Chapter 5
Role of Microbes in Plant Protection Using Intersection of Nanotechnology and Biology

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5.1 Introduction

Plant diseases have escalated internationally which results in about 50% estimated loss caused by insect pests, and the value of this crop disaster was assessed to be US $2000 billion per year (van Lenteren and Martin 1999). Pathogens and pests trim growth of plants due to several adverse soil and climatic factors and thus lead to diminish the overall crop productivity. Habitually, worldwide the crops are protected from pathogens and pests with pesticide operations. This extensive usage of pesticides raises the resistance of crop pathogens and pests (Dzhavakhiya et al. 2012; Alghuthaymi et al. 2015), recedes nitrogen fixation, and scales down soil biodiversity which later contributes to the bioaggregation of pesticides in environment. Moreover, maximum share of the pesticides and fertilizers that are utilized are lost to air during operational hours and some as runoff which results in ecotoxicity (Chen et al. 2015; Vu et al. 2015). Also, concerning the global food security and climate change objections, crop protection is benefited from technological modernizations such as synthetic chemicals and hybrid varieties, but researchers are now focusing on green technology in particular. To tackle these situations, attempts have been made for secure crop productions with best management practices under varying circumstances (Leake et al. 2002). Biocontrol management of phytopathogens utilizing microbes or their derived products is useful as some of them are natural antagonists of pathogens with various inherent challenges (Frampton et al. 2012). Worldwide molecular techniques involving enzymes and primers are also widely used in laboratories for identification and control of specific plant pathogens due to their high degree of specificity (harmless to nontarget organisms), environmental compatibility, and applicability in integrated pest management. Still demerits exist for on-site detection of phytopathogens, shorter shelf

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life, and high diagnostic price especially in developing countries which caps the application of these conventional molecular approaches. Therefore, the crop up nanobiotechnology sounds to be of preeminent attention for the detection and control of phytopathogens especially in prior detection of disease and smart delivery of biocontrol agents to crop (Prasad et al. 2014, 2017a, b). In general, nanobiotechnology refers to the intersection of nanotechnology and biology which involves bio-fabrication, manipulation, and utilization of submicron or nano-objects (≤100 nm). Ample of products are used to construct nanoparticles, such as lipids, polymers, ceramics, quantum dots, silicates, dendrimers, and metal oxides (silver, gold, platinum, silica, selenium, titania, zirconia, etc.) (Oskam 2006; Puoci et al. 2008). Usually nanoparticles are synthesized chemically which is curbed by high cost, toxicity, and complexity of the technique. Therefore, microbes are considered as the ideal architect of these diversified nanostructures due to their small size, control culturability, genetic manipulability, regeneration, high biosorption capacity, wide diversity, selective adsorption of metal ions, and indeed allowing an easier downstream purification procedure. Accordingly, the links between nanotechnologies and microbiology are bolstering in the last few years with continuous revealing the potentials of microbes as nanoparticle industries. Also, higher surface area to volume ratio and unique optical properties of nanoparticles enables more interaction chances with pathogens and destroys them. Nanoparticle biosynthesis by microbes can be classified into intracellular and extracellular synthesis according to the site (Simkiss and Wilbur 1989; Mann 2001; Prasad et al. 2016). Transportation of ions occurs into microbial cell to form nanoparticles in the existence of enzymes during intracellular method. Trapping of metal ions on cell surface and reducing ions again in the companionship of enzymes appear during extracellular synthesis of nanoparticles (Zhang et al. 2011; Prasad et al. 2016). Known fact of bacteria is to use an enzyme and metabolize oxygen for sustaining life. Silver ions stop this oxygen metabolization by debilitating the enzyme which results in suffocation to fungi and bacteria and ultimately death (Puebla et al. 2004). If the concentrations of nanoparticles exceed a limit, some bacterial genera such as *Geobacter sulfurreducens* and *Shewanella oneidensis* reduce and precipitate the cations as nanoparticles that are counterpart of metabolic processes enabling nanoparticle synthesis as a protective mechanism (Tanzil et al. 2016). Magnetotactic bacteria (*Magneto spirillum*) that are known to generate natural magnetic nanoparticles due to their size, homogeneity, and stabilization have also been used for bioremediation, sensor development, and agrochemical degradation (Jacob and Suthindhiran 2016). Also, polyhydroxyalkanoate (PHA) nanobead biosynthesis proved to be a less costly technique due to accessible downstream procedure of PHA intracellular granules (Dinjaski and Prieto 2015). There are the evidences which provide in vitro efficacy of distinct nanoparticles (Guo et al. 2015) and biodegradable nanoparticles that can be opted for plant disease management (Chowdappa and Gowda 2013). Unique nanosize of nanoparticles makes them suitable in gene transfer (Rai et al. 2012a, b) and management of pathogens in crop production (Mishra and Singh 2015). Efficacy of nano-based materials provides the quick on-site detection of phytopathogens, addresses toxicity issues to manage different plant diseases, and allows

only minor doses to be used (Prasad et al. 2017b). Many researchers over the globe reported development of nanoparticles for the diseases detection and phytopathogen management employing bacteria (Tiwari et al. 2014), fungi (Potara et al. 2015), actinomycetes (Golinska et al. 2014), algae (El-Kassas and El-Sheekh 2014), and plants (Mallikarjuna et al. 2015).

This chapter provides a brief overview of the concepts and current state of the nanobiotechnology applications in disease management and plant protection involving nanoparticles that either incorporate biological elements or are entirely biological in origin. Next, biosynthesis mechanisms along with examples and current applications of diversified nanoparticles biosynthesized in the agricultural fields are presented. The chapter concludes with discussions on the current limitations and prospects of nanobiotechnology in sustainable agriculture.

