Sustainable Agriculture Reviews 60

Nirbhay K. Singh
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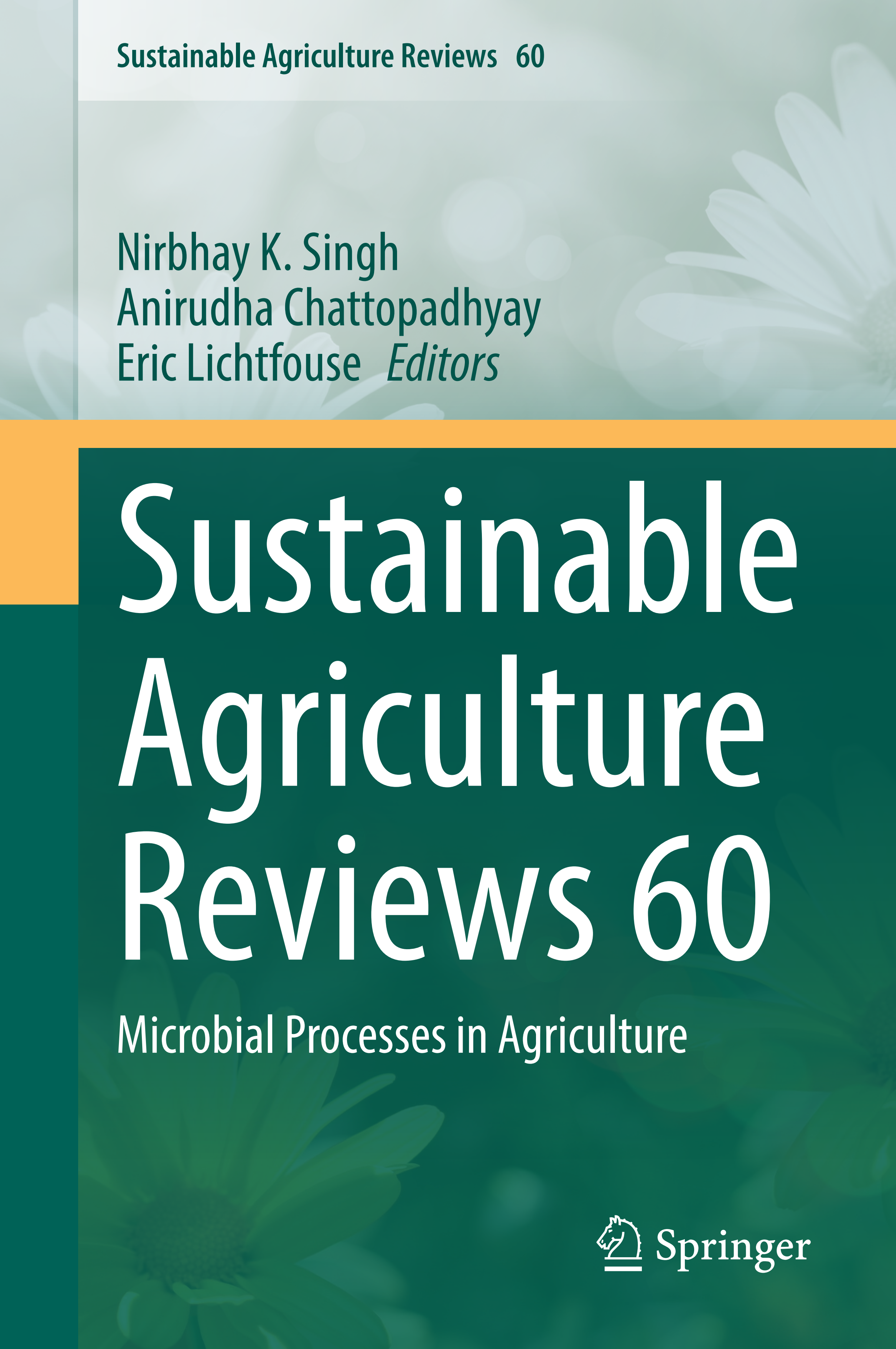
Microbial Processes in Agriculture



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Sustainable agriculture is a rapidly growing field aiming at producing food and energy in a sustainable way for humans and their children. Sustainable agriculture is a discipline that addresses current issues such as climate change, increasing food and fuel prices, poor-nation starvation, rich-nation obesity, water pollution, soil erosion, fertility loss, pest control, and biodiversity depletion.

