanium dioxide NPs. The results indicated that alcohols, phenols, alkanes, primary amines, and aliphatic amines in *E. prostrata* might be participating in the process of nanoparticle synthesis. Authors mentioned that the *E. prostrata* leaves contained beta-amyrin, wedelolactone, triterpenoids, flavonoids, luteolin-7-o-glucoside, L-terthienyl methanol and stigmasterol. Water-soluble heterocyclic compounds such as flavones were the reducing and capping ligands of the NPs. Functional groups associated with these the cause for the bioreduction of $TiO(OH)_2$ to TiO_2 NPs. Field

emission scanning electron microscopy analysis showed that the synthesized NPs were spherical clusters, quite polydisperse and they ranged in size from 36 to 68 nm with calculated average size of 49.5 nm (Rajakumar et al. 2012).

11.4 Reaction Conditions

The important challenges frequently encountered in the green synthesis of NPs are to control the shape and size of the nanosized particles as well as to achieve the monodispersity in solution phase. Moreover, the rate of nanoparticle synthesis and stability of the NPs are very important especially in industrial production. Therefore, effects of reaction conditions should be checked. In this section, we mention the effects of some important parameters including pH, temperature, amount of biomass or plant extract, substrate concentration, and reaction time:

11.4.1 pH

The pH of the solution is an important factor in controlling the size and morphology of NPs, and in the location of nanoparticle deposition (Diao and Yao 2009; Machado et al. 2013; Herrera-Becerra et al. 2008). For instance, it was demonstrated that in the case of *Cocos nucifera*, high monodispersity was obtained when the reaction was conducted at pH 11, with an average size of 23 nm, while at pH 2 no reaction occurred. It was observed that upon adjusting the pH of the reactants with NaOH, the light brown extract and colorless silver nitrate solution developed murky brown, respectively. It was revealed that when silver nitrate solution was mixed with NaOH solution first it would favor the formation a pure silver oxide (Ag_2O) precipitate and if a reducing reagent (e.g., *C. nucifera* coir extract) was also added, then the product became pure silver and that the reduction of Ag^{+}–$Ag^{(0)}$ occurred on the surface of the existing colloids in the system (Roopan et al. 2013). Sathishkumar et al. (2009b) reported the effect of pH on the formation of silver NPs using *C. zeylanicum* powder and bark extract over a wider pH range (1–11) and concluded that the pH of the solution dropped in most of the cases after the synthesis of silver NPs. As a result, small NPs were formed at high pH. Moreover, the formation of large-sized ellipsoidal silver NPs was observed at lower or acidic pH, while at higher or alkaline pH highly dispersed, small-sized, and spherical NPs tended to form. Andreescu et al. (2007) reported the acceleration in the rate of silver nanoparticle synthesis (rapid and complete reduction of silver ions) by using elevated pH. They reported that increase in pH results in increase the absolute value of the negative zeta potential which led to the formation of highly dispersed NPs. This phenomenon could be related to the electrostatic repulsion at high pH or attributed to the high absolute value of the negative zeta potential (Sadowski et al. 2008). Dubey et al. (2010b) have shown

the effect of pH on zeta potential of produced NPs. Zeta potential values reveal details about the surface charge and stability of the synthesized metal NPs (Dubey et al. 2010b). Actually, stability of produced metal NPs are evaluated with zeta potentiometer and corresponding surface Plasmon spectra (Dwivedi and Gopal 2010; Parashar et al. 2009a, b). Ag NPs demonstrated lower zeta potential value at strongly acidic pH, but when the pH increased higher zeta potential values were obtained. Furthermore, larger particle size could be achieved by decreasing the pH. Gardea-Torresdey et al. (1999) found that pH is an important factor in the biosynthesis of colloidal gold using alfalfa biomass and concluded that the size of NPs varied with the change in pH. Mock et al. (2002) also have reached to similar conclusions and reported that pH is responsible for the formation of NPs of various shapes and size. As different plant extracts and even the extracts coming from different parts of the same plant may have different pH values which further need optimization for the efficient synthesis of NPs, it has been reported by several researchers that larger NPs formed at lower pH (2–4) as compared to higher pH. Moreover, Armendariz et al. (2004a) reported that the size of gold nanoparticle produced by *A. sativa* was highly dependent on the pH value. At pH 2, large size NPs (~25–85 nm) were formed albeit in a small quantity but at pH 3 and 4, smaller sized NPs were formed in a large quantity. They speculated that at low pH (pH 2), the gold NPs prefer to aggregate to form larger NPs rather than to nucleate and form new NPs. In contrast, at pH 3 and 4, more functional groups (carbonyl and hydroxyl) are available for gold binding; thus a higher number of Au(III) complexes would bind to the biomass at the same time. Dwivedi and Gopal (2010) revealed that silver and gold NPs are stable in a wider range of pH as they observed very small variation in the zeta potential values between pH 2–10 in their study using *Chenopodium album*. Veerasamy et al. (2011) while working on mangosteen leaf extract reported that at low pH, aggregation of silver NPs is favored over the nucleation. However, higher pH facilitates the nucleation and subsequent formation of large number of NPs with smaller diameter. Nalawade et al. (2014) reported the synthesis of spherical silver NPs (~20–150 nm) using an aqueous extract of *Acacia auriculiformis* (as the reducing and capping agents). FTIR indicated the role played by hydroxyl groups of polyphenols and ester groups of terpenoids in the reduction of Ag^+ ions. Increase in the pH of the extract caused the formation of the NPs immediately after addition of Ag^+ ions. At pH 11, instant formations of silver NPs was observed. The color of the silver solution was also found to be changed from brown to yellow when pH of the extract was increased up to pH 11. It was observed that as pH of the solution increased above 2.3, the wavelength of absorption maximum due to silver NPs also decreased linearly from 432 to 403 nm. It was also observed that the silver NPs obtained at pH higher than 9.5 have shown enhanced colloidal stability compared to those obtained at lower pH. Moreover, the silver NPs obtained at pH 11 were stable for more than 2 months at room temperature and above 7 months at 4 °C. Further, it was observed that the NPs formed at higher pH are nearly monodispersed with polygonal morphology (Nalawade et al. 2014).

In case of *Pinus eldarica*, by increasing the pH of the reaction mixture, an increase in absorbance was observed, which could be due to the increase in production of colloidal silver NPs and reduction rate. It seems that pH affected the amount of nanoparticle formation and stability of them. Furthermore, pH influenced the rate of the reduction reaction. The reaction mixture turned brown when silver was reduced, and the reaction mixture coloring accelerated with increasing pH. Furthermore, the formation of large-sized silver NPs was observed at lower or acidic pH; while higher or alkaline pH highly dispersed, small-sized NPs tended to form (Iravani and Zolfaghari 2013).

11.4.2 Temperature

Temperature might be one of the crucial factors dominating the size and shape of NPs. It was reported that when the reaction temperature was increased from 25 to 150 °C, the size of silver and gold NPs became smaller which resulted into sharpness of plasmon resonance band of them (Dubey et al. 2010b). This is so because with the increase in temperature the rate of reaction will be increased which thus enhances the synthesis of NPs (Dwivedi and Gopal 2010; Philip 2009). Since the sharpness in absorbance peak depends on the size of the synthesized NPs, as with higher temperature, the particle size may be smaller, which results into the sharpening of the plasmon resonance band of silver and gold NPs (Shaligram et al. 2009). Andreescu et al. (2007) reported a rapid synthesis rate of silver NPs at higher temperatures. The increase in the reaction rate due to increasing the reaction temperature have been also reported by Rai et al. (2006) in case of gold nanotriangles biosynthesis using lemongrass extract as well as Song et al. (2009) in case of silver nanoparticle synthesis using persimmon and magnolia leaf extract. Song et al. (2009) have shown that silver NPs could be rapidly synthesized by using Magnolia leaf extract requiring 11 min for up to 90 % conversion at a reaction temperature of 95 °C. It can be concluded that the rate of synthesis of the NPs was related to the reaction and incubation temperature, and increased temperature levels allowed nanoparticle growth at a faster rate. Gericke and Pinches (2006) observed that nanorod and platelet-shaped gold NPs were synthesized at higher temperatures while the spherical-shaped NPs formed at lower temperatures. Moreover, Sathiskumar et al. (2010) realized the increase in the surface plasmon resonance with the increase in temperature, confirming the positive correlation between the yield of the NPs and the temperature.

11.4.3 Amount of Biomass/Plant Extract

Song et al. (2009) reported that the use of a low concentration of phyllanthin extract reacting with $HAuCl_4$ led to synthesis of hexagonal or triangular gold NPs,

but spherical NPs could be formed by addition of higher concentrations of the extract. Dubey et al. (2010b) have used different quantities of fruit extracts. They found that larger quantities of fruit extract leads to an increase in the peak absorbance in UV/Vis spectrum. Moreover, a decrease in particle size of Ag and Au NPs has been reported due to an increase in the extract amount. Dwivedi and Gopal (2010) demonstrated that with increasing ratio of leaf extract, the color of reaction mixture changed from reddish-yellow to deep red and pink to reddish pink for silver and gold NPs, respectively. In addition, the influences of the initial concentrations of the tea extract on the silver NPs productivity were studied at 25 °C. The stock solution of tea extract was diluted to 1, 5, 10, 25, 50, and 100 % (*v*/v) and was used as working solution, with the total organic carbon (TOC) of 1.0, 2.0, 5.0, 10.1, and 20.2 g/L, respectively. Consequently, the synthesis efficiencies of silver NPs were 99.1, 99.7, 99.9, 99.8, 94.6, and 95.3 % (w/w) with 1, 5, 10, 25, 50, and 100 % (*v*/v) tea extract, respectively. The production efficiencies of silver NPs were all above 94 % (w/w), while the highest nanosilver synthesis efficiency was achieved with 5 % (*v*/v) tea extract (Sun et al. 2014).

11.4.4 Substrate Concentration

One of the important measures to make the reaction more economical and efficient is finding the maximum concentration of the substrate which could be converted to the final product. We demonstrated green synthesis of spherical silver NPs using *P. eldarica* bark extract. The results obtained from time course of reaction indicated that by gradual increase in concentration of silver nitrate, the nanoparticle synthesis was also increased (Iravani and Zolfaghari 2013). In another study, Kora et al. (2012) reported the synthesis of spherical silver NPs (~7.5 ± 3.8 nm) using the aqueous extract of gum olibanum (*Boswellia serrata*), a renewable natural plant biopolymer. The water-soluble compounds in the gum serve as dual functional reducing and stabilizing agents. It was demonstrated that upon increasing the concentration of metal precursor, the intensity of the absorption band was increased, due to an enhancement in the nuclei formation, resulted in the synthesis of larger number of NPs.

11.4.5 Reaction Time

It was observed that the contact time may play a crucial role in the synthesis of NPs. It seems that due to the instability of the NPs formed, an optimum duration is required for complete nucleation and subsequent stability of the synthesized NPs. For instance, in one study, it was reported that the optimum time required for the completion of the reaction for silver nanoparticle synthesis was 60 min (Veerasamy et al. 2011). The requirement of optimum reaction time for

the stability of synthesized silver and gold NPs using *Rosa damascene* has been reported, as well (Ghoreishi et al. 2011). Dwivedi and Gopal (2010) reported an increase in the sharpness of UV absorption spectra peaks with the increase in the contact time, while working with *Chenopodium* leaf extract. They reported that NPs appeared within 15 min of the reaction and were increased up to 2 h, but after that only slight variation was occurred. Furthermore, Dubey et al. (2010b) observed that in *T. vulgare* mediated synthesis, the formation of silver and gold NPs started with in 10 min of the reaction. In addition, they found that the increase in the contact time is responsible for the sharpening of the peaks in both silver and gold NPs. Roopan et al. (2012b) studied the effect of duration for the formation of titanium dioxide NPs using aqueous extract of *A. squamosa* fruit peel. Consequently, at each and every 2 h interval the reaction mixture was subjected to UV-Vis analysis. It was found that the sample which was heated for 6 h gave the higher absorbance value and that the absorbance value decreased with further increase in the time of heating. Therefore, in order to form titanium dioxide NPs, 6 h was found to be most effective (Roopan et al. 2012b).

11.5 Mechanistic Aspects

In general, several mechanisms have been proposed (summarized in Table 11.2). Shankar et al. (2004a) reported that gold nanoparticle synthesis takes place due to the reduction of aqueous chloroaurate by reducing aldoses and ketones. Richardson et al. (2006) also reported that leaf extracts containing carbohydrates and proteins serve as reducing agents for silver ions. Jacob et al. (2012) reported a cost-effective and eco-friendly technique for green synthesis of spherical silver NPs (~17.6–41 nm) from silver nitrate solution (1 mM) using the extract of *Piper longum* leaf as reducing as well as capping agent. The synthesized NPs were also exhibiting excellent cytotoxic effect on HEp-2 cell lines. The FTIR spectrum of silver NPs showed distinct peaks 1,660 cm^{-1}, which represent the involvement of C=N in the plane vibrations of amino acids, 1,031–1,225 cm^{-1} represent the involvement of C–N in the plane vibrations of aliphatic amines. The above bonds commonly occur in proteins indicating the presence of proteins as ligands for silver NPs, which increase the stability of synthesized NPs. A peak at 1,225 cm^{-1} was assigned to the C–O group of polyols, indicating that polyols are playing a major role in the reduction of silver NPs. Two new alkaloids, piperlongumine and piperlonguminine, were isolated from *Piper longum*, and might be responsible for the reduction of silver nitrate into silver NPs. Rest of the bands showed resemblance to alkenes (617–702 cm^{-1}) and aromatic (900 cm^{-1}) groups, which is present in the plant extract and might have an effect in the synthesis of NPs (Jacob et al. 2012). Huang et al. (2007) reported green synthesis of silver NPs in the presence of reductive biomolecules present in *C. camphora* leaf extract. In the FTIR spectra, the presence of functional groups like –C–O–C, –C–O–, –C=C, and –C=O–, derived from several heterocyclics, was observed. These bioactive

Table 11.2 Phytochemicals and biomolecules involved in the synthesis of NPs using plants

Plants	Nanoparticle	Phytochemicals/ biomolecules	References
A. indica	Zinc oxide	Flavonoids, terpenoids	Gnanasangeetha and SaralaThambavani (2013a)
Achyranthus aspera	Silver	Polyols	Daniel et al. (2011)
A.cepa	Gold	Vitamin C	Parida et al. (2011)
A. sativum	Silver	Sucrose, fructose	White II et al. (2012)
A. sativum	Gold	Proteins	Coman et al. (2013)
Aloe barbadensis	Copper oxide	Phenolic compounds, terpenoids, proteins	Gunalan et al. (2012a)
Alternant he ra sessilis L.	Silver	Alkaloids, tannins, ascorbic acid, carbohydrates, proteins	Niraimathi et al. (2013)
Anacardium occidentale	Gold, silver, gold–silver alloy, gold core-silver shell	Polyols, proteins	Sheny et al. (2011)
Andrographis paniculata Nees.	Silver	Hydroxyflavones, catechins	Sulochana et al. (2012)
Annona squamosa	Palladium	Secondary metabolites contained –OH group	Roopan et al. (2012a)
Arachis hypogaea	Chromium oxide	Reducing sugars	Ramesh et al. (2012)
Astragalus gummifer L.	Silver	Proteins	Kora and Arunachala (2012)
Azadirachta indica	Silver	Reducing sugars, terpenoids	Shankar et al. (2004a)
Azadirachta indica A. Juss.	Gold	Salanin, nimbin, azadirone, azadirachtins	Thirumurugan et al. (2010)
Benincasa hispida	Gold	Polyols	Aswathy Aromal and Philip (2012a)
Bryophyllum sp.	Silver	Flavones, quinones, organic acids (e.g. oxalic, malic, tartaric, and protocatechuic acid)	Jha et al. (2009b)
Cacumen platycladi	Gold–palladium bimetallic	Water-soluble polyhydroxy biomolecules	Zhan et al. (2011)
Calotropis procera L.	Copper	Cysteine protease and tryptophan with functional groups of amines, alcohols, ketones, aldehydes, and carboxylic acids	Harne et al. (2012)

(continued)

Table 11.2 (continued)