5.2 Synthesis and Application of Nanoparticles

Nanoparticles are synthesized by a variety of physical, chemical, and biological methods which involve size reduction, high-pressure homogenization, sonication, reactive precipitation, and solvent displacement (Sasson et al. 2007). Nanoparticles generated from various natural resources such as plant extracts (pectin, cellulose, and chitin) are more expedient in terms of biodegradability, environmental safety, and biodegradation. Microbiocidal nanoparticles such as AgO and MgO due to their high stability, versatility, and biocompatibility are employed for agrochemical degradation, soil remediation, detection, and control of food spoilage (Baruah and Dutta 2009). Immunomolecules labeled ultrasensitive QD bioconjugates are developed as sensors for disease detection crops due to their potential for recognition of specific antibodies or antigens (Vinayaka et al. 2009). Other inorganic materials such as montmorillonite and other clay nanoparticles have a structure of stacked platelets with one dimension of the platelet in the nanoscale. Several investigations also demonstrated the potential use of nanoclays in the deployment of agrochemicals such as plant growth promoters, pesticides, and fertilizers (Bin et al. 2009).

5.2.1 Microbial Synthesis of Nanoparticles

Development of efficient and green nanoparticles is a crucial facet of nanobiotechnology. To achieve this objective, soil microorganisms and natural bioresources (plant−/marine-based material) are important options. However, soil microorganisms have gained preferences over plant material due to their natural ability of biocontrol agents. Also, the macro matter can be reformed into nanomaterials with the use of microbes through either intracellular or extracellular routes (Table 5.1). A diverse range of microbes have been found to produce nanoparticles in the substrate (El-rafie et al. 2012).

Table 5.1 Synthesis of nanoparticles by various microbes

Mode	Microbe	Nanoparticle
Extracellular	**Bacteria**	
	Penicillium sp.	Ag
	Klebsiella pneumonia	Ag
	Pseudomonas aeruginosa	Au
	Thermomonospora spp.	Au
	Rhodopseudomonas capsulate	Au
	Bacillus subtilis	Ag, Au
	Shewanella oneidensis	U
	Clostridium thermoaceticum	CdS
	Fungi	
	Aspergillus fumigates	Ag
	Aspergillus flavus	Ag
	Phanerochaete chrysosporium	Ag
	Phoma sp.	Ag
	Verticillium	Ag
	Trichoderma asperellum	Ag
	Fusarium oxysporum	Ag, magnetite
Intracellular	**Bacteria**	
	Bacillus licheniformis	Ag
	Pseudomonas stutzeri	Ag
	Streptomyces albidoflavus	Ag
	Bacillus subtilis	Ag, Au
	Lactobacillus sp.	Ag, Au
	Escherichia coli	Au, CdS
	Clostridium thermoaceticum	CdS
	Shewanella oneidensis	Magnetite
	Yeast	
	Pichia jadinii	Au
	Candida glabrata	CdS
	Schizosaccharomyces pombe	CdS
	Torulopsis	CdS

Various microbes (e.g., culture supernatant of *E.coli*) reduce the Ag^+ ions to form silver nanoparticles, most of which are found to be spherical particles (Mukherjee et al. 2001; Ahmad et al. 2003a, b; Fayaz et al. 2010). Further purification is done by using sucrose density gradient centrifugation, and purified sample was characterized by fluorescence spectroscopy, TEM, and UV-vis spectra. *Pseudomonas stutzeri* AG259 isolated from silver mines, when placed in a concentrated aqueous solution of silver nitrate, reduce Ag^+ ions and form silver nanoparticles (AgNPs) of well-defined size within the periplasmic space of the bacteria (Klaus et al. 1999). *E. coli* was used for the extracellular biosynthesis of AgNPs and characterized by UV-vis spectra, FTIR, and SEM of nanoparticles (Manonmani and Juliet 2011). Some researchers also focused on optimum reaction conditions which resulted in the reduction of particle size and maximize the synthesis of AgNPs (Gurunathan et al. 2009) using different medium (nitrate medium as the most efficient one), varying concentrations (5 mM

$AgNO_3$), pH (10.00), and reaction temperatures (60 °C). Utilizing these ideal conditions, 95% conversion was obtained with culture supernatant of *E. coli* in a time interval of 30 min. Mourato et al. (2011) investigated the biosynthesis of AgNPs by utilizing extremophilic yeast strain isolated from acid drainage mine. A mechanism involving c-type cytochromes reduces Ag(I) as insoluble AgCl or Ag^+ ions, precipitating extracellular nanoscale silver by *Geobacter sulfurreducens* (Law et al. 2008). Silver nanoparticles are also isolated from *Morganella morganii*, Gram-positive bacteria (Abd et al. 2013). Fungi are considered as sometimes advantageous in the synthesis of nanoparticles due to their ease of handling in laboratory and production of large quantities of enzymes (Mandal et al. 2006; Mohanpuria et al. 2007). When nanoparticles are synthesized outside the cell (extracellularly), it becomes easy to purify and can be used as direct applications (Mukherjee et al. 2008; Gaikwad et al. 2013). Also, fungal mycelial mesh can withstand flow pressure in bioreactors in resemblance to bacteria or plant extract (Narayanan and Sakthivel 2010). AgNPs were synthesized in film or solution (intracellulary) or accumulated on the cell surface (extracellulary) when fungi, *Verticillium*, *Fusarium oxysporum*, *Aspergillus flavus*, and *Penicillium*, were employed (Senapati et al. 2004; Bhainsa and D'Souza 2006; Vigneshwaran et al. 2007; Jain et al. 2011). Extracellular biosynthesis of AgNPs has been also achieved by using bacterium *Bacillus licheniformis* (Kalimuthu et al. 2008) and fungus *Aspergillus niger* (Gade et al. 2008). *F. oxysporum* and *Verticillium* sp. synthesized magnetite nanoparticles in the presence of ferric and ferrous salts. *F. oxysporum* f. sp. *lycopersici* was found to be efficiently producer of inter- and extracellular platinum nanoparticles and AgNPs using nitrate reductase-mediated technique. Biosynthesis of spherical and silver colloidal NPs was also investigated using *F. oxysporum* (Ahmad et al. 2003a, b). Biosynthesis of AgNPs was obtained by *Penicillium fellutanum* using $AgNO_3$ from coastal mangrove sediment as a substrate (Shaligram et al. 2009). Culture supernatants of *A. niger*, *A. fumigatus*, *A. clavatus*, and *A. terreus* were utilized for extracellular biosynthesis of AgNPs (Bhainsa and D'Souza 2006; Saravanan and Nanda 2010; Verma et al. 2010; Jaidev and Narasimha 2010; Li et al. 2012) that effectively suppress different plant pathogens. Stable forms of AgNPs were synthesized extracellularly with aqueous silver nitrate solution employing *Phoma sorghina*; *Phanerochaete chrysosporium*, the white rot fungus (Birla et al. 2009; Gade et al. 2013); and *Mucor* (Aziz et al. 2016). Single or aggregated AgNPs (8–60 nm) with round and uniform shape were produced by the biocontrol agent, *Trichoderma asperellum*, and other five *Trichoderma* species, viz., *T. asperellum*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii*, and *T. virens*, which remain stable for several months (Devi et al. 2013). $AgNO_3$ were reduced to polydispersed and spherical-shaped AgNPs with extracellular solution of *Cladosporium cladosporioides* revealed by TEM analysis (Balaji et al. 2009). Biosynthesis of AgNPs was also investigated using *Pleurotus* sp. (Gade et al. 2007) and *Pleurotus sajor-caju* (Nithya and Ragunathan 2009) with antimicrobial activities.

A superb quality of gold nanoparticles was synthesized extracellularly by fungus *Fusarium oxysporum* and extremophilic bacteria *Thermomonospora* sp., respectively (Ahmad et al. 2003a, b), and intracellularly by fungus *Verticillium* sp. Mesophilic bacterium, *Shewanella*, was utilized for synthesis of gold nanoparticles using H_2 as an electron donor. Monodisperse gold nanoparticles of size 10–20 nm

have been synthesized by using *Rhodopseudomonas capsulata* (He et al. 2007) and alkalotolerant *Rhodococcus* sp. under intense alkaline and exalted temperature conditions. Synthesis of different shapes (spherical, cubic, and octahedral), gold nanostructures, and analysis of their formation mechanisms by filamentous cyanobacteria from Au(I)-thiosulfate and Au(III)-chloride complexes were identified by various researchers (Lengke et al. 2006). Intracellular synthesis of gold nanoparticles of various morphologies and sizes were obtained by fungi *V. luteoalbum* and *Penicillium* sp. (Kathiresan et al. 2009). Particle formation rate and size can be manipulated by controlling various factors such as concentration, pH, temperature, and exposure time to $AuCl^{4-}$. *Hormoconis resinae* also proved as an excellent source with high stability for extracellular synthesis of gold NPs (Mishra et al. 2010).

On the other hand, Au-Ag alloy nanoparticles were synthesized via an extracellular approach as marked by TEM and fluorescence microscopy. Some workers reported the synthesis of bimetallic Au-Ag alloy by fungus *F. oxysporum* and contend that the secreted cofactor NADH plays an influential role in determining the configuration of Au-Ag alloy nanoparticles (Senapati et al. 2005). Biosynthesis of Au-Ag alloy nanoparticles by yeast cells were also studied by Zheng et al. (2010a, b). Sawle et al. (2008) also proved the synthesis of core-shell Au-Ag alloy nanoparticles from fungal strains *Fusarium semitectum* and revealed that the nanoparticle suspensions remain highly stable for weeks. Au, Ag, and Au-AgNPs as reducing and protecting agents were also synthesized by a newly developed extracellular synthesis method utilizing an extract from edible mushroom, *Volvariella volvacea* (Philip 2009).

Several studies were conducted successfully to achieve the nanoparticles using heavy metals. Many heavy metals such as U(VI), Tc(VII), Cr(VI), Co(III), and Mn(IV) were reported to reduce by *Pyrobaculum islandicum*, an anaerobic hyperthermophilic bacterium. Platinum nanoparticles were accomplished using the metal ion-reducing bacterium *Shewanella algae* (Konishi et al. 2007) which reduce aqueous $PtCl_6^{2-}$ ions into elemental platinum at room temperature and neutral pH within 60 min with lactate as the electron donor. Uniform-sized (2–5 nm) and monodispersed intracellular mercury nanoparticles can also be synthesized by *Enterobacter* sp. cells (Sinha and Khare 2011) at pH 8.0 and lower concentration of mercury. Sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, and metal ion-reducing bacterium, *S. oneidensis*, enable the synthesis of palladium nanoparticles (DeWindt et al. 2005). Long-term studies immobilized fungus *Coriolus versicolor* which bioremediates cadmium and is utilized for synthesizing stable CdS NPs in continuous column mode (Sanghi and Verma 2009). Mono- and bimetallic Au/AgNPs are synthesized from a biological agent *Neurospora crassa*, a filamentous fungus (Castro-Longoria et al. 2011). Mycelia-free culture filtrates of *Nigrospora oryzae* with gold chloride were used for mycosynthesis of AuNPs of size 6–18 nm (Kar et al. 2014).

Many magnetotactic bacteria also played a crucial role in the synthesis of oxide nanoparticles due to their super paramagnetic and high coercive force properties and unique micro-configurations. Magnetic nanoparticles such as Fe_3O_4 (magnetite) and Fe_2O_3 (maghemite) are found to be highly biocompatible, synthesized intracellularly by magnetotactic bacteria, and are referred to as bacterial magnetic particles (BacMPs) (Arakaki et al. 2008). BacMPs consist of phospholipids and

proteins and thus smoothly spread in aqueous solutions. BacMPs aligned in chains within the bacterium, which enable the bacterium to shift along oxygen gradients in aquatic environments, with the clout of the Earth's geomagnetic field. Furthermore, magnetotactic bacteria are observed to possess various morphological types (ovoid bacteria, rod-shaped bacteria, cocci, vibrios, spirilla) and to inhabit various aquatic environments such as aquatic sediments, salt marshes, freshwater sediments, etc. Already established magnetic isolation techniques and different growth medium resulted in the isolation of a considerable number of the magnetotactic bacteria including *Magnetospirillum magnetotacticum* strain MS-1, *Magnetospirillum gryphiswaldense* strain MSR-1, and *Magnetospirillum magneticum* AMB-1 (Arakaki et al. 2008), and various others are uncultured. Among these isolated magnetotactic bacteria, the cultured ones are mostly mesophilic in nature and only a few reports describing thermophilic magnetotactic bacteria. Magnetotactic bacteria HSMV-1 were found in samples from springs that can grow at temperatures ranged from 32 to 63 °C (Lefevre et al. 2010). Besides magnetic oxide nanoparticles, a green low-cost and reproducible *Saccharomyces cerevisiae*-mediated biosynthesis of Sb_2O_3 nanoparticles was also investigated with a spherical aggregate of 2–10 nm size (Jha et al. 2009). Several other workers prepared tetragonal $BaTiO_3$ (4–5 nm) and quasispherical ZrO_2 nanoparticles (3–11 nm) from *F. oxysporum* (Bansal et al. 2006).