Plants	Nanoparticle	Phytochemicals/ biomolecules	References
Camellia sinensis	Gold	Polyphenolic compounds	Boruah et al. (2012)
Carica papaya (fruit extract)	Silver	Hydroxyflavones, catechins	Jain et al. (2009)
Carica papaya (callus extract)	Silver	Proteins and other ligands	Mude et al. (2009)
Chenopodium album	Gold, silver	Oxalic acid, lignin	Dwivedi and Gopal (2010, 2011)
Cinnamomum zeylanicum	Palladium	Terpenoids (e.g. linalool, methyl chavicol, and eugenol)	Sathishkumar et al. (2009a, b)
Cinnamomum camphora	Palladium	Polyol components, water-soluble heterocyclic components	Yang et al. (2010)
Citrullus colocynthis	Silver	Polyphenols with aromatic ring and bound amide region	Satyavani et al. (2011)
Coleus aromaticus Lour.	Silver	Flavonoids, lignin	Vanaja and Annadurai (2012)
Corriandrum sativum	Zinc oxide	Phytoconstituents such as alcohol, aldehyde and amine	Gnanasangeetha and SaralaThambavani (2013b)
Cycas leaf	Silver	Polyphenols, glutathiones, metallothioneins, Ascorbates	Jha and Prasad (2010)
Cyperus sp.	Silver	Flavones, quinones, organic acids (e.g. oxalic, malic, tartaric, and protocatechuic acid)	Jha et al. (2009b)
Datura metel	Silver	Plastohydroquinone, plastrocohydroquinol	Kesharwani et al. (2009)
Delonix elata	Silver	Phenolic compounds, flavonoids	Sathiya and Akilandeswari (2014)
Desmodium triflorum	Silver	H^+ ions produced a long with NAD during glycolysis, Water-soluble antioxidative agents like ascorbic acids	Ahmad et al. (2011)
Diopyros kaki	Platinum	Terpenoids, reducing sugars	Song et al. (2010)

(continued)

Table 11.2 (continued)

Plants	Nanoparticle	Phytochemicals/ biomolecules	References
Dioscorea bulbifera	Silver	Polyphenols, flavonoids	Ghosh et al. (2012b)
Dioscorea oppositifolia	Silver	Polyphenols with aromatic ring and bound amide region	Maheswari et al. (2012)
Eclipta prostrata	Titanium dioxide	Heterocyclic compounds such as flavones	Rajakumar et al. (2012)
Elettaria cardamomom	Silver	Alcohols, carboxylic acids, ethers, esters, aliphatic amines	Gnanajobitha et al. (2012)
Eucalyptus hybrida	Silver	Flavanoid and terpenoid constituents	Dubey et al. (2009)
Euphorbia nivulia	Copper	Peptides, terpenoids	Valodkar et al. (2011)
Festuca rubra	Silver	Reducing sugars, antioxidant compounds, ascorbic acid	Marchiol et al. (2014)
Gardenia jasminoides E.	Palladium	Geniposide, chlorogenic acid, crocins, crocetin	Jia et al. (2009)
Glycine max	Palladium	Proteins, amino acids	Petla et al. (2012)
Glycyrrhiza Glabra	Silver	Flavonoids, terpenoids, thiamine	Dinesh et al. (2012)
Hibiscus cannabinus	Silver	Ascorbic acid	Bindhu and Umadevi (2012)
Hydrilla sp.	Silver	Flavones, quinones, and organic acids (e.g. oxalic, malic, tartaric, and protocatechuic acid)	Jha et al. (2009b)
Hydrilla verticilata	Silver	Proteins	Sable et al. (2012)
Jatropha curcas	Zinc sulfide, lead	Curcacycline A (an octapeptide), Curcacycline B (a nonapeptide) Curcain (an enzyme)	Hudlikar et al. (2012), Joglekar et al. (2011)
Justicia gendarussa	Gold	Polyphenol, flavonoid compounds	Fazaludeena et al. (2012)
Lantana camara	Silver	Carbohydrates, glycosides, flavonoids	Sivakumar et al. (2012)
Leonuri herba	Silver	Polyphenols, hydroxyl groups	Im et al. (2012)

(continued)

Table 11.2 (continued)

Plants	Nanoparticle	Phytochemicals/ biomolecules	References
Magnolia kobus	Gold	Proteins and metabolites (e.g. terpenoids having functional groups of amines, aldehydes, carboxylic acid, and alcohols)	Song et al. (2009)
Mentha piperita	Gold, silver	Menthol	Ali et al. (2011)
Mirabilis jalapa	Gold	Polyols	Vankar and Bajpai (2010)
Morinda pubescens	Silver	Hydroxy flavones, catechins	Jancy and Inbathamizh (2012)
Ocimum sanctum	Silver, platinum	Phenolic and flavanoid compounds, proteins, ascorbic acid, gallic acid, terpenoids, proteins and amino acids	Soundarrajan et al. (2012), Ramteke et al. (2013)
Ocimum tenuiflorum	Silver	Polysaccharides	Vignesh et al. (2013)
Parthenium hysterophorus	Silver	Hydroxy flavones, catechins	Kumar (2012)
Pear fruit extract	Gold	Organic acids, peptides, proteins, amino acids, accharides	Ghodake et al. (2010)
Pedilanthus tithymaloides	Silver	Proteins, enzymes	Sundaravadivelan and Nalini (2011)
Pelargonium graveolens	Silver	Proteins, terpenoids and other bio-organic compounds	Shankar et al. (2003a)
Phoma glomerata	Silver	Proteins, amino acids	Gade et al. (2014)
Pinus eldarica	Silver	Polyphenolic compounds	Iravani and Zolfaghari (2013)
Pinus resinosa	Platinum	Lignin	Coccia et al. (2012)
Piper betle	Silver	Proteins	Mallikarjuna et al. (2012)
P. betle	Palladium	Flavonoids, terpenoids, proteins	Mallikarjuna et al. (2013)
Piper nigrum	Silver	Proteins	Garg (2012)
Plumeria rubra	Silver	Proteins	Patil et al. (2012)
Saraca indica	Gold	Polyphenolic compounds	Dash et al. (2014)
Sesuvium portulacastrum	Silver	Proteins, flavones, terpenoids	Nabikhan et al. (2010)

(continued)

Table 11.2 (continued)

Plants	Nanoparticle	Phytochemicals/ biomolecules	References
Solanum lycopersicums	Gold, silver	Flavonoids, alkaloids, antioxidant vitamins, carotenoids (lycopene), polyphenols	Bindhu and Umadevi (2014)
Solanum xanthocarpum	Silver	Phenolics, alkaloids, sugars	Amin et al. (2012)
Sorghum bicolor Moench	Silver, iron	Polyphenols	Njagi et al. (2011)
Swietenia mahogany	Silver, gold, bimetallic alloy gold–silver	Polyhydroxy limonoids	Mondal et al. (2011)
Syzygium aromaticum	Gold	Flavonoids	Deshpande et al. (2010)
Terminalia arjuna	Copper	Flavonones, terpenoids	Yallappa et al. (2013)
Terminalia catappa	Gold	Hydrolysable tannins	Ankamwar (2010)
Terminalia chebula	Silver	Polyphenols present in the form of hydrolysable tannins	Kumar et al. (2012)
Trianthema decandra	Silver	Hydroxyflavones, catechins	Geethalakshmi and Sarada (2010)
Tridax procumbens	Copper oxide	Water-soluble carbohydrates	Gopalakrishnan et al. (2012)
Vitus vinifera	Lead	Flavone, anthocyanins	Pavani et al. (2012)
Zingiber officinale	Gold, silver	Alkanoids, flavonoids	Singh et al. (2011)

compounds are presumed to act as reducing and capping agents for the silver NPs. Li et al. (2007) proposed recognition reduction-limited nucleation besides a growth model to explain the silver NPs production. In the recognition phase, the silver ions were trapped on the surface of proteins present in the *Capsicum annum* extract through electrostatic interaction, then, probably the proteins reduce the silver ions, resulting in nucleation of silver. Afterward, the proteins and biomolecules present in the reaction mixture lead to isotropic growth of silver nuclei with stabilization of silver NPs.

Sathishkumar et al. (2012) reported the green synthesis of FCC crystalline silver NPs (~10–60 nm) using leaf extract of *Morinda citrifolia* L. under different temperatures and reaction periods. It was observed that these NPs had moderate inhibitory effects against human pathogens than the crude plant extract, demonstrating its antimicrobial value against pathogenic diseases. The FTIR spectrum analysis of the synthesized silver NPs and the plant extract shows variation in transmittance and modification stretches at 3394, 2078, 1408, 1025, 1638, and

672 cm^{-1} in *M. citrifolia* plant extract, which might be due to the interaction of biomolecules with silver NPs. The results depicted the presence of phenolic compounds such as flavonoids, triterphenoids present in the leaf extract which might possibly influence the reduction and stabilization of the synthesized silver NPs (Sathishkumar et al. 2012). Shankar et al. (2003a) reported the presence of proteins and secondary metabolites in the water-soluble fractions of geranium leaves and postulated that terpenoids contributes in the reduction of silver ions and are oxidized to carbonyl groups. They suggested that hydroxyls in the terpenoids present in geranium leaf extract, like citronellol and geraniol, are oxidized to carbonyl groups and hence act as a reducing agent for silver ions. They have assigned a band of 1,748 cm^{-1} to ester C=O group of chlorophyll in FTIR analysis supporting their hypothesis. In another study, the proteins were found to be involved in the surface capping of gold NPs synthesized using geranium leaf extract (Shankar et al. 2003b). Vanaja and Annadurai (2012) suggested that the presence of aromatic amine, amide(I) group, phenolic groups, and secondary alcohols in *Coleus aromaticus* leaf extract may act as reducing agents for the synthesis of silver NPs. Moreover, Safaepour et al. (2009) used geraniol extract for the reduction of silver ions and found that geraniol possesses the ability to synthesize silver NPs by reducing silver ions. The study reported synthesis of uniformly dispersed silver NPs in the size range of 1–100 nm. Kasthuri et al. (2009a) isolated phyllanthin from the plant *Phyllanthus amarus* and used different concentrations of phyllanthin extract for the synthesis of gold and silver NPs. The FTIR studies demonstrated a shift in the methoxy ($–OCH_3$) band (1,088 cm^{-1}) of the phyllanthin extract after silver or gold nanoparticle formation. This change can be attributed to the binding of the $–OCH_3$ group with the NPs. This shift, in gold NPs, was slightly higher than with the silver NPs, indicating that the adsorption of phyllanthin on the surface of gold NPs was more efficient than on silver NPs. Depending on the phyllanthin concentration, different shapes of the gold and silver NPs were obtained.

Haverkamp and Marshall (2009) reported that plants have limited capacities for reducing the metal ions and this capacity relies on the reduction potential of the metal species. During their work on the uptake of various silver salts solution by hydroponically grown *Barssica juncea*, they proposed that metal nanoparticle formation by plants is restricted to precious and semiprecious metals (Haverkamp and Marshall 2009). Jatropha latex obtained from *J. curcas* was used as reducing and capping agent to form silver NPs (~20–30 nm). The data based on this plant revealed that the major peptide constituents of the latex are curcacycline A (cyclic octapeptide) and curcacycline B (cyclic nonapeptide) (Bar et al. 2009a). The FTIR analysis before and after silver nanoparticle formation showed decreased intensity of the secondary amine band and a shift of the stretching vibration of the (NH) C=O group. These changes can be attributed to the reduction of silver ions and to the binding of the (NH) C=O group to the NPs, respectively. These results indicated that the cyclic peptides curcacycline A and B were involved in the reduction of silver ions to silver NPs and in their stabilization (Bar et al. 2009a). The authors of this article suggest a possible mechanism to form silver NPs. In this

mechanism, first of all the silver ions are entrapped in the core structure of the cyclic peptide. Then, the ions are reduced and stabilized in situ by the amide group of the host peptide under the experimental conditions. The sizes of these silver NPs were similar to the radius of the cavity of the cyclic peptides, confirming this mechanism (Bar et al. 2009a).

Ganeshkumar et al. (2013) developed a method to synthesize monodispersed gold NPs by mixing gold solution with fruit peel extract of *Punica granutum* without using any surfactant or external energy. Casein, being a biocompatible polymer, is used to couple the prepared gold NPs for functionalization of folic acid, which is highly expressed in cancer cells. These functionalized gold NPs could be used for targeted drug delivery for cancer therapy with enhanced therapeutic efficacy and minimal side effects. Hemocompatibility of the synthesized gold NPs was evaluated in human blood samples and found that the particles were hemocompatible. The gold NPs were facilely obtained via the addition of plant extract into Au^{3+} solution within 30 s. The preparation of gold NPs was so efficient that the synthetic process could be completed within 60 s, which indicated a great practical value for the large-scale preparation of gold NPs. Epicalocatechine present in the plant extract could be a reason for the reduction of Au^{3+} to form Au^0. The authors of this study mentioned that the gold NPs prepared using tea extract did not show much stability, but the gold NPs prepared by this method were found to be very stable, which might be due to the presence of ellagitannins like punicalagin. The punicalagin molecule first chelated with Au^{3+} through its adjacent phenolic hydroxyls to form a five-membered chelate ring. Due to the high oxidation-reduction potential of Au^{3+}, the reacted adjacent phenolic hydroxyls of punicalagin were inductively oxidated to the corresponding quinones, and thus the Au^{3+} were reduced to Au^0 atoms. These neighbor Au^0 atoms further collided with each other to form gold NPs with high stability. The FTIR spectrum of pomegranate extract showed a peak at 3,380 cm^{-1} which could be attributed to the stretching vibration of phenolic hydroxyls (O–H bond) in extracts, the absorption peaks at 1445, and 1623 cm^{-1} represent aromatic ring present in the extract. However, in the FTIR spectrum of the synthesized gold NPs, the peak at 3,400 cm^{-1} becomes relatively narrow which could be attributed to the stretching vibration of phenolic hydroxyls (O–H bond), confirm the interactions of phenolic hydroxyls to gold NPs, resulting in the partial destruction of hydrogen bonds among pomegranate extract molecules. A shift in the peak at 1,346–1,384 cm^{-1} also indicated that pomegranate extract interacted with gold NPs through its adjacent phenolic hydroxyls and/or formed quinones (Ganeshkumar et al. 2013).

Silver NPs were prepared by using plant extracts from xerophytes (*Bryophyllum* sp.), mesophytes (*Cyperus* sp.), and hydrophytes (*Hydrilla* sp.). For xerophytes (like *Bryophyllum* sp.), a mechanistic scheme was suggested. In this mechanism, malic acid undergoes oxidative decarboxylation to produce pyruvate in the presence of $NAD^+/NADP^+$-dependent malic enzyme. Furthermore, pyruvate in the presence of phosphoenolpyruvate carboxykinase produces phosphoenolpyruvate that is used in the glycolytic pathway. Also, emodin, which is an anthraquinone, undergoes redial tautomerization that leads to reduction of

silver ions (Jha et al. 2009b). A different mechanism was proposed for mesophytes (*Cyperus* sp.) which have well-defined metabolic pathways. In this mechanism, silver nanoparticle synthesis might result due to tautomerization of quinones. Benzoquinones, for example, cyperquinone (type I), dietchequinone (type II), and remirin (type III), are reported to undergo redial tautomerization for reduction of silver ions (Jha et al. 2009b). In hydrophytes (*Potamogeton* sp. or *Hydrilla* sp.), the antioxidant ascorbate is oxidized in antioxidative reactions and the enzyme dehydroascorbate reductase catalyzes the re-reduction of dehydroascorbate to ascorbate. Apart from the antioxidants, the hydrophytes are also known to possess catechol and protocatechuic acid. In alkaline conditions, catechol oxidizes to protocatechuic acid through protocatechaldehyde, presumably with hydrogen participation in the reduction of silver ions and synthesis of silver NPs. Jha et al. (2009b) suggested possible mechanisms of silver nanoparticle synthesis using plant extracts based on different metabolites or metabolic pathways. In the same direction, using leaf extract from *Psidium guajava* on an aqueous gold chloride solution gave stable poly-shaped gold NPs. Zeta potential experiments showed that the biogenic colloidal functionalized NPs kept their stabilities up to 30 weeks of storage. It was found that flavonoids in the extracellular solution from the leaves were responsible for the biosynthesis of the gold NPs (Raghunandan et al. 2009). A biosynthesis of silver and gold NPs from aqueous leaf extract of *Chenopodium album*, an obnoxious weed, was carried out given quasi-spherical-shaped NPs (~10–30 nm), probably through some chemical constituent in the extract acting as reductant (e.g., oxalic acid and aldehyde groups) (Dwivedi and Gopal 2010). During the biomimetic synthesis of silver NPs by *Citrus lemon*, Prathna et al. (2011) demonstrated the ability of citric acid as both the principal reducing agent for nanoparticle synthesis process and the probable stabilizing agent. In another study with tamarind leaf broth, Ankamwar et al. (2005a) investigated the possibility of the acid group (tartaric acid) to act as a capping agent and to become responsible for the stability of bio-reduced gold NPs.

We reported the formation of spherical silver NPs (~10–40 nm) using *Pinus eldarica* bark extract (Iravani and Zolfaghari 2013). *P. eldarica* bark contains phenolic compounds such as catechin, taxifolin, caffeic acid, and ferulic acid (Iravani and Zolfaghari 2011). We determined the chemical composition of *P.eldarica* bark extract. A reversed-phase high pressure liquid chromatography (RP-HPLC) method for the determination of catechin, caffeic acid, ferulic acid, and taxifolin in *P. eldarica* bark extract was developed. A mixture of 0.1 % formic acid in deionized water and 0.1 % formic acid in acetonitrile was used as the mobile phase, and chromatographic separation was achieved on a Nova pack C18 at 280 nm. Among four marker compounds, the main substances identified in *P. eldarica* was catechin (Iravani and Zolfaghari 2014). These phenolic compounds were possibly responsible for the synthesis of NPs (Iravani and Zolfaghari 2013).