In addition to nanoparticles mentioned above, $SrCO_3$ crystals were retrieved with *Fusarium oxysporum* when incubated with aqueous Sr^{2+} ions and zinc phosphate nanopowders with yeasts as biotemplates (Pandian et al. 2009). Other researchers showed that highly luminescent CdSe quantum dots can be generated by *F. oxysporum* at room temperature (Kumar et al. 2007a, b). *Clostridium thermoaceticum* precipitate CdS on the surface of cell in existence of cysteine hydrochloride in growth medium where cysteine acts as the source of sulfide. 20–200 nm CdS was found on the cell surface when *Klebsiella pneumoniae* was exposed to Cd^{2+} ions in the growth medium. When *Escherichia coli* is incubated with $CdCl_2$ and Na_2SO_4 intracellular CdS nanocrystals are formed which poised a wurtzite crystal phase (Sweeney et al. 2004). *S. pombe* and *C. glabrata* (yeasts) have also successfully utilized to produce intracellular CdS nanoparticles with cadmium salt solution. ZnS and PbS were the other successfully synthesized nanoparticles by different microbes. ZnS nanoparticles were generated from *Rhodobacter sphaeroides* and *Desulfobacteraceae* intracellularly with 8 nm and 2–5 nm size, respectively (Bai et al. 2006; Bai and Zhang 2009). Eukaryotic organisms such as fungi (*F. oxysporum*) were also found for the synthesis of metal sulfide nanoparticles by extracellular means when exposed to aqueous solution of metal sulfate.

5.2.2 Mechanism of Nanoparticle Synthesis by Microbes

In general, nanoparticles are synthesized by microbes through entrapment of metal ions on the surface of cell (extracellular) or inside the cell (intracellular) followed by reduction with the help of enzymes (Fig. 5.1). Absorption and reduction of these metal ions are done with the help of fungal cell wall and cell wall sugars. However,

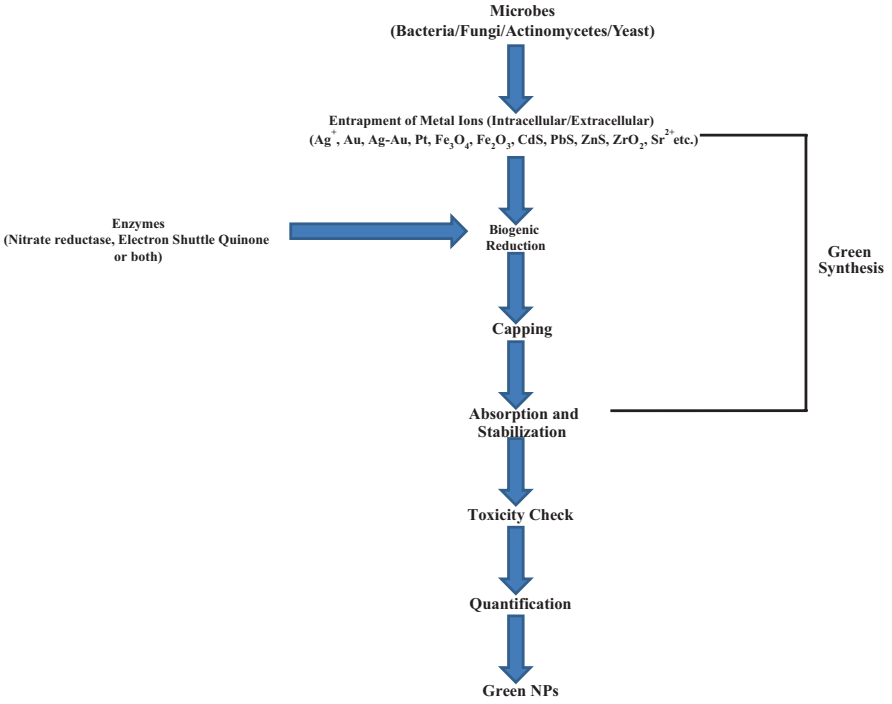

Fig. 5.1 Microbial synthesis of nanoparticles

mechanisms of nanoparticles synthesis vary with different microorganisms. For instance, synthesis of nanoparticles by extracellular means includes three possible mechanisms, viz., nitrate reductase action, electron shuttle quinones, or both. Nanoparticle synthesis was initiated by nitrate reductase utilizing *Penicillium* species and many other fungal species (Perez-de-Luque et al. 2008; Deepa and Panda 2014). The nitrate reductase assay was performed by the reaction of nitrite with 2,3-diaminophthalene (Duran et al. 2005; Kumar et al. 2007a, b). AgNP synthesis for *F. oxysporum* was involved with extracellular shuttle quinone, a-NADPH-dependent reductases, and nitrate-dependent reductases. Investigation also revealed that AgNP synthesis for *A. flavus* takes place earlier with a "33 kDa" protein and then by a protein (cysteine and free amine groups) which forms a capping agent and stabilizes the NPs (Soni and Prakash 2011).

During intracellular synthesis, metal ions were first entrapped at the cell surface of fungi via electrostatic interaction which further reduced by the enzymes within the cell wall, which leads to the accumulation and development of NPs (Singh et al. 2014). Synthesis of silver nanoparticles in *B. licheniformis* was involved with nitrate reductase enzyme (Kalishwaralal et al. 2008). *B. licheniformis* secretes the NADH and NADH-dependent enzymes that are responsible for the bioreduction of Ag^{+} and the subsequent formation of AgNPs (Husseiny et al. 2007). Reduction of Ag^{+} involves electron shuttle enzymatic metal reduction mechanism convinced with nitrate ions and dwindle silver ions to metallic silver.

Heavy metal (Co^{2+}, CrO_4^{2+}, Pb^{2+}, Zn^{2+}, Hg^{2+}, and Cd^{2+}) nanoparticles are synthesized by the microbes that developed genetic and proteomic responses to strictly regulate metal homeostasis and counter the toxic effects (Reith et al. 2007). On the other hand, synthesis of magnetite using *Shewanella oneidensis* involves both passive and active mechanisms. Initially, when bacteria utilize ferrihydrite, pH value rises due to amino acid metabolism, and active production of Fe^{2+} occurs followed by passive concentration of Fe^{2+} and Fe^{3+} which enabled the magnetite phase to precipitate. Investigation was done for the synthesis of CdS NPs through disulfide (cysteine) bridges which causes cleavage of S–H bond and generation of a new bond (Cd–S–CH_2COOH) complex on nanoparticle (Sanghi and Verma 2009). The –COOH groups from cadmium–thiolate complex react with hydrogen bond resulted in the capping of CdS nanoparticles bonding to –NH_2 groups (Tang et al. 2005). One oxygen atoms of carboxylic group (–COOH) formed coordinate bond between oxygen atom and Cd^{2+} ions, which competes with thiol group to accumulate on the CdS nanoparticles surfaces (Li et al. 2011).

5.2.3 Microbial-Based Nanoparticle Applications for Crop Protection

Nanobiotechnology can have ample of utilizations such as the identification and control of phytopathogens, protecting food from pathogens through nanosensors fabrications, biofertilizers, and biopesticide delivery in agriculture and highly competent gene transfer approach (Fig. 5.2). NP application is considered as successful for crop protection if it remains active in extreme environments (such as temperature fluctuations); penetrates the target pathogen; resists defense of phytopathogens; has low cost to formulate, preferably with advanced mode of action; and caters to social benefits and economic returns (Smith et al. 2008). NPs are also playing a dominant act for heighten the efficacy and stability of cells and enzymes used. The integration of biomolecules (enzymes, metabolites, etc.) or whole cells with nanomaterials leads to hybrid systems with numerous applications in agriculture (Bailey et al. 2010). Nanoparticles embedded with microbes offered the advantage of improved biological efficacy, easy attachment through larger surface area, higher solubility and mobility, lower toxicity, and enhanced mass delivery systems. After the entrapment of NPs and bonding of the nanomaterials, controlled release of the active ingredient is accomplished. The use of NP assisted delivery will require a targeted delivery approach focused on the behavior of phytopathogens and environmental conditions. For instance, for bombardment of plant cells and tissues to achieve gene transfer, DNA-coated AuNPs were used as bullets in "gene gun" system (Vijayakumar et al. 2010). Microbes (bacteria, fungi) and their products (enzymes, inhibitors, antibiotics, and toxins) can serve as biocontrol agents from decades for crop protection from phytopathogens as well as productivity boost. Coating of polymeric NPs offered advanced paths to enhance the efficiency and stability of these biocontrol agents as biofertilizer preparations to yield

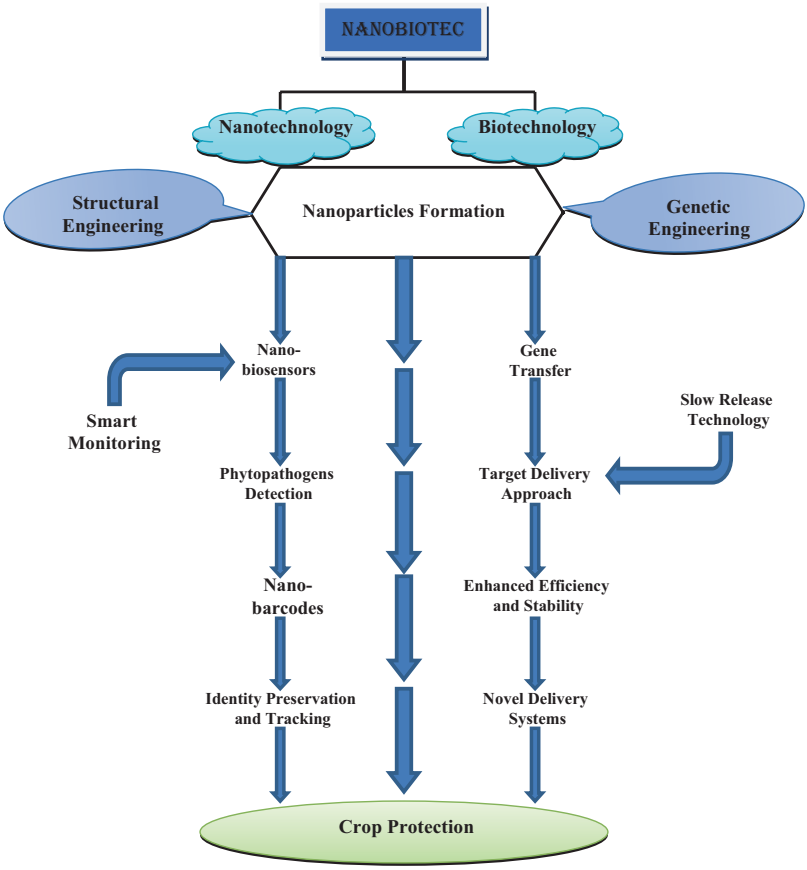

Fig. 5.2 Overview of nanoparticles impact on crop protection

formulations with directed delivery mechanism toward targeted pathogens. Furthermore, nanomaterial entrapped products would promote plant growth and soil health (Peteu et al. 2010).