FTIR analysis was carried out to identify possible functional groups responsible for the synthesis of silver NPs by using *G. jasminoides* E. extract (Fenfen et al. 2014). Consequently, through the peak identification, it was reported that the compounds responsible for the synthesis of silver NPs might include saccharides,

polyphenols, aldehydes, alcohol ketones, and carbonyl compounds. The authors of this study speculated that the synthesis of silver NPs was due to the reduction of aqueous silver nitrate by the saccharides, carbonyl compounds or phenolic hydroxyl group, and the aldehydes ketones binding to silver nanoparticles act as protective group. Results from the component analysis showed that proteins, flavonoid, reducing sugar, polyphenol, geniposide and chlorogenic acid were important for the formation of silver NPs. Especially, the content of flavonoid, polyphenols and chlorogenic acid had a significant drop after reaction. To further ascertain the role of the components in *G. jasminoides* E. extract, pure chemicals of these active components were utilized and corresponding identifying experiments showed that rutin, gallic acid and chlorogenic acid had reducing and protecting capacities, and bovine albumin was a potential capping agent. Moreover, geniposide exhibited good shape-directing capacity for the formation of silver nanowires (Fenfen et al. 2014).

In the case of tea extract, results from FTIR spectroscopy showed a shift in peaks: 3,420–3,371 (due to N–H stretching, amides), 2,931–2,925 (due to C–H stretching, alkanes), 1,383–1,371 (characteristic of hydroxyl groups, phenolic hydroxyl), and 1,051–1,044 cm^{-1} (due to C-stretching, ether groups). Further, the synthesized silver NPs showed peaks at 1695, 1452, 1241, and 926 cm^{-1} related to alkene groups (C=C stretching), tertiary ammonium ions, poly phenols, aliphatic amines (C–N stretching vibrations), and alkene groups (C–H stretching), respectively. The FTIR analysis indicated the involvement of amides, carboxyl, amino groups and poly phenols in the synthesized silver NPs (Sun et al. 2014). Moreover, Begum et al. (2009) suggested that polyphenols or flavonoids present in black tea leaf extracts were responsible for silver and gold nanoparticle synthesis. They reached to this conclusion silver or gold nanoparticle was not observed in the leaf extract lacking polyphenols/flavonoids. Sathishkumar et al. (2009c) published similar results during palladium nanoparticle synthesis using *C. longa* tuber extract. Aswathy Armol and Philip (2012b) reported that fenugreek (*Trigonella foenum-graecum*) seed extract has the ability to perform dual function of reduction and stabilization of gold NPs (~15–25 nm). They elaborated that flavonoids present in seed extract are powerful reducing agents which may be responsible for the reduction of chloroauric acid, whereas the carboxylate group present in proteins can act as surfactant to attach on the surface of gold NPs and stabilize them through electrostatic stabilization. Kasthuri et al. (2009c) used apiin, which is a flavonoid glycoside isolated from *L. inermis* leaves for the synthesis of gold and silver NPs. Results obtained from FTIR spectroscopy confirmed that the carbonyl group of apiin contributes to the interaction between the NPs and apiin. Moreover, Raghunandan et al. (2009) found that flavonoids in the guava (*Psidium guajava*) leaf extract were responsible for the synthesis of gold NPs. Song et al. (2009) also reported that various plant extracts can be used to synthesize the metallic NPs owing to the existence of terpenoids and reducing sugars in them. During their study on the synthesis of gold nanoparticle with *M. kobus*, they reported that terpenoids having the functional groups of amines, alcohols, ketones, aldehydes, and carboxylic acids were responsible for the stabilization of gold NPs. Furthermore,

Song et al. reported that platinum nanoparticle synthesis using *Diopyros kaki* leaf extract is not an enzyme mediated process because the rate of nanoparticle synthesis was found to be greatest at the temperature as high as 95 °C and there were no peaks associated with protein/enzymes on the FTIR analysis (Song et al. 2010). Moreover, Narayanan and Sakthivel (2011) reported that plant-mediated synthesis could not be happened by enzymes as generally the plant extract is heated up to 90 °C during the synthesis process. According to them, phytochemicals such as phenolics, terpenoids, sesquiterpenes, and flavonoids and the functional groups present in these phytochemicals are involved in the reduction and capping of NPs. Similarly, saponins present in the aqueous leaf extract of *Memecylon edule* also contributed to the reduction of silver and gold ions to nanosized silver and gold particles respectively (Elavazhagan and Arunachalam 2011). Mesocarp layer extract of *C. nucifera* was used and assessed for the synthesis of silver NPs (~23 ± 2 nm) (Roopan et al. 2013). The reduction of silver ions occurred when silver nitrate solution was treated with aqueous extract of *C. nucifera* coir at 60 °C. In this study, GC–MS of coir extract confirmed the presence of hydrocarbon such as nonacosane and heptacosane which may possibly influence the reduction process and stabilization of silver NPs. Synthesized silver NPs were effective antilarvicidal agents against *Anopheles stephensi* and *Culex quinquefasciatus* (Roopan et al. 2013). Nabikhan et al. (2010) reported the effect of extracts from tissue culture-derived callus and leaf of the saltmarsh plant, *Sesuvium portulacastrum* L. on synthesis of antimicrobial silver NPs using silver nitrate as a substrate. It was demonstrated that the callus extract could be able to produce silver NPs, better than leaf extract. There were prominent peaks in the extracts corresponding to amide I, II, and III indicating the presence of the protein, as revealed by FTIR spectroscopy measurement. There were also peaks that were corresponding to aromatic rings, geminal methyls, and ether linkages, indicating the presence of flavones and terpenoids responsible for the stabilization of the silver NPs (Nabikhan et al. 2010).

11.6 Conclusion

In general, different methods have been developed to obtain NPs of various shapes and sizes, but most of them can cause contamination, from precursor chemicals, organic solvents, and hazardous by-products, which often raise environmental concerns. Therefore, there is a significant benefit in developing nontoxic, cheap, and eco-friendly processes for nanoparticle synthesis. Plants are one of the best candidates for synthesizing NPs. Green synthesis of NPs using plants is regarded as a safe, cost-effective, sustainable, and clean process. Plants have successfully been used to produce NPs such as magnetite, silica, silver, gold, titania, selenium, platinum, palladium, quantum dots, zirconia, indium oxide, zinc oxide, titanium dioxide, silver–gold alloy and etc. The use of plants in green nanotechnology and nanobiotechnology is developing rapidly because of the ease of handling

and formation of NPs. By using the plants (biomass or extracts) in the reaction mixture, the formation of NPs with controlled morphologies and sizes can be achieved. The synthesized NPs have wide applications in catalysis, biomedicine, electronics, sensing, photonics, environmental clean-up, imaging, bio-labeling, drug and gene delivery, and pharmaceutical sciences. Concluding remarks and research challenges for phytosynthesis of NPs using plants are mentioned below:

1. Actually, most of the researches focused their attention on silver and gold nanoparticle synthesis, and it seems that more efforts are needed for green synthesis of other metallic NPs.
2. Major drawbacks associated with phytosynthesis of NPs are tedious purification steps and poor understanding of the mechanisms. Some investigations have put forward hypothetical mechanisms proposing that the reduction of the metal ions to metal NPs may be due to the distinct compounds present in the extracts including reducing sugars, phenolic compounds, proteins/enzymes, amino acids, flavonoids, polysaccharides, alkaloids, tartaric acid, tannic acid, alcoholic compounds, vitamins, citric acids, phyllanthin, pralines, tartaric acid, tannic acid, and etc. In addition to use of whole plant extracts, the pure natural products (small molecules or macromolecules) can be used for reducing and stabilizing the metallic NPs. It seems that by using these natural products, there is better control over the size, shape, and agglomeration. Then the synthesized NPs are stable and destined for many potential applications. In this context, there have been significant advances.
3. Qualitative routine-based spectrophotometric analysis such as FTIR, colorimetric assays, and UV/Vis spectroscopy can give information about reducing agents responsible for metallic ions reduction. However, these methods cannot unambiguously differentiate the presence or absence of the different families of compounds in the extracts and the resulting NPs. Thus, the role of the various components of plant extracts in the metal ions reduction or stabilization has not been clearly understood or addressed. Therefore, more efforts such as using advanced chromatographic techniques (e.g., HPLC-MS and NMR) are needed to find the exact mechanisms of nanoparticle formation which may lead to fine tuning of the process ultimately leading to the synthesis of NPs with a strict control over the size and shape parameters. In some studies, advanced chromatographic techniques have been used.
4. Phytosynthesis of NPs is still in a developing stage, and the stability and aggregation of NPs, control of crystal growth, morphology, size, and size distribution are the most experienced problems. The important challenges frequently encountered in the synthesis of NPs are to control the shapes and sizes of the particles as well as to achieve the monodispersity in solution phase. Several important parameters including substrate concentration, electron donor (e.g., glucose or fructose), reaction or incubation time, pH, temperature, buffer strength, mixing speed, and light should be optimized. It seems that by optimization of these critical parameters, highly stable NPs with desired sizes and morphologies can be achieved. In addition, purification, isolation, and stabilization of the synthesized NPs are very important, and challenges in this regard must be overcome.

5. It seems that several important challenges, and technical problems and otherwise, should be overcome before this green method will be a successful and competitive alternative for industrial synthesis of NPs. An important challenge is scaling up for production level processing. Furthermore, little is known about the mechanistic aspects, and information in this regard is necessary for economic and rational development of phytosynthesis of NPs. The large-scale phytosynthesis of NPs is interesting because it does not need any hazardous, toxic, and expensive chemicals for synthesis and stabilization of the synthesized NPs.

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Chapter 12
Phytosynthesis of Metal Nanoparticles

Alka Yadav and Mahendra Rai

Abstract The field of nanotechnology has created great interest among researchers due to its remarkable outcomes in different fields of optoelectronics, medical, pharmaceuticals, chemical, and agricultural importance. It is an emerging cutting-edge technology involving different methodologies for the synthesis of nanoparticles of particular size and shapes. Development of experimental protocols for synthesis of metal nanoparticles of specific size and shape is a necessary advancement of nanotechnology. Although physical and chemical methods have been successfully used to synthesize metal nanoparticles, there is a persistent necessity to develop eco-friendly and sustainable techniques for the synthesis of nanoparticles. Biosynthesis of nanoparticles using a number of fungi, bacteria, actinomycetes, lichen, and viruses have been reported till date but the plant system has emerged as an efficient system due to its distinctive characters like easy availability, low cost, green approach, simpler downstream processing, etc. In the plant system, biosynthesis process is more useful if nanoparticles are produced extracellularly using plants or their extracts and in a controlled approach related to their size, dispersity, and shape. Plant system can also be suitably scaled up for large-scale synthesis of nanoparticles. However, some aspects like role of different biomolecules in synthesis of nanoparticles, understanding the biological mechanism of synthesis process needs to be considered elaborately. In this chapter, we have discussed briefly about plants as a prominent tool for the synthesis of metal nanoparticles. Moreover, different methods of synthesis of nanoparticles, different mechanisms involved in the synthesis process, and also the potential applications of metal nanoparticles have also been discussed.

Keywords Nanotechnology · Metalnanoparticles · Biosynthesis · Plants · Mechanism

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12.1 Introduction

Nanotechnology is an interdisciplinary field of science including physics, chemistry, biology, and material science. The nanoparticles are an indispensable part of nanotechnology (Parashar et al. 2009a; Bankar et al. 2010; Zhang et al. 2011; Mahdavi et al. 2013). Engineered metal nanoparticles are produced by a number of physical and chemical methods. However, these methods are harmful as the chemicals used are generally toxic, flammable, and not easily disposable due to environmental issues, expensive, and have low production rate (Kasthuri et al. 2008; Bankar et al. 2010; Nagajyothi and Lee 2011). Thus, instead of using toxic chemicals for the synthesis of metal nanoparticles, the use of biological entities has received substantial consideration in the field of nanobiotechnology (Logeshwari et al. 2013). The biological methods for the synthesis of metal nanoparticles are regarded as safe, cost-efficient, sustainable, and toward greener approach (Marchiol 2012). Hence, extensive contribution have been made to employ biological systems for the synthesis of metal nanoparticles at ambient temperature and pressure conditions without the use of any toxic chemicals and also without production of any poisonous byproducts (Kumar and Yadav 2009; Satyavathi et al. 2010; Gopalkrishnan et al. 2012). A variety of microorganisms including bacteria, fungi, and yeasts have been harnessed as potential nanofactories for intra and extracellular synthesis of metal nanoparticles (Sharma et al. 2009; Mallikarjuna et al. 2011; Renugadevi and Aswini 2012; Iravani and Zolfaghari 2013). However, the use of plant system for the production of metal nanoparticles is an upcoming research field (Iravani 2011). The use of plants for the synthesis of metal nanoparticles offers an environment friendly, cost-effective, and legitimate alternative for large-scale production of metal nanoparticles (Marchiol 2012; Logeshwari et al. 2013).

The present chapter deals with the use of plants for the synthesis of metal nanoparticles and the several aspects related to the process, the mechanism of synthesis in plants, and the applications of the system.

12.2 Plants as the System of Choice

Among the different living systems harnessed for the synthesis of metal nanoparticles, plants have found predominant application in the synthesis process as the use of plants for the biosynthesis of metal nanoparticles could be beneficial compared to other biological agents (Rai et al. 2008; Mude et al. 2009; Jha and Prasad 2010; Duran et al. 2011; Renugadevi and Aswini 2012; Dinesh et al. 2012). In the case of plant systems, the elaborate process of maintaining cell cultures is eliminated (Marchiol 2012). Also, biological synthesis of metal nanoparticles involves synthesis in a controlled manner according to their size, dispersity, and shape (Shankar et al. 2004; Ankamwar et al. 2005; Parashar et al. 2009a, b).

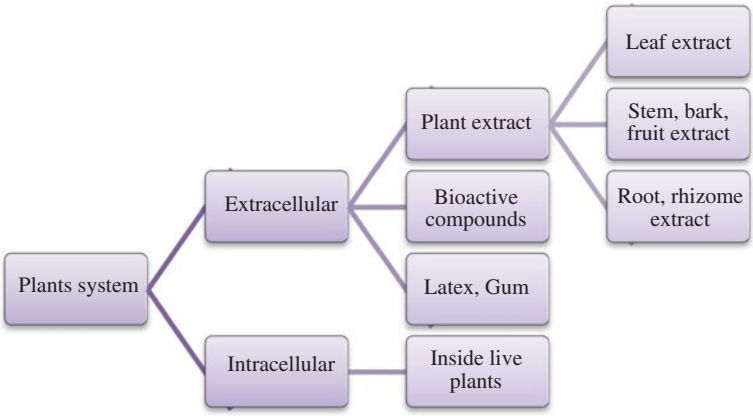

Fig. 12.1 Different methods of synthesis of metal nanoparticles using plant system

The plant system can also be duly scaled up for large-scale synthesis of nanoparticles (Rai et al. 2008). Different metals especially silver and gold have been extensively studied for the phytosynthesis of metal nanoparticles employing plant extracts and plant biomass (Marchiol 2012; Mahdavi et al. 2013) (Fig. 12.1). It has been depicted that many plant species can actively uptake and bioreduce metal ions from soil and solutions during detoxification process and thereby form insoluble complexes with the metal ion in the form of nanoparticles (Goldsbrough 2000). This natural phenomenon of heavy metal tolerance of plants attracted researchers to explore the related biological mechanisms as well as physiology and genetics of metal tolerance in hyperaccumulator plants (Baker and Brooks 1989; Memon and Schröder 2008). Thus, the researchers concentrated on the use of plants with potential in phytomining and phytoremediation of heavy metals in order to phytosynthesize metallic nanoparticles. Gardea-Torresdey et al. (2002) presented the first report of synthesis of nanoparticles using alfalfa seedlings which is considered as a hyperaccumulator plant. It was revealed that gold nanoparticles, ranging in size from 2 to 20 nm, could be synthesized inside live plants.

Also, trending research in biosynthesis of nanometals using plant extracts, fruit extract, and bark and root extracts has opened a new era in easy, fast, and ecofriendly methods for the synthesis of metal nanoparticles (Sharma et al. 2009; Thakkar et al. 2010; Iravani et al. 2011; Iravani and Zolfaghari 2013). Many researchers have explored the phytosynthesis of metal nanoparticles using different plant extracts and their potential applications (Gardea-Torresdey et al. 2002; Shankar et al. 2004; Chandran et al. 2006; Harris and Bali 2007; Haverkamp and Marshall 2009; Mude et al. 2009; Jha and Prasad 2010; Duran et al. 2011; Renugadevi and Aswini 2012; Dinesh et al. 2012; Mahdavi et al. 2013).