Fungal biocontrol agents are relatively specific, act by contact without ingestion, and are easy for mass production. Some fungal genera (*Nomuraea*, *Beauveria*, and *Verticillium*) spread infection through conidia that require the moisture for their germination to activate host pathogenesis (Kulkarni et al. 2008). In oil emulsion formulation of *L. giganteum* (a water mold), inclusion of hydrophobic silica NPs (7–14 nm) to water in oil mycelium diminishes the desiccation, which reduced cell sedimentation and imparted N95% efficiency (Vandergheynst et al. 2007). A saprophytic fungus (*Myrothecium verrucaria*) produced endochitinase that assassinates mosquito larvae within 48 h of *Aedes aegypti* (Chavan 2009). Nanoformulation was prepared using chitosan and montmorillonite clay NPs to stabilize *Myrothecium* enzyme complex and observed for the biocontrol activity against *Fusarium* spp. and *Phenacoccus gossypiphilous* (cotton mealy bug) with

slow discharge of the enzymes. Plants of *Curcuma longa* treated with chitosan nanoparticles induce antifungal hydrolases and increased chitinases and chitosanases enzymes responsible for the defense of host plants which enabled them to become resistant to *Pythium aphanidermatum*, the causal organism of rhizome rot of turmeric (Anusuya and Sathiyabama 2013). Sensitive detection of phytopathogens (even for a single bacterial cell) was demonstrated with silica-based NPs (60 nm) filled with a fluorescent dye and conjugated to an antibody specific to a surface antigen of the microbe of interest. With the assistance of endomycorrhizal fungi, the common wetland plants (*Phragmites australis* and *Iris pseudacorus*) transform copper into metallic NPs when grown in contaminated soil (Manceau et al. 2008) to prevent copper biomagnification. Ag_2S nanocrystals and $ZnTiO_3$ exhibited higher growth inhibition efficacy against *A. niger* (Fateixa et al. 2009; Jo et al. 2009; Ruffolo et al. 2010). Higher resistance against *Fusarium oxysporum* and *Aspergillus niger* was demonstrated in maize plants treated with silica NPs of 20–40 nm (Suriyaprabha et al. 2014). Titania NPs increased bacterial population (*Bacillus amyloliquefaciens*) in the rhizosphere of *Brassica napus* and provides protection against *Alternaria brassicae* (Palmqvist et al. 2015). ZnO NPs proved for the inhibition of conidiophores and conidia of *Penicillium expansum* resulting in the disappearance of fungal mats (He et al. 2010). A highly specific and more sensitive method employs magnetic nanoparticle-based reverse. Transcription loop-mediated isothermal amplification (RTLAMP) was developed for the prompt detection of *Prunus necrotic ringspot virus* (Zong et al. 2014).

Highly fluorescent CdSe QDs and CdTe QDs were biosynthesized with *F. oxysporum* when incubated with a mixture of $CdCl_2$ + $SeCl_4$ and $CdCl_2$ + $TeCl_2$, respectively, at ambient conditions by transmission electron microscopy (TEM) and selected area electron diffraction (SAED) (Shaligram et al. 2009). Yeast cells were also utilized for biosynthesis of biocompatible cadmium telluride (CdTe) QDs with tunable fluorescence emission (Nayak et al. 2010). Nucleic acid probe attached with the surface of a quartz crystal microbalance biosensor can also be combined with fast PCR protocols to reduce the time for specific detection of phytopathogens (Maliszewska et al. 2013).

Recently, nanobiotechnology received a greater effectiveness against various phytopathogens with the use of AgNPs. Contacts of AgNPs with microbes increase due to their larger surface area-to-volume ratio and thus more ability to permeate (Kim et al. 2008). When aqueous silver (Ag^+) ions exposed to a filtrate of *Trichoderma viride*, it reduced in solution resulted in the formation of extremely stable AgNPs with 5–40 nm size (Fayaz et al. 2010). When evaluated for antimicrobial activities, it was observed that combination of antibiotics with AgNPs has better antimicrobial effects against various Gram-positive and Gram-negative bacteria (Aziz et al. 2014, 2015, 2016). Pathogenic bacterial (*Staphylococcus aureus*) infection was minimized into textile fabrics with extracellularly produced AgNPs incorporating *Fusarium oxysporum* (Duran et al. 2007). AgNPs were also demonstrated for highest-disease inhibition rate against six species of *Colletotrichum* (*C. acutatum*, *C.dematium*, *C. gloeosporioides*, *C. higginsianum*, *C. nigrum*, and *C. orbiculare*) and powdery mildew in cucumbers and pumpkins (Lamsal et al. 2011).

Leaf-spot disease caused by *Xanthomonas perforans* can be suppressed by DNA-directed AgNPs (Ocsoy et al. 2013). In another study, biogenically synthesized silver nanoparticles impregnated antibiotic discs (chloramphenicol) and reported excellent antibacterial activity against two bacteria (*Citrobacter freundii* and *Erwinia cacticida*) causing diseases on *Abelmoschus esculentus* and *Citrullus lanatus* (Paulkumar et al. 2014). AgNPs using *Serratia* spp. were biosynthesized and demonstrated for remarkable antifungal activity against spot blotch disease in wheat caused by *Bipolaris sorokiniana* (Mishra et al. 2014). *Xanthomonas axonopodis* pv. *vesicatoria* causing bacterial spot disease in tomatoes and peppers was successfully detected by fluorescent silica nanoparticles (FSNP) combined with antibody molecules (Mishra et al. 2010). The nanoparticles embedded with antibodies are used for detection of *Xanthomonas axonopodis* (Yao et al. 2009). AgNPs as antimicrobial agents are rapidly growing attraction toward researchers worldwide to make their production more economical and profitable for the control of plant diseases. Various studies also revealed strong impact of AgNPs when used in consortium with other nanocrystals. *Botrytis cinerea* was diminished by strong antifungal effect when used with Ag-SiO_2 NPs (Oh et al. 2006). AgNPs were evaluated for antifungal activity along with fluconazole by disc diffusion technique against *Phoma glomerata*, *Phoma herbarum*, *F. semitectum*, *Trichoderma* spp., and *C. albicans* (Gajbhiye et al. 2009). *Colletotrichum gloeosporioides* (responsible for anthracnose), *Bipolaris sorokiniana*, *Magnaporthe grisea*, *Sclerotium cepivorum*, and sclerotium-forming phytopathogenic fungi were significantly suppressed in a dose-dependent manner with the existence of Ag NPs (Jung et al. 2010; Aguilar-Mendez et al. 2010). Fungistatic and fungicidal effect of the AgNPs was assayed against ambrosia fungus *Raffaelea* spp., *F. culmorum*, and certain pathogenic yeasts (*Candida tropicalis*, *C. albicans*, and *C. parapsilosis*) (Kasprowicz et al. 2010). It was also demonstrated that 15 mg concentration of AgNPs showed excellent inhibitory activity against *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, *B. cinerea*, and *Curvularia lunata* (Krishnaraj et al. 2012).

5.3 Microbial-Based Nanoparticles Delivery Systems

With the utilization of NPs, nanofibers and nanocapsules, nanobiotechnology offers a novel set of procedures to multiply genes and upgrade plant resistance (Rai et al. 2012a, b). Delivery of genetic material (DNA and small interfering RNA) is important for the development of phytopathogen-resistant strains of plants by alteration of gene expression (Price and Gatehouse 2008). Gene expressions confirmed the successful insertion and integration of plasmid DNA in plant genome (Filipenko et al. 2007) and thus have more potential to develop disease resistance embedded with genetic modifications. The methods (microinjection, *Agrobacterium*-mediated transformation, and microprojectile bombardment) applied for gene delivery systems have very low efficiency (0.01%–20%) and

were mostly applied for dicotyledonous plant transformation (Sivamani et al. 2009). When NPs were employed, these technologies expand to both dicotyledonous and monocotyledonous plants with tissue specificity. During transformation of plant cells, delivery of genetic material (DNA) was employed with AuNPs (5–25 nm) embedded carbon matrices in both monocotyledonous *Oryza sativa* and dicotyledonous *Nicotiana tabacum*. This resulted in the easy access to plant cell due to its increased size transformation efficiency. In comparison to the commercial micrometer-sized gold particles, NPs also reduced plasmid and gold requirement (Vijayakumar et al. 2010) with minimal plant-cell damage and enhanced plant regeneration. Gene expressions were also triggered in the plants when uncapped with AuNPs by disulfide reduction in cellular environment under controlled release conditions (Torney et al. 2007). Starch NPs (50–100 nm) embedded with fluorescent material Tris-(2,2′-bipyridine) ruthenium-(Ru (bpy)3)2^{+} were used to transfer plasmid DNA through *Dioscorea* spp. plant cell wall, cell membrane, and nuclear membrane (Liu et al. 2008).

Polymer/DNA complexes are more stable for the protection against nuclease degradation. Also, cationic polymers (chitosan) are used to condense and deliver DNA both in vitro and in vivo (Kim et al. 2007) because of biocompatibility and low cytotoxicity. Biocontrol of crops from the phytopathogens that feed upon the double-stranded RNA (dsRNA)-producing plants was improved by RNA-based silencing (Auer and Frederick 2009). Due to the properties of binding with RNA in a highly efficient manner, safety, and ability to invade the cell membrane, chitosan NPs can prove to be competent in dsRNA delivery. In *C. elegans*, delivery of dsRNA was followed by the feeding of bacteria-expressing dsRNA or even soaking in dsRNA solution (Tomoyasu and Denell 2004). For siRNA delivery vehicles, chitosan NPs (<500 nm) entrapped with siRNA performed well as vectors (Katas and Alpar 2006). Studies also demonstrated that chitosan NPs (100–200 nm) stabilized and delivered dsRNA (against chitin synthase genes) resulted in enhanced larval susceptibilities, in existence of chitin synthase inhibitors (Zhang et al. 2010). Nucleic acid sensors were fabricated via immobilization of ssDNA onto a chitosan nanobiocomposite film containing Fe_3O_4 NPs (22 nm) for cypermethrin and permethrin detection at 0.0025 ppm (Kaushik et al. 2009).

5.3.1 Nanosensors for Crop Protection

In addition to crop protection by NPs, nanosensors help farmers in maintaining farm with precise control, crop monitoring, accurate analysis of nutrients, report of plant needs (Mousavi and Rezaei 2011), as well as detection of phytopathogens to foster a smart agriculture (Prasad et al. 2014, 2017a, b; Bhattacharyya et al. 2016; Sangeetha et al. 2017a, b). More accurately, nanosensors can be used to monitor presence and identification of phytopathogens in order to carry out remedial activities timely for crop protection. Nanosensors located in cultivated fields will provide a real-time and comprehensive monitoring of the crop growth with

high-quality data for best management practices (ElBeyrouthya and ElAzzi 2014). Some of the strategies such as antibody–antigen, adhesion receptor, antibiotic, and complementary DNA sequence recognitions are used for a specific detection between target phytopathogenic cells and bio-functionalized nanomaterials (Sastry et al. 2010; Duran et al. 2010). Gold nanoparticles are used in biosensors to develop biomolecular detection with DNA or protein-functionalized gold nanoparticles as the target-specific probes (Khosravi and Shojaosadati 2009). *Bacillus subtilis* were known to form spherical selenium nanoparticles with diameters of 50–400 nm employed for building HRP (*horse radish peroxidase*) biosensor (Wang et al. 2010). Yeast cells were used for biosynthesis of Au-Ag alloy nanoparticles to fabricate a sensitive electrochemical vanillin sensor (Zheng et al. (2010a, b). AuNP-based glucose oxidase (GOx) biosensors were synthesized for the enhancement of enzyme activity of GOx (Zheng et al. (2010a, b). Reaction rates in microbiology have also been enhanced using magnetic nanoparticles. Magnetic (Fe_3O_4)-coated nanoparticles with cells of *Pseudomonas delafieldii* were utilized to fulfill desulfurization of dibenzothiophene (Shan et al. 2005). Various researchers also emphasize on the incorporation of nanobiotechnology to emerging biotechnological methods for mycotoxin bioassays (Babu and Gunasekaran 2009) and utilization of nanosensors to reduce the fungal pathogen detection time with high sensitivity. *Escherichia coli* also rapidly determined using flow-injection system approach in which electrochemical measurement of $K_3Fe(CN)_6$ was shortened by microbial metabolism and thus allowed the quantitative determination of bacteria within 20 min. A new biosensor system utilizing equal quantities of two different microbes was developed for the rapid diagnosis of soil-borne diseases in which each microbe is individually immobilized on an electrode (Perez-Gonzalez et al. 2010). Copper oxide (CuO) nanoparticles and nanostructural layer biosensors fabricated by sol-gel and spray pyrolysis methods, respectively, were employed for detecting the *A. niger* fungi (Bao et al. 2003).