Thus, green nanotechnology has involved a lot of interest of researchers compared to other biological systems and includes a wide range of procedures that reduce or eliminate toxic substances to restore the environment. Also, the

phytosynthesis of metal nanoparticles using plant extracts and other parts of living plants has become a current substitute for the production of metal nanoparticles. As phytosynthesis of metal nanoparticles involves use of environmental friendly, nontoxic, and safe reagents.

12.2.1 Mechanism of Synthesis

The precise mechanism for the formation of metal nanoparticles using plants is not yet known, nor investigated in depth (Rai et al. 2008; Haverkamp and Marshall 2009). Biosynthesis of metal nanoparticles is a bottom-up approach of synthesis where reduction/oxidation is the main reaction by which synthesis takes place (Marchiol 2012). Various microorganisms such as bacteria, fungi, and yeasts are considered as nanofactories for intra- and extracellular synthesis of metal nanoparticles (Lovley et al. 1987; Ahmad et al. 2003; Husseiny et al. 2007; Singaravelua et al. 2007). Whereas, use of plant system for biosynthesis of metal nanoparticle is a comparatively new and under advancement research technique (Marchiol 2012).

The bioreduction of metal nanoparticles in plants occurs by a combination of bioactive compounds present in plant extracts like enzymes, proteins, amino acids, vitamins, polysaccharides, etc. (Iravani 2011). Several researchers have reported efficient and rapid extracellular synthesis of silver, gold, copper, and gold nanoparticles using extracts of several plants; for example, *Aloe vera* (Chandran et al. 2006), *Medicago sativa* (Gardea-Torresdey et al. 2002), *Azadirachta indica* (Shankar et al. 2004), *Avena sativa* (Armendariz et al. 2004), *Emblica officinalis* (Ankamwar et al. 2005), *Humulus lupulus* (Rai et al. 2006), *Spinacia oleracea* and *Lactuca sativa* (Kanchana et al. 2011), *Capsicum annum* (Jha and Prasad 2011), *Tridax procumbens* (Gopalkrishnan et al. 2012), and *Sargassum muticum* (Mahdavi et al. 2013).

Shankar et al. (2004) employed neem (*A. indica*) leaf extract for the synthesis of silver nanoparticles. The FTIR spectra showed the presence of reducing sugars and flavones or terpenoids in the sample. Hence, it was supposed that the reducing sugars are responsible for the reduction of silver ion to silver nanoparticles while the flavones or terpenoids act as the capping agent. The TEM images of the reaction mixture gave a picture of synthesis of polydisperse spherical nanoparticles. While, the XRD spectra confirmed the crystalline nature of nanoparticles.

Capsicum annum extract was used by Li et al. (2007) for the synthesis of silver nanoparticles. The fruit extract depicted rapid change in coloration from green to dark-brown marking synthesis of silver nanoparticles. The UV-visible spectra demonstrated peak at 440 nm and the TEM images confirmed the synthesis of spherical nanoparticles. In this study, mechanism of recognition-reduction-limited nucleation and growth for the synthesis of nanoparticles was proposed by the authors. It was projected that the silver ions undergo electrostatic interaction with the proteins present in the extract which leads to the formation of silver complex. Further, the flexible linkages of proteins and other biomolecules lead to the synthesis of stable spherical nanoparticles (Fig. 12.2).

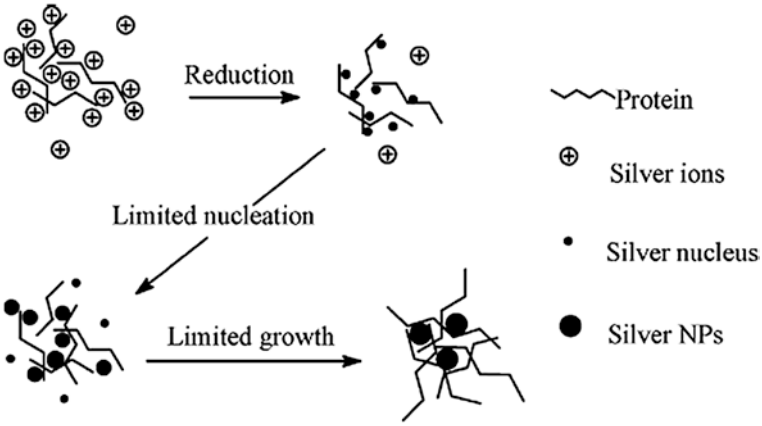

Fig. 12.2 Schematic representation of mechanism of synthesis of silver nanoparticles (Li et al. 2007, Reproduced with permission from Royal Society of Chemistry)

Bioactive compound phyllanthin isolated from *Phyllanthus amarus* was harnessed by Kasthuri et al. (2008) for the biosynthesis of silver nanoparticles. In the study, UV-visible absorbance peak at 439 nm with a shift at 446 nm was observed and the TEM analysis of the nanoparticles depicted synthesis of quasispherical nanoparticles with average size about 30 nm. The cyclic voltammetry measurements showed that upon addition of phyllanthin extract to the reaction medium, the cathodic peak gets shifted toward the negative direction suggesting that the silver nanoparticles gets stabilized by the phyllanthin extract. The FTIR spectra also revealed that the $–OCH_3$ group of the phyllanthin extract plays a leading role in the formation and stabilization of nanoparticles (Fig. 12.3).

In a similar way, latex extract of *Jatropha curcas* was also harnessed for the synthesis of silver nanoparticles by Bar et al. (2009a). The HRTEM images of the study illustrated two broad range distributions of nanoparticles, with diameter 20–30 nm and some larger diameter an uneven shapes. The XRD spectra of the biosynthesized silver nanoparticles revealed the crystalline nature of silver nanoparticles, the EDX spectra also showed strong signal of silver. The latex of *J. curcas* was observed to curcacycline A, curcacycline B, and curcain. Thus, it was hypothesized that the silver ions get entrapped into the core structure of curcacycline A or curcacycline B and get reduced and stabilized in situ by the amide group which results in the formation of silver nanoparticles and the enzyme curcain functions as a stabilizing agent of the nanoparticles.

Bar et al. (2009b) used the seed extract of *J. curcas* for the synthesis of silver nanoparticles. The UV-visible spectra of the silver nanoparticles depicted an absorbance peak at 425 nm and the HRTEM and XRD studies also showed predominant synthesis of spherical nanoparticles with polycrystalline nature. The FTIR study of the biosynthesized silver nanoparticles demonstrated that the amide groups were responsible for the reduction of silver ions while, the proteins acted as stabilizing agent for the nanoparticles.

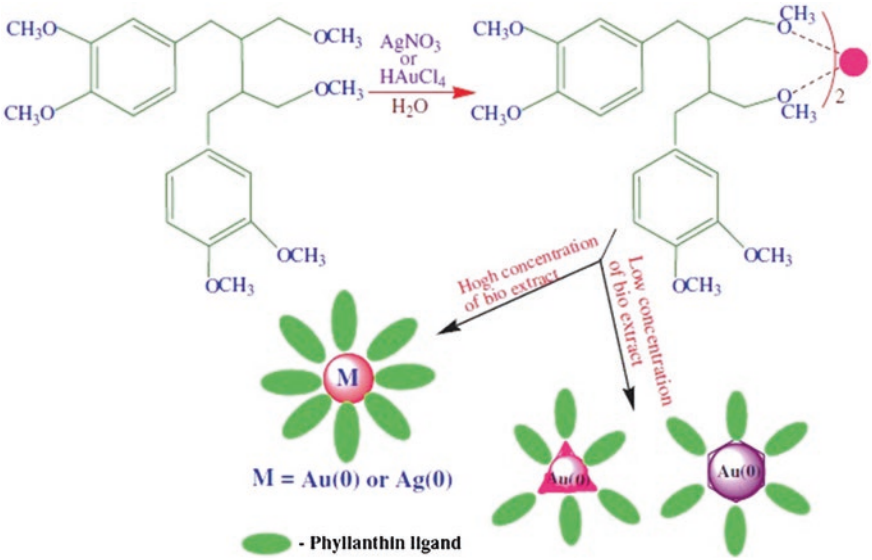

Fig. 12.3 Schematic representation of formation of phyllanthin stabilized gold and silver nanoparticles (Kasthuri et al. 2008, Reproduced with permission from Springer.com)

Singh et al. (2011) also harnessed latex of *Calotropis procera* for biosynthesis of zinc oxide (ZnO) nanoparticles. The TEM and SEM studies of the biosynthesized nanoparticles depicted formation of spherical-shaped nanoparticles, granular in nature, and average size 5–40 nm. The XRD study also revealed the presence of crystalline-natured zinc oxide nanoparticles. In the above study, latex of *C. procera* plant was supposed to be the reducing as well as the stabilizing agent for the synthesis of zinc oxide nanoparticles.

Yadav and Rai (2011) demonstrated the synthesis of silver nanoparticles using *Holarrhena antidysenterica* and studied the mechanistic aspects related to it. In the study, the authors proposed the possible role of terpenoids for the bioreduction of silver ions. The proteins were observed to act as an encapsulating and stabilizing agent to protect agglomeration of silver nanoparticles. The ESI (Elemental Spectroscopy Imaging) analysis of the silver nanoparticles also confirmed the stabilization of nanoparticles by proteins. The FTIR spectra of the silver nanoparticles depicted well-known signatures of amide linkages in proteins.

Green synthesis of palladium (Pd) nanoparticles was depicted by Petla et al. (2012) using *Glycine max* (soyabean) leaf extract. The change in coloration of the soyabean leaf extract after treatment with palladium ions from orange to dark-brown was marked as the synthesis of Pd nanoparticles. The UV-vis spectra at 420 nm also confirmed the formation of nanoparticles. The authors believed that the proteins and some of the amino acids present in the leaf extract were responsible for the synthesis of Pd nanoparticles. The FTIR analysis also corroborated that the amino acids were not only involved in the synthesis process but also acted as surfactants inhibiting rapid agglomeration of nanoparticles.

Tridax procumbens was exploited for the synthesis of copper oxide nanoparticles by Gopalkrishnan et al. (2012). In the study, the authors observed that the water soluble carbohydrates present in plants were responsible for the reduction of copper ions and formation of copper oxide nanoparticles. The antibacterial activity of copper oxide nanoparticles was also checked against *E. coli*. It was found that nanoparticles at concentration of 20 μg cm^{-3} inhibited 65 % bacterial growth while, nanoparticles at a concentration of 30 μg cm^{-3} inhibited 100 % bacterial growth.

Mahdavi et al. (2013) exploited green biosynthesis method for reduction of ferric chloride solution with brown seaweed (BS*, S. muticum*). The water extract of brown seaweed containing sulfated polysaccharides was considered as the main factor which acted as the reducing agent and efficient stabilizer for iron oxide nanoparticles. The structure and properties of the iron oxide nanoparticles were investigated using X-ray Diffraction, Fourier Transform Infrared Spectroscopy, Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-ray Fluorescence Spectrometry (EDXRF), Vibrating Sample Magnetometry (VSM), and Transmission Electron Microscopy. The average particle diameter of iron oxide nanoparticles was found to be 18 ± 4 nm. The X-ray diffraction study showed that the nanoparticles are crystalline in nature, with a cubic shape.

Iravani and Zolfaghari (2013) synthesized silver nanoparticles using *Pinus eldarica* bark extract. The effects of quantity of the extract, substrate concentration, temperature, and pH on the formation of silver nanoparticles were also studied. The TEM images depicted that biosynthesized silver nanoparticles were predominantly spherical in shape with approximately size range of 10–40 nm.

Logeswari et al. (2013) reported biosynthesis of silver nanoparticles by commercially available plant powders, such as *Solanum tricobatum*, *Syzygium cumini*, *Centella asiatica*, and *Citrus sinensis*. The characterization of silver nanoparticles was done by UV–Vis Spectrophotometer, X-Ray Diffractometer (XRD), Atomic Force Microscopy (AFM), and Fourier Transform Infrared (FTIR) Spectroscopy. The AFM study showed irregular shapes of silver nanoparticles, and the size was found to be 53, 41, 52, and 42 nm, corresponding to *S. cumini*, *C. sinensis*, *S. tricobatum* and *C. asiatica*, respectively. The FTIR Spectroscopy confirmed the presence of protein as the stabilizing agent surrounding the silver nanoparticles.

12.3 Applications

- Metal nanoparticles offer great interest in different disciplines including biotechnology/biomedicine, bioremediation, agriculture, catalyst, biosensors, etc. Functionalized nanoparticles present immense potential in catalysis, bio-labeling, and bioseparation (Gupta et al. 2012).
- Nanotube membranes are harnessed as channels for separation of molecules and ions between solutions hence, used as biomembranes. These nanotube biomembranes separate nanoparticles based on their size while, membrane with dimension 20–60 nm can be used to separate proteins (Gupta et al. 2012).

- Magnetic nanoparticles are used as effective molecular carrier for gene separation. Magnetic nanoparticles also show potential application in drug delivery. In this process, magnetic nanoparticles are injected to drug molecule to be attached, these particles are then guided toward the chosen site under localized magnetic field and can carry large doses of drugs (Lu et al. 2007; Perez-Martinez et al. 2012).
- Application of metal nanoparticles as catalyst is an immensely growing field. Nanoparticles due to their distinctive properties form an ideal component for catalyst. Platinum and gold bimetallic nanoparticles are used as electrocatalyst for polyelectrolyte fuel cells for the conversion of exhaust heat to energy (Toshima 2013). Titanium, gold, and silver heterostructures have also depicted electrochemical properties and are thus used as a photocatalyst (Zhang et al. 2013; Kawamura et al. 2013).
- Metal nanoparticles have also depicted biological applications like silica-coated nanoparticles are biocompatible structures used in artificial implants and drug delivery due to their high stability, surface properties, and compatibility (Dikpati et al. 2012; Perez-Martinez et al. 2012). Polyethylenimine-derived (PEI) nanoparticles and dendrimers have also shown applications like gene delivery, catalysis, and electronics (Perez-Martinez et al. 2012; Dikpati et al. 2012).
- Magnetite nanoparticles demonstrate application in wastewater treatment and removal of heavy metals from water through single-step removal of some model organophosphorus pesticide from water along with some microorganisms (Das et al. 2009). Magnetite nanoparticles are also harnessed as adsorbents for separating and removing the contaminants in water by applying external magnetic fields (Carlos et al. 2013).
- Metal nanoparticles with unique properties also offer use in the detection and destroying of pesticides (Argay et al. 2012). The optical properties of nanoparticles related to their size and surface helps in the detection of pesticides. However, for destruction of pesticides photocatalytic oxidation method employing titanium nanoparticles is used (Argay et al. 2012).

12.4 Future Prospects and Conclusion

Varying number of chemical, physical, and biological methods are used for the production of metal nanoparticles. But, most of these methods are still in the development stage and thus problems are faced regarding the stability and aggregation of metal nanoparticles and morphology and size distribution. It is observed that the metal nanoparticles synthesized by plants are more stable in comparison with those produced by other organisms. Plants reduce metal ions faster than fungi or bacteria. In addition, use of plants offers an easy and safe green method to scale-up production of well-dispersed metal nanoparticles. Hence, researchers have focused their attention on understanding the biological mechanisms and enzymatic processes for synthesis of metal nanoparticle using plants and detection of biomolecules involved

in the synthesis of metallic nanoparticles. Many biomolecules present in plant extracts like proteins/enzymes, amino acids, polysaccharides, alkaloids, alcoholic compounds, and vitamins are found to be involved in bioreduction, formation, and stabilization of metal nanoparticles. The future investigations related to the use of plant system would focus toward the optimization of reaction conditions and engineering the recombinant organisms for production of high amounts of proteins, enzymes, and biomolecules involved in biosynthesis and stabilization of nanoparticles. Understanding the biochemical processes/pathways involved in plant heavy metal detoxification, accumulation, and resistance will also be studied to improve nanoparticle production. Genetic modification of plants with improved metal tolerance and accumulation capacities is also a future approach to increase the production of metal nanoparticle synthesis.

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Chapter 13
Plant-Based Synthesis of Silver Nanoparticles and Their Characterization

Poonam Patel, Priti Agarwal, Sajjan Kanawaria, Sumita Kachhwaha and S.L. Kothari

Abstract Nanotechnology is a very promising area of research which involves the production of nanomaterials as the basic strategy. Although artificial synthesis of nanomaterials were initiated by using chemical and physical approaches, but recently the biological synthesis methods are being widely used as ecofriendly alternatives. Plant-based synthesis of nanomaterials is better because of its ease of handling, rapidity, and cost-effective nature along with environmental friendliness. A wide range of applications of silver nanoparticles (AgNPs) creates a focal point for attention of researchers. In view of published studies, in this chapter, we critically assess the role of plants in the synthesis of AgNPs, the characterization methods, applications of biologically synthesized AgNPs in various fields and future perspectives.

Keywords Atomic force microscopy · Electron microscopy · Silver nanoparticles · Synthesis mechanism · XRD-analysis

13.1 Introduction

Nanoparticles are the mandatory constituents of nanotechnology. They exhibit a distinct property of larger surface-area-to-volume ratio, which makes them better than their bulk counterparts in the sense of their activity (Annamalai et al. 2011; Raimondi et al. 2005). Nanoparticle synthesis is usually carried out by physical and chemical methods. Both these methods suffer from high energy demand or the use of toxic chemicals. The biological method of synthesis involves the use of microorganisms (Pugazhenthiran et al. 2009), fungus (Dhillon et al. 2012), algae (Prasad et al. 2013) and plants (Iravani 2011). The development of bio-inspired synthesis

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of nanoparticles has received immense attention in the last few years in progressive manner due to the tremendous advantages it offers in terms of cost and eco-friendliness. It is evident from the earlier reports that plants are the better candidates for synthesis of nanoparticles as nanoparticles produced by using plants parts are more stable and also the rate of synthesis is faster than in the case of microorganisms (Iravani 2011). Thus, they are suitable for large-scale biosynthesis of nanoparticles.

Nowadays researchers are concentrating on green synthesis of nanoparticles of nobel metals viz. gold, silver, platinum, and palladium because of their applications in medical and pharmaceutical products, besides their use in consumer goods such as shampoos, soaps, detergents, shoes, cosmetic products and toothpaste (Kim and Song 2010).

This chapter provides an overview about the phytosynthesis of AgNPs, proposed mechanisms, factors affecting the reaction, characterization methods, published reports and applications of AgNPs in various areas with future perspectives.

13.2 Plant-Based Synthesis of AgNPs

Silver nanoparticles have been produced by physical and chemical methods for a long time, but recent developments explored the critical role of biological systems for this purpose (Fig. 13.1).

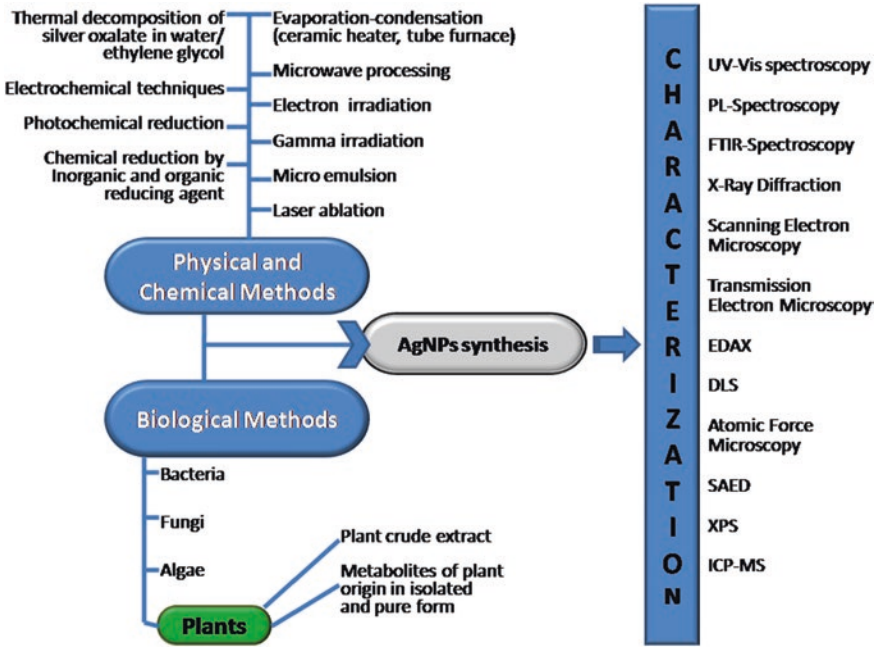

Fig. 13.1 Commonly used methods (chemical, physical, and biological) for synthesis of silver nanoparticles and the techniques used for the characterization of synthesized nanoparticles

Physical and chemical methods are energy intensive processes which mean high expenditure. Production of silver nanoparticles by chemical reduction (e.g., hydrazine hydrate, sodium borohydride, DMF, and ethylene glycol) may lead to absorption of harsh chemicals on the surfaces of nanoparticles raising the toxicity issue (Iravani 2011). Moreover, nanocrystalline silver colloids produced by such aqua-chemical routes exhibit aggregation with time, thereby compromising with the size factor upon storage.

Among the bio-inspired synthesis of AgNPs, plant extracts are found to be more suitable candidates over other biological entities (microorganisms and fungi) because they do not require toxic reducing and capping agents, radiation, high temperature, microbial/fungal strains and costly media for microbial/fungal growth as well as for nanoparticles production. They also avoid the chances of infection/contamination during synthesis and application section (Borase et al. 2014).

These demerits recommend the plant-mediated synthesis of AgNPs which involves synthesis at biological pH. Furthermore, due to slower kinetics, it offers better manipulation and control over crystal growth and their stabilization. An economical point of view also prioritizes to plant extracts as they are ubiquitous and easily available. Besides this, the process of extract preparation is cheap and simple.

13.2.1 Mechanism Behind Synthesis

The silver nanocrystals are usually grown from Ag^+ solutions. These silver ions come from a silver salt. The ions are first reduced to atoms by means of a reducing agent followed by nucleation in small clusters that grow into particles. If this reduction of silver ions is mediated by plant extracts, the process is known as bio-reduction. Nanoparticle formation (size and shape) depends on the availability of atoms, which in turn depends on the silver salt to reducing agent concentration ratio.

Reduction of silver (I) by green chemical methods proceeds through one-step process to produce a colored silver sol. After reduction, produced particles show nucleation due to the hyperreactivity and thus continuous increase in the size. To get nanoparticles of smaller size, it is necessary to add some capping agents to reaction mixture as soon as possible. Sometimes, used plant extract itself act as capping agent. During reaction, appearance of an intense color between red and black in reaction mixture is the signatory feature of AgNPs formation (Fig. 13.2).

Besides this, the role of plant metabolites as reducing and capping agents in AgNPs synthesis has not been well documented yet. In one of the reports on phytosynthesis of AgNPs, working with the leaf broth of *Azadirachta indica,* the flavanone and terpenoid constituents of the broth were believed to be the surface active molecules stabilizing the nanoparticles, while the formation of pure Au, Ag, and bimetallic Au core–Ag shell nanoparticles was facilitated by reducing sugars and/or terpenoids present in the plant extract (Shanker et al. 2004). Later on, Li et al. (2007) found that the extract of *Capsicum annuum* is also suitable for phytosynthesis of AgNPs and the reduction of silver ions and stabilization of the AgNPs was thought to occur through the participation of proteins. In another study, Huang et al. (2007)

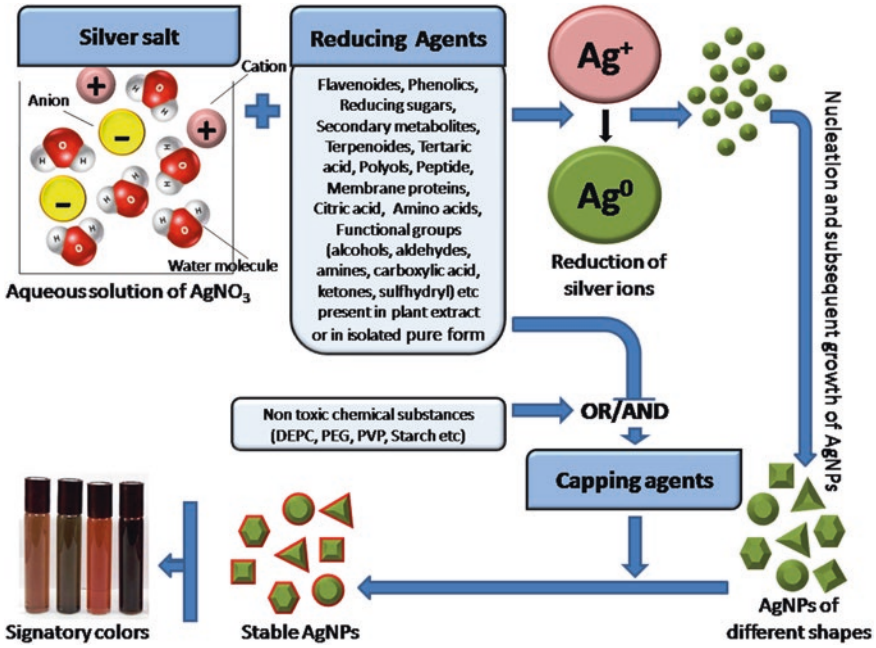

Fig. 13.2 Schematic diagram showing the mechanism behind the formation of AgNPs by using plant extracts or responsible reducing agents in isolated pure form

reported that polyols present in the leaf extract of *Cinnamomum camphora* were mainly responsible for the reduction of silver and/or chloroaurate ions and formation of silver and gold nanoparticles. While during the study with *Ananas comosus*, it was believed that the biomolecules responsible for the reduction and stabilization of AgNPs are antioxidants including phenols probably because different types of antioxidants are present in the pineapple juice (Ahmad and Sharma 2012). Several other researchers have also suggested the involvement of various plant metabolites such as terpenoids, flavonoids, polyphenols (Marimuthu et al. 2011), amines (Prasad et al. 2011), saponins (Elavazhagan and Arunachalam 2011), aldehydes, ketones (Chandran et al. 2006), arabinose and galactose (Kora et al. 2010), and starch (Vigneshwaran et al. 2006) in AgNPs synthesis.

Currently, the field of phytosynthesis of AgNPs is under exploitation but only limited reports have been published with a proposed mechanism and almost all of them are just reasonable hypotheses without any convincing experimental support.

13.2.2 Factors Affecting the Synthesis Process

In order to synthesize the AgNPs at industrial scale by using the plants, the yield and the production rate are most important issues to be considered. In the field of phytosynthesis, the challenges encountered are the control of shape and size of the

particles as well as to achieve monodispersity in solution phase. Therefore, the ultimate need is the further research to optimize the bioreduction conditions in the reaction mixture. The substrate concentration, biocatalyst concentration, electron donor and its concentration, pH, exposure time, temperature, buffer strength, mixing speed and light need to be controlled and optimized (Iravani 2011) for this purpose.

13.3 Characterization of Synthesized AgNPs

The bioreduction and formation of AgNPs can be monitored by sampling the reaction mixture at regular intervals during the experiment. The formation of AgNPs and characteristics of prepared nanoparticles can be examined by the following methods (also shown in Fig. 13.1).

13.3.1 UV/Vis Spectrophotometry

Absorbance spectroscopy is used to determine the optical properties of a solution (Skoog et al. 2007). Light is passed through the sample and the amount of absorbed light is measured. However, for examination of nanoparticles, the optical properties are much more complicated and require an individually developed theory. As illustrated in Fig. 13.3a, UV-Vis spectra of AgNPs have absorbance peak near 420 nm where broadening of peak indicate that the particles are polydispersed (Mohanpuria et al. 2008; Shankar et al. 2003).

13.3.2 Photoluminescence (PL) Spectroscopy

The luminescence of Ag and that of noble metal is generally attributed to excitation of electron from occupied d bands into states above the Fermi level (Smitha et al. 2008). The synthesized AgNPs are found to be photoluminescent. Figure 13.3b shows the luminescence spectrum of freshly prepared AgNPs, which exhibited a sharp and strong peak near 365 nm and a broadened band between 500 and 600 nm.

13.3.3 Fourier Transform Infrared (FTIR) Spectroscopy

Nanomaterials are often surface passivated with organic molecules or organic materials. Identification of such molecules throws light on the possible mechanism behind synthesis which shows the utility of the techniques in the study of phytosynthesis (Kulkarni 2009). FTIR measurement is carried out to identify the

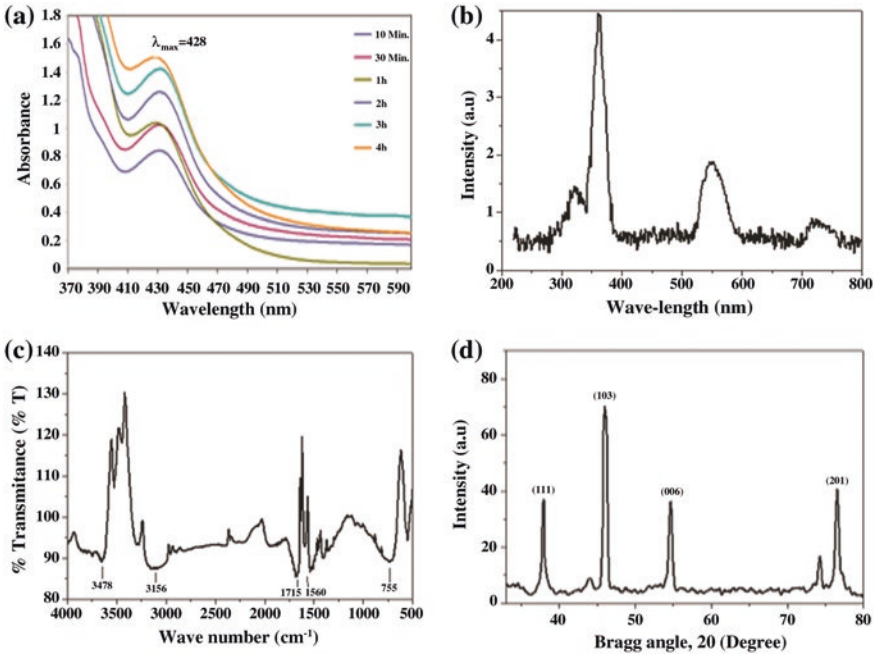

Fig. 13.3 Various characterization graphs of AgNPs synthesized by the plant-based methods. Formation of AgNPs can be confirmed and monitored by characterization methods. **a** UV-Vis spectra. **b** PL spectrum of SNPs synthesized from *Cucumis sativus*. **c** FTIR Spectrum. **d** XRD spectrum

possible interaction between bio molecule and AgNPs. As supported by Fig. 13.3c, the FTIR measurements of biosynthesized silver nanoparticles show bands at around 755, 1715, 3156, and 3678 cm^{-1}. In the IR spectra of synthesized AgNPs by all the parts of *Datura stramonium*, the peak at 465–876 cm^{-1} was assigned to the O–H group from sugar alcohols (Rozenberg et al. 1999), while the absorption peak at 1,715 cm^{-1} was exhibited to the carbonyl functional group in unsaturated/aromatic carboxylic acids (Akhter et al. 2010). The band at 3,156 cm^{-1} is characteristic to hydroxyl functional group in alcohol and phenols present in high concentration, while the low concentration is shown by the band at 3,678 cm^{-1} (Silverstein et al. 1981).

13.3.4 X-ray Diffraction (XRD) Analysis

X-ray diffraction shows the crystalline nature of the particles. Diffraction is essentially due to the existence of certain phase relations between two or more waves (Kulkarni 2009). A comparison of obtained XRD spectrum with the standard confirms that the

AgNPs formed in the experiment are in which form of nanocrystals. As illustrated in the Fig. 13.3d, the peaks at 2θ values of 38.01°, 46°, 54.5°, and 77.62° corresponding to 111, 103, 006, and 201 planes, respectively, for silver. This indicates that the sample contained a mixed phase, cubic and hexagonal structures of silver nanoparticles (Krause 1979).

13.3.5 Scanning Electron Microscopy (SEM)

The SEM is a valuable instrument for obtaining high-resolution images of the surface of a sample because it measures the electrons scattered from the sample, making the instrument very useful in determining the size distribution of nanoparticles. Electrons are accelerated by an electric potential so the wavelength are made shorter than the one of photons. This makes the SEM capable of magnifying images up to 200,000 times (Kulkarni 2009). In Fig. 13.4a SEM micrographs of AgNPs synthesized using *Capsicum annum* callus extract are shown with spherical AgNPs of sizes between 30 and 40 nm.

13.3.6 Transmission Electron Microscopy (TEM)

In Transmission Electron Microscopy, a beam of electrons is transmitted through an ultra-thin specimen, interacting with the specimen as it passes through and image is formed. Figure 13.4c shows the TEM micrograph of AgNPs synthesized using *D. stramonium* root extract. It was observed that AgNPs were spherical and found in the range of 10–20 nm.