5.4 Nanotoxicity: A Major Obstacle

Although nanoparticles proved to have very huge applicability for the biocontrol of phytopathogens and thus management of various plant diseases, this increased application emerges the possibilities of getting nanoparticle accumulation in the environment which results in the toxicity of soil ecosystem (with more effect on soil microflora) leading to various harmful impacts. Activities of soil enzymes (*protease*, *catalase*, and *peroxidase*) were inhibited by TiO_2 and ZnO nanoparticles, thereby affecting the soil quality and health, and also have negative impact on the biomass of wheat growth (Du et al. 2011). Root elongation of *Zea mays*, *Cucumis sativus, Glycine max*, *Brassica oleracea*, and *Daucus carota* was affected with Al_2O_3, Al, Zn, and ZnO when conjugated with and without phenanthrene (Yang and Watts 2005) which resulted in suppression of plant germination. TiO_2 was observed for the reduction of the water usage in *Z. mays* which

changes the path of apoplast (Asli and Neumann 2009). Accumulations of Fe_2O_3 and Pd nanoparticles were found in tissue of pumpkin and leaves of barley, respectively (Battke et al. 2008; Zhu et al. 2008). One hundred percent control was achieved for powdery mildew diseases of cucurbits with silica-silver nanoparticles but become phytotoxic at 3200 ppm (Park et al. 2006). ZnO and TiO_2 nanoparticles also affected diversity of soil microbial community and biomass. Soybean exposed to ZnO and TiO_2 nanoparticles impacted directly on biomass or plant-microbe interactions, including N_2-fixing symbiosis (Ge et al. 2011). ZnO NP toxic effects on rhizospheric population might be reduced with soil–plant interactive system (Lee et al. 2012). NPs also have an impact on plant sensitivity to bacterial infection, bacterial growth and stress resistance, and the interactions occurring between host plants and associated bacterial community (Degrassi et al. 2012).

5.5 Advantages of Microbial-Based Nanoparticles

Biosynthesis of microbial-based nanoparticles is considered as nontoxic, clean, and environmentally green. Biosynthesis mechanism by microbes (bacteria, fungi, and yeast) can be classified according to the location where NPs are formed, i.e., intracellular or extracellular. Various parameters (pH, substrate concentration, reaction temperature, etc.) influence the rate of NP biosynthesis and thus can be manipulated as per needs.

NP biosynthesis can also be manipulated through gene expression by genetic delivery materials (DNA and small interfering RNA). Below are the some of the advantages of microbial NPs in crop protection: highly effective and on time response for integrated plant disease management, quick and reliable response on phytopathogens with antifungal and bactericidal effects, smart delivery along with modification as genomic and proteomic levels, development of nanobiosensor for biodetection and management of plant pathogens in field, advanced nanosystem procedures for sampling of soil and plant, and enhancement of microbiological reaction rates.

5.6 Current Prospects and Way Forward for more Efficient Microbial-Based Nanoparticles

Till date nanotechnology has a wide range of potential applications in agriculture, enabling intense research at academic and industrial levels (Chen and Yada 2011; Dasgupta et al. 2015; Parisi et al. 2015). Besides the numerous advantages of NPs, still, there are many issues that remain to be resolved for this technology to make significant contributions in sustainable agriculture. Some aspects require immediate

attention such as mode of uptake, impact of size, agglomeration, penetration, transport and stability of nanoparticles on crops, and consequences of their exposure to the environment. These factors indicate the promises of NPs for suppression/inhibition to various phytopathogens. Phytotoxicity study of NPs to determine root length, germination effect, and adsorption into the plant systems needs to be addressed (Kumari et al. 2012). Biocompatibility and biodegradability studies of nanoparticles are also desirable. Attention may also be given on the development of specific hybrid carriers for delivering active agents in order to maximize their efficiency (DeOliveira et al. 2014), upscaling of technology, risk assessment, and advancement in the regulations for the use of nanoparticles (Amenta et al. 2015). Nanosensors developed are also very specific, fast, and inexpensive, but their commercialization is again a major concern. Mechanisms of nanoparticles should be taken care to understand the interactions with target (Oliveira et al. 2015a) and nontarget organisms (Oliveira et al. 2015b) as well as risk-assessment analysis (Sadiko et al. 2014). Quantification at different concentrations in the localized environment (Kah et al. 2014) and advances in life cycle assessment (Kookana et al. 2014) are other two important factors to consider while developing any new nanoparticles. Isolation and characterization of the compounds responsible for reduction of NPs can be carried out at genomic and proteomic levels to expect shorter reaction time and high biosynthesis efficiency. Efforts are also required in defining the interactions of nanoparticles with microbes, plants, and soil for both constructive and catastrophic impacts on plant and soil health. In this context, expertise among the researchers from multidisciplinary fields would be imperative (Malysheva et al. 2015).

5.7 Conclusion

Nanobiotechnology has potential applications in the multiple detection and control of phytopathogens utilizing microbial-based nanoparticles, nanosensors, and quantum dots which greatly contribute to address the issue of sustainability. Biosynthesis of nanoparticles by microbes is eco-friendly, green, and economical viable, and issue of phytotoxicity and microbes resistant to microbicides or fungicides is also resolved. Moreover, nano-based diagnostic kits enhanced the rate of specific pathogen detection in field with high diagnosis accuracy. Additionally, nanosensors are able to detect phytopathogens at early stages of plant disease before these can be observed by farmers. Apart from detection, nanobiotechnology can be applied to know the interaction between plant and phytopathogens as well as solutions for soil remediation. Thus, application of green nanobiotechnology can greatly contribute to the management of phytopathogens and appropriate disease management action. However, experimental field trials at large scale are required to figure out the host and phytopathogen synergy, infection steps, and disease interpretation which will help in developing modern disease management approach to make agriculture as smart systems.

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