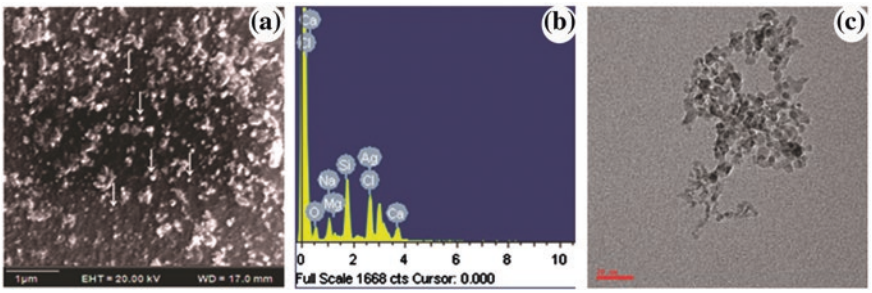

Fig. 13.4 Characterization of AgNPs, synthesized by plant-based methods using Electron microscopy (SEM, TEM) and its attachments like EDAX. This exercise can provide the knowledge about size, shape, and elemental constituents of prepared AgNPs. **a** SEM micrograph of AgNPs synthesized using *Capsicum annum* callus extract. **b** EDAX spectrum of AgNPs prepared by using *Cucumis sativus* plant extract. **c** TEM micrograph of AgNPs synthesized using *Datura stramonium* root extract

13.3.7 Energy Dispersive X-ray (EDAX) Analysis

The elemental analysis of silver nanoparticles was performed using EDS or EDAX on to SEM. Figure 13.4b exhibits the EDS spectrum of the spherical nanoparticles synthesized using *Cucumis sativus* plant extract as reducing agent. Strong signals from silver, while weak signals from Cl, P, Na, Mg, and Ca atoms were observed, which may be due to X-ray emission from proteins/enzymes present in the plant extract. The presence of Si signal was due to sample preparation on the glass substrates.

13.3.8 Dynamic Light Scattering (DLS) Analysis

Dynamic Light Scattering (DLS) is a measuring technique which is used for the determination of particle size and particle size distribution (Frisken 2001). The technique makes use of the shift of the frequency of light when it interacts with moving particles in the solution and become scattered, and the fact that this change depends on the particle size; the smaller the particles, the greater the shift in the light frequency (Berne and Pecora 2000).

13.3.9 Atomic Force Microscopy (AFM)

The AFM is an instrument capable of measuring the topography of a given sample of nanomaterial and provide a computer-generated 3D image of the sample (Lang et al. 2004). Thus, the AFM makes it possible to determine the height also of the particles and this is the advantage of the AFM over SEM.

13.3.10 Selected Area Electron Diffraction (SAED) Analysis

Selected Area (Electron) Diffraction (abbreviated as SAD or SAED), is a crystallographic experimental technique that can be performed inside a transmission electron microscope (TEM). SAD of nanoparticles or nanocrystals gives ring patterns analogous to those from X-ray powder diffraction, and can be used to identify texture and discriminate nanocrystalline from amorphous phases. SAED patterns can be used to identify crystal structures and measure lattice parameters (Lábár 2005).

13.3.11 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) is a surface-sensitive quantitative spectroscopic technique that measures the elemental composition at the parts per thousand range, empirical formula, chemical state, and electronic state of the elements

that exist within a material (Turner and Jobory 1962). XPS is used for determining the interaction between capping agents and nanoparticles.

13.3.12 Inductively Coupled Plasma with Mass Spectrometry/Atomic Emission Spectrometry (ICP-MS/ICP-AES)

ICP-MS (inductively coupled plasma with mass spectrometry) method offers rapid, sensitive, accurate, and simultaneous determination of chemical elements with atomic mass ranged from 7 to 250 (except C, N, O, F, Cl) in biological samples and aqueous media in a single run. The limits of detection are at the level of nanograms per liter (Ahrends 2007). ICP-AES is widely used technique as good alternative with higher detection capabilities. The technique is employed to calculate the yield of the nanoparticle in the synthesis reaction, even if yield is low in the experiment.

13.4 Inspiring Bricks of Literature on Phytosynthesis of AgNPs

Numerous plants have been identified and screened for the production of AgNPs because they are known to harbor a broad range of metabolites which provide them the potential to synthesize AgNPs. In Table 13.1 we have reviewed the inspiring experimental approaches and elucidations of the field of phytosynthesis of AgNPs with the aim to create the scenario of the present status.

13.5 Applications of AgNPs

The wider range of applications of AgNPs makes the field never endings and this statement is supported by the earlier reported findings on different activities of AgNPs.

13.5.1 Inhibitory Effects on Different Organisms

Silver nanoparticles have shown their potential as toxic/inhibitory agent against bacteria, fungus, viruses, protozoa, and arthropods because of their high surface to volume ratio. Researchers have used these features of AgNPs for such applications.

Table 13.1 Some reports on plants investigated for AgNPs synthesis and features of synthesized nanoparticles

Plants	Plant parts	AgNPs specifications	References
Allium cepa	Leaves	Spherical, 33.6 nm	Saxena et al. (2010)
Allium sativum	Garlic cloves	Spherical, 7.3 nm	Rastogi and Arunachalam (2011)
Aloe vera	Leaves	Spherical, 15.2 nm	Chandran et al. (2006)
Ananas comosus	Leaves	12.4 nm	Emeka et al. (2014)
Argemone maxicana	Leaves	Spherical, uniform and crystalline	Arokiyaraj et al. (2013)
Artemisia nilagirica	Leaves	70–90 nm	Vijayakumar et al. (2013)
Azadirachta indica	Leaves	Spherical, 5–35 nm	Shankar et al. (2004)
Capsicum annuum	Fruit	Spherical and crystalline, 10–70 nm	Li et al. (2007)
Carica papaya	Fruit	Cubic, 15 nm	Jain et al. (2009)
Catharanthus roseus	Leaves	Crystalline and face-centered- cubic, 35–55 nm	Ponarulselvam et al. (2012)
Citrus sinensis	Peel	10–35 nm	Kaviya et al. (2011)
Cocos nucifera	Coir	Face-centered-cubic and crystalline, 23 nm	Roopan et al. (2013)
Curcuma longa	Tuber powder	Quasi-spherical, triangular and small rods, l–50 nm	Sathishkumar et al. (2010)
Daucus carrota	Tap root	Spherical, 31–52 nm	Mukunthan and Balaji (2012)
Elaeocarpus ganitrus, Terminalia arjuna, Pseudotsuga menzi-etii, Prosopis spicigera, Ficus religiosa, Ocimum sanctum and *Curcuma longa*	Rudraksha beads, bark, leaves and fruits, leaves, leaves, leaves and sacred fig, leaves and rhi-zome, respectively	–	Dwivedi et al. (2014)
Eucalyptus globulus	Bark	5–50 nm	Astalakshmi et al. (2013)
Hibiscus rosa sinensis	Leaves	Nearly spherical, ~13 nm	Philip (2010)
Jatropha curcas	Latex	Face-centered-cubic, 10–20 nm	Bar et al. (2009)
Mangifera indica	Leaves	Triangular, hexagonal and nearly spherical, 20 nm	Philip (2011)

(continued)

Table 13.1 (continued)

Plants	Plant parts	AgNPs specifications	References
Mentha piperita	Leaves	Spherical, 90 nm	Ali et al. (2011)
Mulberry plant	Leaves	20–40 nm	Awwad and Salem (2012)
Nicotiana tobaccum	Leaves	Crystalline, 8 nm	Prasad et al. (2011)
Ocimum sanctum	Root and stem	5–10 nm	Ahmad et al. (2010)
Parthenium hystrophorus	Leaves	20–70 nm	Anwar et al. (2013)
Pepper plant	Leaves	5–60 nm	Mallikarjuna et al. (2012)
Pinus desiflora, Diopyros kaki, Ginko biloba, Magnolia kobus and Platanus orientalis	Leaves	Cubic, 15–500 nm	Song and Kim (2009)
Piper betle	Leaves	17–120 nm	Rani and Rajasekharreddy (2011)
Plumbago indica	Roots	Spherical, 50–70 nm	Kumar et al. (2013)
Rosa rugosa	Leaves	Spherical, 12 nm	Dubey et al. (2010)
Sesbania grandiflora	Leaves	Spherical, 10–25 nm	Das et al. (2013)
Shorea roxburghii	Stem bark	Spherical, 4–50 nm	Subramanian et al. (2013)
Solanum lycopersicum	Fruit	Spherical, 10 nm	Umadevi et al. (2013)
Sorghum spp	Bran	Face-centered- cubic and crystalline, 50 nm	Njagi et al. (2011)
Svensonia hyderobadensis	Roots	24 nm	Rao and Savithramma (2013)
Terminalia chebula	Fruit	Pentagons, spherical, triangular, hexagon, crystalline and face- centered-cubic, <100 nm	Kumar et al. (2012a)
Trachyspermum ammi and *Papaver somniferum*	Seeds	60–80 nm	Vijayaraghavan et al. (2012)
Triticum aestivum	Seeds	Face-centered-cubic, crystalline and spherical, 5.06–5.42 nm	Waghmode et al. (2013)
Withania somnifera	Roots	25.02 nm	Subbaiah and Savithramma (2013)
Zingiber offcinale	Rhizome	6–20 nm	Kumar et al. (2012b)

Antibacterial activity—Literature shows that a great percentage of the reported papers on phytosynthesis represents the antimicrobial activity by prepared AgNPs. For example, Suresh et al. (2010) compared chemically and biologically synthesized AgNPs and found that biologically synthesized AgNPs showed higher inhibition effect on Gram-negative (*Escherichia coli* and *Shewanella oneidensis*) and Gram-positive (*Bacillus subtilis*) bacteria.

Antifungal activity—A research conducted by Kim et al. (2008) demonstrated antifungal effect of AgNPs against skin pathogen (ATCC strains of *Trichophyton mentagrophytes* and *Candida* species) and found comparable with amphotericin-B and fluconazole. Kaur et al. (2012) also supported the antifungal activity of nanosized silver chitosan nanoformulation and silver/chitosan nanocomposite against seed borne plant fungal pathogen by inhibiting mycelium growth.

Antiviral activity—Recently, it has been found that AgNPs can interact with viral biomolecules. A study suggested that the AgNPs can bind to external viral coat of HIV and prevent viral infection (Lara et al. 2011). AgNPs have proven their potential as antiviral agents against other viruses also, including herpes simplex virus, respiratory syncytial virus, monkey pox virus, human immunodeficiency virus and hepatitis B virus and provide a novel platform for antiviral therapies (Galdiero et al. 2011).

Antiprotozoal activity—The anti-giardia was concluded by Said et al. (2012) and found that toxicity against parasite was highest with the combination of curcumin-NPs and chitosan-NPs. Likewise the anti leishmanial activity was observed by Rossi-Bergmann et al. (2012) against *Leishmania amazonensis* who reported the action of AgNPs comparable to amphotericin-B against parasitemia at lower concentration.

Anti arthropods activity—Biologically synthesized silver nanoparticles/gold nanoparticles can also be used as an ecofriendly approach to mosquito control (Adhikari et al. 2013). This kind of study provides a new platform for the use of AgNPs as insecticides.

13.5.2 Silver Nanoparticles in Medicine

Biomedical applications—The antimicrobial activity of AgNPs has drawn considerable attention of researchers of medical and pharmacological biology. Agarwal et al. (2013) examined the phytosynthesized AgNPs against a wide range of drug sensitive (DS) and drug-resistant (DR) bacteria. AgNPs can also be used as antibacterial coatings on medical devices to reduce nosocomial infection rates. Many kinds of new fabrication methods of AgNPs have emerged for medical application (Chaloupka et al. 2010).

Antimicrobial surface functionalization of plastic catheter—Silver nanoparticles coated plastic catheters are being used for a targeted delivery and sustained

release of silver at implantation site because of their antimicrobial properties which reduces the risk of infectious complication in patient (Roe et al. 2008).

Therapeutics applications—Anti inflammatory activity—In a report, Nadworny et al. (2010) showed the potential of nanocrystalline silver-derived solution for a variety of anti-inflammatory treatment applications. Animal studies suggest nanocrystalline silver has potential to alter wound inflammatory events and facilitate the rapid healing (Fong and Wood 2006; Tian et al. 2007). In another study, AgNPs were able to decrease inflammation in peritoneal adhesions without significant toxic effect and provided a novel therapeutic direction for the prevention of postoperative adhesion (Wong et al. 2009).

Anti-proliferative/anti-tumor activity—Asharani et al. (2009) demonstrated the inhibition of proliferation of cancer cells due to AgNPs-induced stress by generating effects of calcium transient and chromosomal aberrations in the cells. By working on Dalton's Lymphoma Ascites (DLA) cell lines, Sriram et al. (2010) concluded that AgNPs act as an anti-tumor agent through activation of caspase 3 enzyme and leads to the apoptosis.

13.5.3 Application in Water Treatment

AgNPs-coated polyurethane (PU) foams are developed and found that these filters are able to reduce the concentration of *E. coli* from water (Jain and Pradeep 2005). For the similar purpose, Mpenyana-Monyatsi et al. (2012) tested the AgNPs-based filters. Biologically synthesized AgNPs also demonstrated the inhibitory effect against biofilms formed by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* during 24-h treatment. Biofilms arise due to bacteria that attach to surfaces and aggregate in a hydrated polymeric matrix which could be responsible for development of microbial keratitis (ocular related infectious diseases) in human (Kalishwaralal et al. 2010).

13.5.4 Application in Agriculture

The biggest application of AgNPs in agriculture is use of AgNPs in the inhibition of the growth of plant pathogens. A research reported that AgNPs showed inhibitory effects against the outbreak of Powdery Mildews Disease on cucumbers and pumpkins (Lamsal et al. 2011a). While in another study, Lamsal et al. (2011b) examined the control of *Colletotrichum* species in vitro and Pepper Anthracnose Disease in field by using AgNPs. With the similar results, Kim et al. (2012) also reported the antifungal activity of colloidal solution of AgNPs against different plant pathogens.

Some research is also going on the effects of AgNPs on plant growth and development and creating new ways towards the field of nanofertilizers.

13.6 Future Research Prospects

Undoubtedly, there was the boom of plant-based synthesis of AgNPs in past decade but it never means that this is the end and there is nothing to do. Plant-based synthesis of metal nanoparticles is a very promising field of research. Some other research areas such as genetic engineering, in vitro plant tissue culture and secondary metabolite extraction are growing profusely. As plant metabolites are the responsible candidates of AgNPs synthesis, the continuous advancements in such fields will improve the procedure of extraction of specific metabolites and yields along with positive feedback to large-scale production of AgNPs. It will generate a new strategy of research where the steps of experiments will be the identification of responsible metabolites, upstream regulation approach for that metabolites and using that approach at industrial level.

The further optimization of production process by using plants can be achieved by cogent experiments on most suitable reaction conditions and elucidation of actual mechanism behind functioning of plant metabolites which may lead to enhanced control over physical parameters of synthesized nanoparticles like size, shape, crystallinity etc. More elaborated research has to be done using isolated pure compounds to explore this field.

This exciting improvement toward the use of plant-based methods for production of AgNPs might facilitate researcher's abilities to overcome many limitations of this field such as toxicity issues related to AgNPs, allergic reactions associated with the use of AgNPs and interaction of AgNPs with the immune system (Borase et al. 2014).

There are so many fields which are still untouched under this concern and can be explored by means of AgNPs application which can prove beneficial. Conclusively, this is just the beginning of the journey, not the end.

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Chapter 14
Nanoparticles Applied to Mass Spectrometry Metabolomics and Pesticide Residue Analysis

Yousef Gholipour, Rosa Erra-Balsells and Hiroshi Nonami

Abstract In this chapter, we review the achievements in application of nanoparticles and nanotubes to mass spectrometry metabolomics of plants. Ultraviolet matrix-assisted laser desorption/ionization mass spectrometry (UV-MALDI-MS) using nanoparticles as the matrices enables biologists to directly analyze metabolite composition of plant tissues. In addition, due to its high sensitivity and low limit of detection, it is applied to plant single-cell analysis for metabolite profiling of pico- to nanoliter of cell microsample with metabolite concentration of femto- to picomolar. In addition to the detection, the technique provides quantitative information of metabolite composition of plant single cells. Another interesting feature of nanoparticle UV-MADI-MS is its applicability to direct analysis of pesticide residues on agricultural products. The technique has been able to detect main component and degradation products of commercial pesticides sprayed on plants in a greenhouse.

Keywords MALDI MS · Single cell · Nanotubes · In situ metabolite profiling · Plant microsample · Pressure probe · Pesticide-residue analysis

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14.1 Introduction

In situ analysis of metabolites by using Nobel Prize awarded technology of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a remarkable tool for biologists, provided suitable matrix capable for laser desorption/ionization (LDI) of favorite metabolites is available (Stahl et al. 1997; Caprioli et al. 1997; Chaurand et al. 1999). UV-MALDI matrix is a compound capable to absorb UV laser photon energy and then to transfer this energy to analyte molecules which results in desorption and ionization of the analyte. If suitable matrix is available biomolecules can desorb/ionize directly from the surface of a biological tissue. Early in the history of introduction of UV-MALDI MS to biological chemistry, applicability of the technique for analyzing carbohydrates and especially oligosaccharides from plants was reported (Karas et al. 1987; Harvey et al. 1996; Stahl et al. 1997).

Provided high absorption in the wavelength region of common MALDI lasers (337 and 355 nm), nanoparticles (NPs) are potential candidates for direct molecular profiling of biological tissues. Furthermore, NPs have size-dependent physical and chemical properties, included interaction with UV light. In addition, unlike organic matrices, NPs have less possible chemical interaction with biological tissues in the ground state (Gholipour et al. 2010). In the case of organic matrices, chemical changes may occur on the surface of a target tissue or the matrix is incorporated into the structure so that no signal of target metabolites may be observed. These characteristics make them prospective matrices for laser desorption ionization (LDI) of metabolites from plant tissues. However, in practice they show different levels of LDI capability which is related with their size (active surface), composition, structure, and chemical and photochemical properties. The usage of NPs as matrix for laser DI was considered in early days of development of soft LDI-MS. Tanaka et al. (1988) used fine powder (20 nm) of inorganic cobalt for protein and polymer analysis. Since then, especially in the last decade, several carbon-related NPs including graphite (Sunner et al. 1995) and carbon nanotubes (CNTs) (Xu et al. 2003; Pan et al. 2009) and metallic NPs such as TiO_2 (Lee et al. 2007; Lorkiewicz and Yappert 2009), Ag (Sluszny et al. 2005; Castellana et al. 2008; Chiu et al. 2008; Shrivas and Wu 2008), silicon (Wen et al. 2007) Au (Castellana et al. 2008; McLean et al. 2005; Su and Tseng 2007), and zinc oxide Watanabe et al. (2008) have been examined in MALDI and surface-ALDI-MS of organic compounds and biomolecules. Several carbon-related and metallic NPs from diamond and graphite to silver and gold have been examined and successfully applied to bio-analyses, included plant metabolomics.

Mass spectrometry was introduced for quantification of biomolecules in early time of its development history (Markey 1981; Harvey 1993); and nowadays, is turning to a well-established reliable technique for comprehensive quantitative analysis in proteomics (Bantscheff et al. 2007) and metabolomics (Soga 2003). Its quantitation capabilities, in addition to its excellent detection power, provides plant biologists a separation (i.e., chromatography)-free tool for both detection and quantitation of metabolites.

Table 14.1 The efficiency of MALDI matrices examined by our group for direct UV-MALDI MS metabolite profiling of in situ plant tissues and single cells

Matrix		Ion mode	Detection suitability	LOD[a]	Linearity signal abundance versus conc.	References
Nanoparticles	Diamond	(+)	Carbohydrates	(+) high	–	Gholipour et al. (2010)
	(SiO_2) (TiO_2)	(+) and (−)	Carbohydrates, organic acids, secondary metabolites	(+) low	Very high	Gholipour et al. (2010)
	(($Ba\text{-}TiO_3$) ($SrTiO_3$)	(+) and (−)	Carbohydrates	(+) low	High	Gholipour et al. (2010)
	TiO_2	(+) and (−)	Carbohydrates	(+) low	Very high	Gholipour et al. (2010)
Nanotubes	CNTs	(+)	Carbohydrates	(+) high	Low	Gholipour et al. (2008a, b, 2010)

[a]Limit of detection (*LOD*). Reproduced with permission from Gholipour et al. (2012a) © Japan Society of Mass Spectrometry

As an interesting and remarkable capability, the technique provides optimum molecular analysis of biological tissues with the least preparation which indeed, is quite suitable for plant growth factories where it is necessary to create speedily a molecular profile of tissue during plant growth. We developed a direct tissue profiling method of MALDI by using some new matrices from inorganic nanoparticles and CNTs. These matrices, unlike organic matrices which were not applicable to plant tissues due to strong chemical interaction with host tissue, are capable of desorbing/ionizing metabolites, especially sugars directly from plant tissues.

UV-MALDI MS can be also applied to plant sciences for direct metabolite profiling of plant single cells by using NPs as the matrices. Its high sensitivity and low limit of detection, and also direct structural elucidation makes UV-MALDI MS a powerful analytical tool for plant cell biology. On the other hands, the low limit of detection and high linearity response of NPs make them a matrix of choice for detecting and quantifying plant metabolites. Consequently, analysis of single-cell extract by NPs UV-MALDI MS can provide comprehensive metabolome analysis of cells, and can be used to verify cell to cell variation, and to understand intracellular variation and changes in metabolic network during specific physiological or environmental events.

Table 14.1 summarized nanoparticles and nanotubes which have been examined by our group for plant tissue and single-cell analyses using UV-MALDI MS (Gholipour et al. 2012a). This group of MALDI MS matrices is quite favorable for the analysis of plant carbohydrates from simple hexoses to oligosaccharides

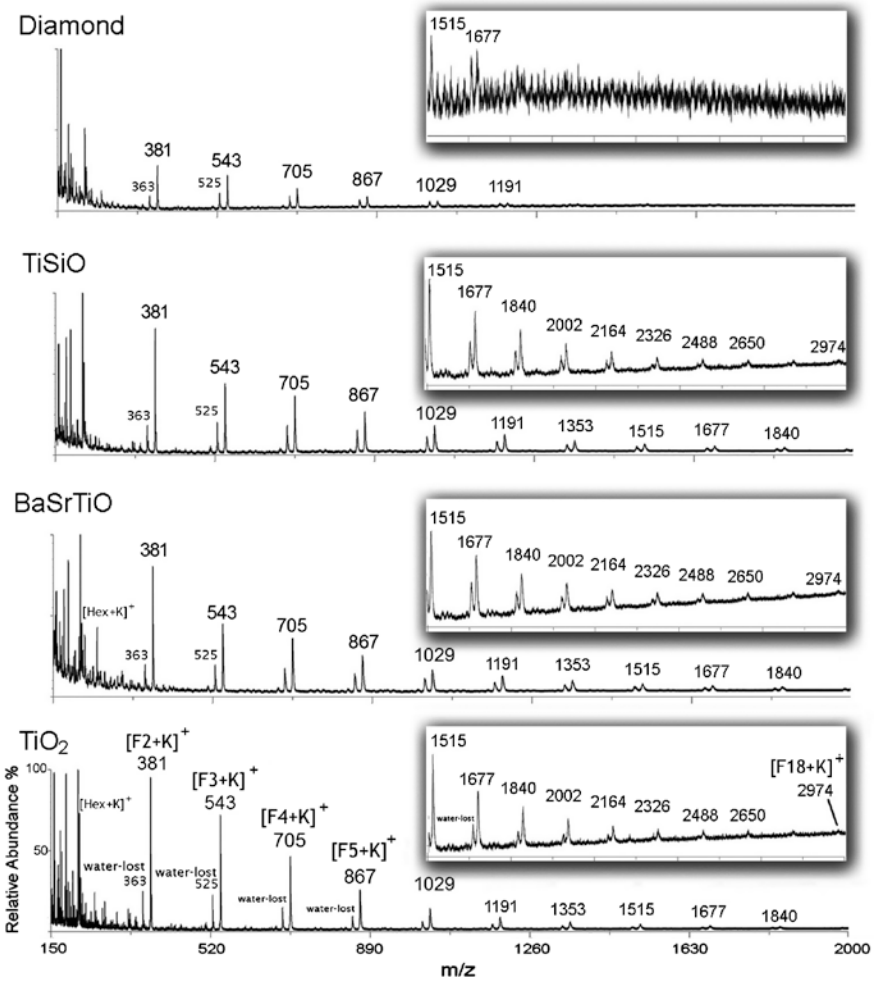

Fig. 14.1 UV-MALDI mass spectra acquired using NPs deposited on air-dried tulip bulb tissue (from bulb stored at 5 °C for 8 weeks) in positive ion mode. *Insets* magnify the m/z 1,000–2,000 region. Here in carbohydrate-rich tulip bulb, fructans (as F_n, where F_1 or hexose is glucose and/or fructose, F_2 as sucrose, F_3 as kestose etc.) are shown. Reproduced with permission from Gholipour et al. (2010) © American Chemical Society

and fructans with high molecular weight (even with 18 sugar moieties; see Fig. 14.1). Meanwhile, we have been able to identify other plant metabolites including organic acids and secondary metabolites (e.g., GABA and tuliposides) in mass spectra acquired by using UV-MALDI MS of plant tissues and single-cell microsamples. NPs vary in the limit of detection and linearity response. In the

case of a plant material with abundant carbohydrates, for example, all NPs can be applied reliably for both characterization and quantitation. In the case of a single-cell microsample, on the other hands, where very small volume of sample with very low quantity of metabolites may exist, titanium NP group has shown to be the choice as the matrix.

14.2 Direct Analysis of Metabolites on Plant Tissues

The techniques which facilitate in situ characterization and localization of metabolites can give fast and easy-to-achieve information about tissues in their native status (Gholipour et al. 2008a). These techniques including NMR and mass spectrometry gradually find potential applications in different biological sciences. An interesting feature of UV-MALDI TOF MS is the possibility of the direct detection of chemicals on a surface without the need of specific preparations or extraction procedures. The only preparation in UV-MALDI MS of in situ biological tissues is uniform (and with minimal disposition of metabolites) deposition of a suitable UV-MALDI matrix on the surface of a target tissue. Availability of the suitable matrix is considered as one of the critical aspect of the technique as there are no clear criteria for the selection of a matrix for a specific analyte and the selection of matrix is experimental (de Hoffmann and Stroobant 2007; Cole 2010). The specificity and availability of matrix are more challenging when the analysis of chemicals on the surface of biological tissues is targeted. Some organic matrices have shown to be able to desorb/ionize metabolites from the surface of plant tissue, including 2,5-dihydroxybenzoic acid (DHB) (Ng et al. 2007; Stahl et al. 1997), α-cyano-4-hydroxycinnamic acid (CHCA) (Ng et al. 2007; Robinson et al. 2007; Wu et al. 2007), and sinapinic acid (Ng et al. 2007). However, as mentioned before, chemical interaction between organic matrices and biological tissue is a barrier to their application to biological tissues from different plants. Nanoparticles including colloidal silver (Sluszny et al. 2005) and graphite (Zhang et al. 2007) have also shown to be useful for in situ plant tissue analyses. Diamond nanoparticles, carbon nanotubes (CNTs), and some metal nanoparticles (NPs) commercially available are capable to desorb/ionize chemicals from the surface of biological tissues (Gholipour et al. 2008b, 2010). Using CNTs and metal NPs we were able to desorb/ionize metabolites from the surface of plant tissues (Fig. 14.1; Gholipour et al. 2008a, 2010).

Figure 14.2 shows how NPs UV-MALDI MS can generate quantitative data from tissues with different physiological statuses.

Cool temperatures triggers synthesis and intracellular accumulation of abundant hexoses, sucroses, and fructans. Relative change in the concentration of carbohydrates is elucidated by direct deposition of NPs on air-dried tissue slices of the bulbs and irradiating UV laser and collecting mass spectra.

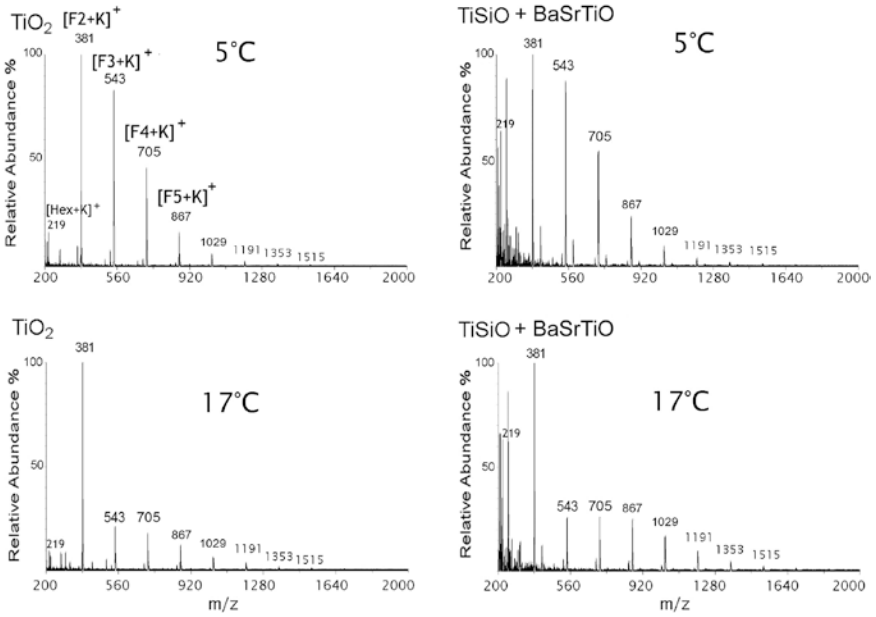

Fig. 14.2 UV-MALDI mass spectra acquired using NPs deposited on tulip bulb tissue (bulbs stored at 5 and 17 °C). During cold storage, synthesis of fructans (here shown as polymeric compounds as F_n) are accelerated and used for cold acclimatization and as energy and carbon source for later growth and flowering. Reproduced with permission from Gholipour et al. (2010) © American Chemical Society

14.3 Plant Cell Mass Spectrometry Metabolite Profiling: Speaking Cell Approach in Plant Growth Factories

The information on the content or quantity of metabolites achieved by tissue extract analysis, so-called bulk biochemistry (Kehr 2003), does not necessarily reflect the status in cell level since metabolite content varies in different parts of cells (e.g., in cytoplasm and apoplast), among cells in a particular tissue (cell to cell variations in different location of tissue), and same cells during developmental or environmental events (Gholipour et al. 2012a, b, 2013). The focus of modern biological research is, therefore, moving from classical organ and tissue-level analyses to single cells, where the most fundamental events of life occur. Therefore, metabolomics with a single-cell resolution can explore the basic aspects of life, cell to cell variations, primary responses to abiotic stresses or various types of biotic attack, and the processes of growth or death; and is the analysis of the phenotype with the highest resolution and has great potential to contribute the enhancement of cell systems biology.

For such a molecular analysis of single cells, a reliable access to the cell sap, the ability to sampling in real time, and sensitive detection are critical. In addition,

for the quantitation of metabolites, femto- to picoliter volume of cell samples must be carefully handled and measured. Otherwise, the interpretation of the signal abundance as the relative natural change of metabolite content in cells cannot be reliable. Accessing and sampling deeper cells with a capillary or a needle may not be also straightforward. A Pressure Probe (PP) is a unique tool in plant biology due to its capability to measure pressure-related properties of plant single cells (Hüsken et al. 1978). The technique works based on the positive or negative pressure created by changing the volume of silicon oil filled inside the capillary glass. After penetration into a plant living turgid cell, some of cytoplasm, particularly from vacuole, enters the capillary because of the turgor pressure inserted by cell membrane and, more important, by cell wall. While, manipulation of meniscus created in the interface between silicon oil of PP and the cell cytoplasm facilitates pressure-related measurements, it is also possible to microsample cell cytoplasm. The picoliter volume of microsample usually contains pico- to femtomole amount of metabolites. PP also facilitates other plant cell measurements such as turgor, osmotic, and water potential, cell wall elastic modulus, cell wall extensibility, and hydraulic conductivity of plasma membrane with which, along with metabolite analysis, a comprehensive physical and chemical profile of plant cell during growth or stresses can be generated.

Different classical techniques such as NMR and mass spectrometry have been applied to metabolome analyses. With its high sensitivity and low limit of detection, and also direct structural elucidation of detected compounds, UV-MALDI MS is great analytical tool for cell metabolomics, where samples are necessarily in pico- to nanoliter volume, with metabolite concentration of femto- to nanomoles (Gholipour et al. 2008a). On the other hands, time ion selector gate and post source decay analysis in reflector mode of MALDI MS can be used for selecting and verifying signals of target metabolites. Combined with PP, UV-MALDI MS with NPs as matrices is a great tool for in situ physical properties measurement, cell sap sampling and handling, and finally, metabolite profiling of plant living single cells. Figure 14.3 shows a workflow of PP-UV-MALDI MS.

In one of our studies on rice and tulip bulbs, which cytoplasm sap was sampled by PP and titanium silicon oxide nanoparticles used as matrices, several metabolites particularly neutral carbohydrates (fructans) which are remarkably involved in many cellular chemical and physical events, were detected (Figs. 14.4 and 14.5). In another study (Nonami et al. 2010) tulip bulb tissue and single cell were analyzed with carbon nanotubes as the laser matrix, and interestingly comparable result on the relative abundance of metabolites was achieved (Fig. 14.6). The joint application of a PP and a UV-MALDI system facilitates obtaining information about physical properties and the molecular composition of in situ living single cells; which means that experimenter can achieve wider and deeper insights to the events during growth or stress responses with a single-cell resolution (Gholipour et al. 2012a). Figure 14.7 shows good linearity response of MALDI MS of picomoles of sucrose and kestose; and also the result of joint application of PP and MALDI MS for quantifying sucrose and kestose content of parenchyma cells located at different depth of a plant tissue. The study was succeeded by reliably

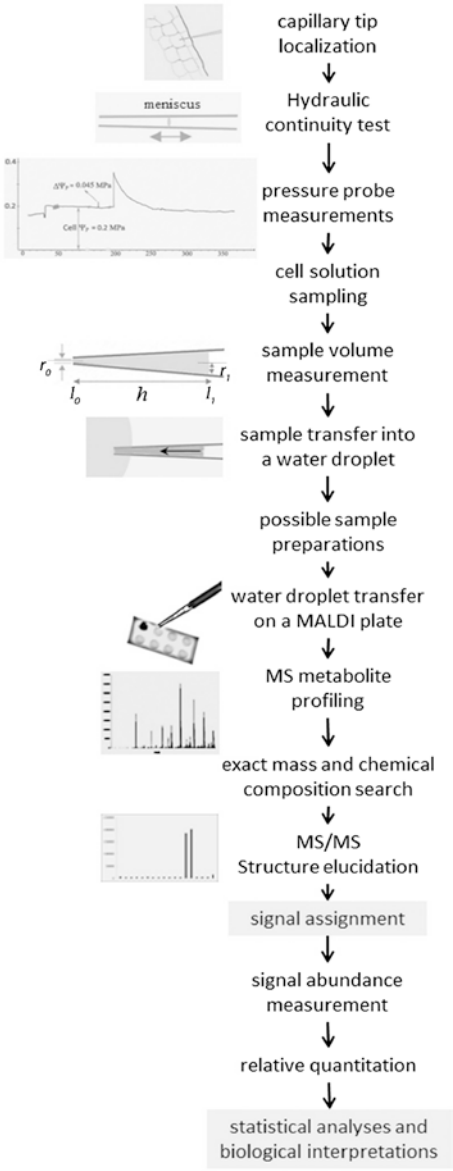

Fig. 14.3 The workflow of in situ single-cell metabolite analyses by pressure probe and UV-MALDI MS combination. Reproduced from Gholipour et al. (2012a) © Japan Society of Mass Spectrometry

accessing deep cells, precise sampling of cell sap, measuring sampled cell sap by PP, followed by identification and quantitation of target molecules by separation free, shotgun UV-MALDI MS. Despite such a great capability of shotgun single-cell MALDI MS, still there is a need for further improvement in the sensitivity of the detection. Additionally, almost all software in the field has been developed for a chromatography-mass spectrometry based data acquisition and processing.

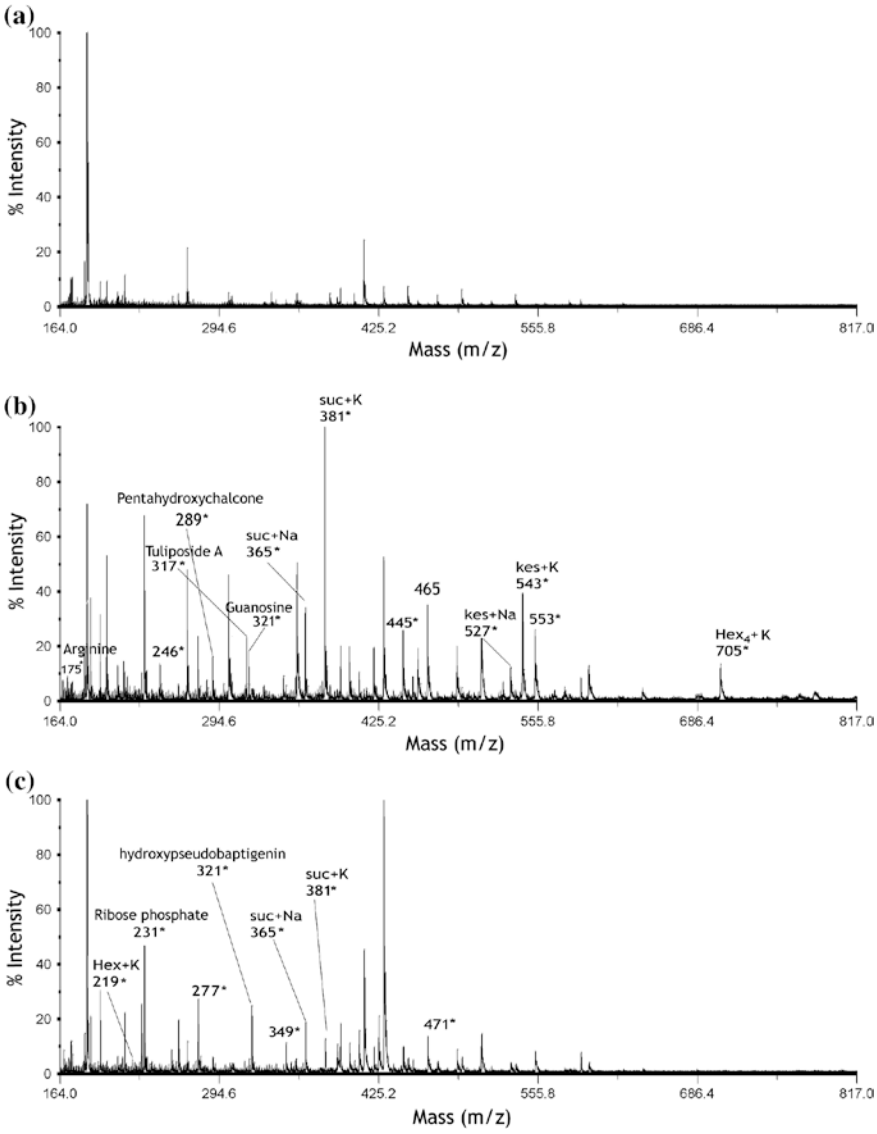

Fig. 14.4 UV-MALDI MS positive ion mode mass spectra of **a** matrix alone (SiO_2) (TiO_2) < 50 nm, **b** microsample of tulip and **c** rice single cells deposited on the matrix. Those metabolites with asterisk were also detected by electrospray mass spectrometry during a complementary study

For shotgun UV-MALDI MS metabolomics, it is critical to develop software which are able to extract information directly from mass spectra, automatically search libraries and databases and finally are able to analyze relative quantitative data. Shotgun approach is very beneficial to omics analyses but the signal yield

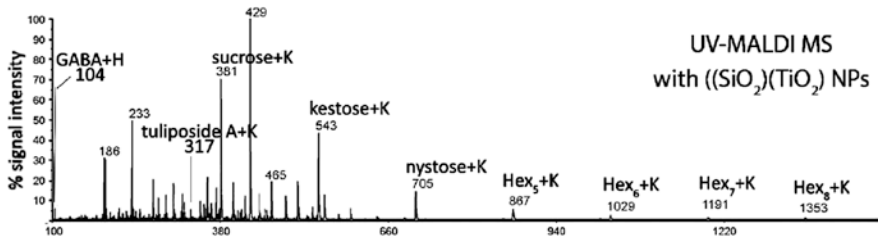

Fig. 14.5 UV-MALDI TOF MS mass spectra of tulip bulb cell sap sampled directly with the pressure probe by using titanium silicon oxide nanoparticles. Fructans along with other metabolites and secondary metabolite could be detected. Reproduced with permission from Gholipour et al. (2012a) © The Mass Spectrometry Society of Japan

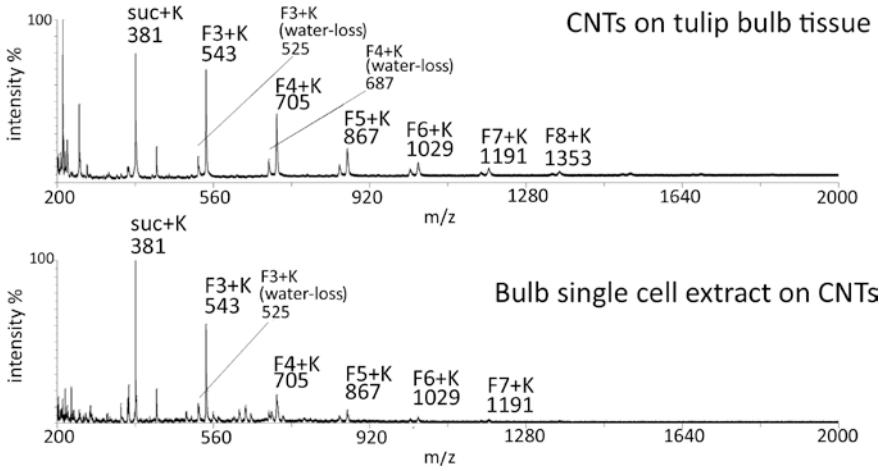

Fig. 14.6 UV-MALDI Mass spectra of a comparative in situ tissue profiling and single-cell microsample analysis of tulip bulb stored at 5 °C for 8 weeks; positive ion mode with carbon nanotubes as matrix. Adapted with permission from Nonami et al. (2010) © International Society for Horticultural Science

of metabolites in the cell mixture samples needs to be improved. It means that different approaches should be applied to detect target compounds, have less interfering signals, and have suitable signal to noise ratio for a reliable quantitation. The purification of cell solutions samples can improve the signal acquisition of targeted metabolites. However, the sample loss should be minimized. Although it seems pessimistic to find a universal matrix for the UV-MALDI-based metabolite profiling, it is worthwhile to examine potential matrices for the laser desorption/ionization of bigger numbers of metabolites and to decrease the limit of detection (Erra-Balsells et al. 2012).

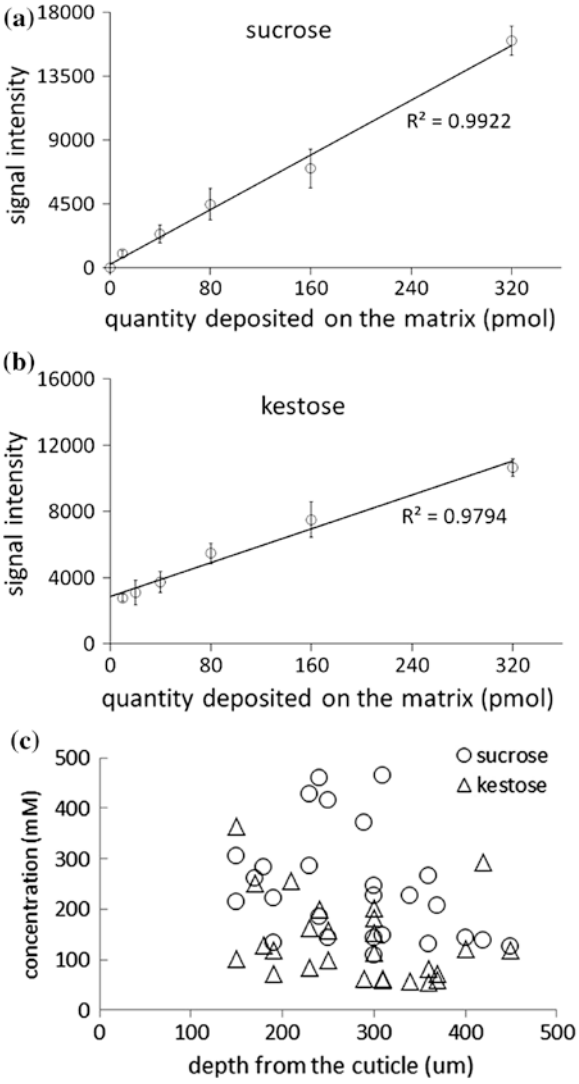

Fig. 14.7 Plots between the signal intensity and the number of moles of standard sucrose **a** and kestose **b** deposited as an aqueous solution on (SiO_2)(TiO_2) NPs, and **c** cell sap analyses with respect to the depth from the cuticle surface of a tulip bulb. About 150–800 pL samples could be obtained from each cell. Sucrose and kestose were abundant in those cells and yielded peaks with big intensities in positive ion mode. Reproduced with permission from Gholipour et al. (2012a) © The Mass Spectrometry Society of Japan

14.4 UV-MALDI MS Analysis of Pesticide Residue on Plant Surfaces Using Carbon Nanotubes

Pesticides are widely used in agriculture throughout the world and have been significantly contributing the crop yield and total food production. However, their residues on edible parts and the chemicals produced by the fragmentation/transformation of active ingredients are big problems seriously taken into account in global trade and countries put strict limit of tolerance when importing or exporting food and food

commodities (Gholipour et al. 2012c). For example, in the case of tomato fruits in the United States, 5 and 8 ppm are the tolerance of benomyl and malathion, respectively (US Environmental Protection Agency, Office of Pesticide Programs 2010). The fragmentation/transformation products of active ingredients and supplementary compounds found in commercial packages of pesticides can be also hazardous to health. It can be expected that greenhouse-grown fruits may have greater amount of residues remained on the surface than field-grown fruits. After spraying pesticides residues and their fragmentation/transformation products on fruit may be in very low quantity. Therefore, high-sensitive analytical techniques capable to detect pesticides a very low amount of the analyte is needed (Herrero et al. 2012).

The techniques able to analyze minute amounts of residues directly on plant surface are of great importance in food chemistry and industry. This complementary exploration contributes to find possible potential biohazards and can improve the quality of food and food commodities used by human and livestock. We applied UV-MALDI TOF MS with carbon nanotubes as matrix for in situ detection of pesticide traces and its transformation products on the surface of tomato fruit sprayed with a mixture of four commercial pesticides (Gholipour et al. 2012c). Sub-femtomoles of active ingredients are deposited on the MALDI plate. The technique showed

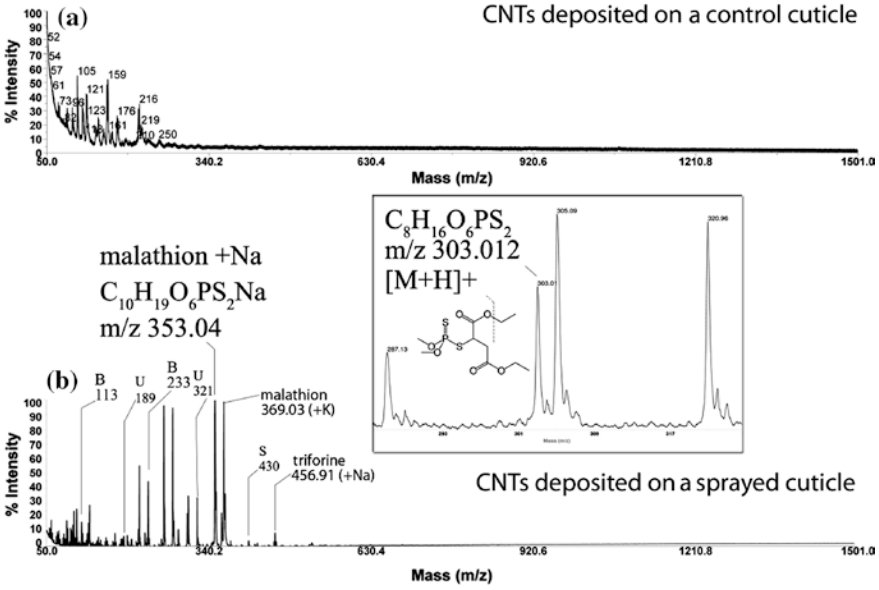

Fig. 14.8 Positive ion mode UV-MALDI mass spectra acquired after depositing carbon nanotubes (CNTs) on the pericarp of the control and sprayed tomato fruits (with a commercial pesticide mixture solution sprayed on intact tomato fruit in greenhouse and picked 1 week later for testing). B, S, C M, and U represent signals originated from Benelate, Sapurol, Chromite, and Malathion solutions, and unidentified compound, respectively. *Inset* shows an identified malathion degradation product. Reproduced with permission from Gholipour et al. (2012c) © The Japanese Society of Agricultural, Biological and Environmental Engineers and Scientists

capability for detecting and analyzing very small amount of pesticides. In all samples taken from control and sprayed fruits signals of pesticide ingredients were observed. However, number of peaks and their signal intensity were not necessarily same for all samples. With CNTs deposited on the tomato fruit pericarp, signals originated from pesticide solutions appeared (Fig. 14.8). Among active ingredients, malathion (as the sodiated peak with m/z of 353.04 and potassiated of m/z 369.03) and triforine (sodiated peak of m/z 456.91) were detected. By shooting the UV laser onto the pericarp surface covered with CNTs, several peaks previously detected in the pesticide solutions were identified, as well. Abundant triforine and malathion peaks showed that these active ingredients were persistent on the tomato cuticle even 1 week after spraying in greenhouse. Importantly, we detected their traces on an edible part. It has been shown that after entering into the human body, malathion translocates to the liver and kidneys and finally affects the nervous system (National Pesticide Information Center 2011). UV-MALDI MS using carbon nanotubes showed that it is able to generate in situ information of pesticides and their degradation products; the technique is unique with its minimal preparation, speed of testing, and detection power.

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