Novel, environmentally-friendly solutions are proposed based on integrated knowledge from sciences as diverse as agronomy, soil science, molecular biology, chemistry, toxicology, ecology, economy, and social sciences. Indeed, sustainable agriculture decipher mechanisms of processes that occur from the molecular level to the farming system to the global level at time scales ranging from seconds to centuries. For that, scientists use the system approach that involves studying components and interactions of a whole system to address scientific, economic and social issues. In that respect, sustainable agriculture is not a classical, narrow science. Instead of solving problems using the classical painkiller approach that treats only negative impacts, sustainable agriculture treats problem sources.

Because most actual society issues are now intertwined, global, and fast-developing, sustainable agriculture will bring solutions to build a safer world. This book series gathers review articles that analyze current agricultural issues and knowledge, then propose alternative solutions. It will therefore help all scientists, decision-makers, professors, farmers and politicians who wish to build a safe agriculture, energy and food system for future generations.

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Preface

Climate change, pesticide pollution, and the need to produce more food in a safe way is calling for advanced methods to manage crops in a sustainable manner, for instance by improving the association of plants with microorganisms. Microbial communities are extremely diverse, interdependent and multi-functional, and are essential in regulating key processes for ecosystem functioning, yet knowledge on soil microbial communities and their interactions with plants is limited. Indeed, microbial communities control ecological processes such as N-fixation, nutrient acquisition, agro-waste management, biodegradation, carbon sequestration, land reclamation, bioremediation, plant growth promotion, pathogen suppression, induced systemic resistance, tolerance against stresses and soil stability (Fig. 1).

This book entitled *Microbial Processes in Agriculture* reviews the microbial processes that modify positively agroecosystems, from microbial evolution to microbial applications. Advances in genomics have uncovered the evolutionary path of

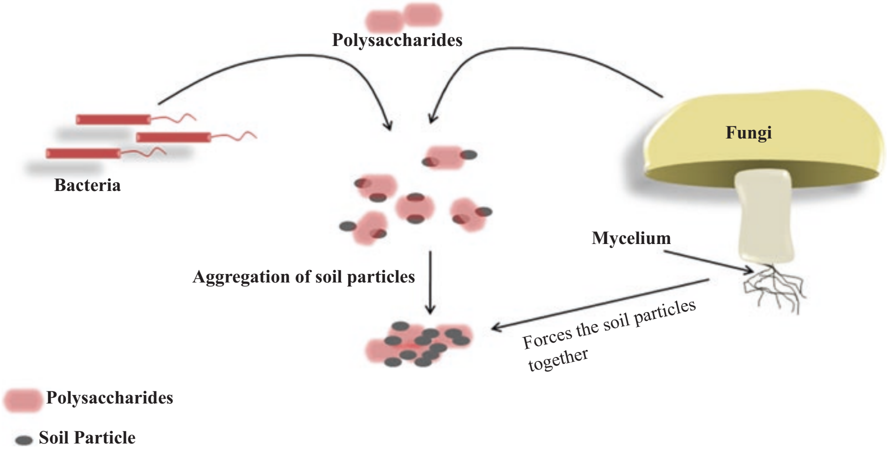

Fig. 1 Positive effect of polysaccharides from bacteria and fungi on soil stability. (From Chap. 2)

microbes, and have unravelled the biogeochemical processes ruling the interactions of plant and microbes. Chapters focus on nutrient cycling, microbial decomposition of agricultural waste and bioremediation of metal contaminants in soils. The benefits of plant-microbe association for the suppression of plant pathogens by induced defence mechanisms are discussed.

Sardarkrushinagar, India Nirbhay K. Singh

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Brief Synopsis of the Book

In the context of rising adverse effects of climate change on agriculture, there is a need for advanced methods and practices to manage soils for production of food and energy. This book presents the latest knowledge about microbial processes that control plant growth, with focus on genomic tools, microbial interactions with the plant and soils habitats, mobilization of plant nutrients, agricultural waste management, biodegradation, bioremediation, carbon sequestration, land reclamation, plant growth promotion, suppression of plant pathogens, induced systemic resistance and tolerance against biotic and abiotic stresses.

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Nirbhay K. Singh is an agricultural microbiologist and is presently working as professor and head, at the Department of Microbiology, C.P. College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University, Gujarat, India. His areas of specialization are biofertilizer technology, microbial inoculant, microbial ecology, environmental microbiology, biological control, microbial characterization and microbial diversity.

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Eric Lichtfouse is professor of environmental chemistry and scientific writing at Xi'an Jiaotong University, China. He has invented carbon-13 dating, a method allowing to measure the relative age of organic molecules in complex media. He has proved for the first time the occurrence of temporal pools of a single organic substance in the same environmental sample, e.g. a soil sample, by isotope tracing, thus opening the field of chronochemistry. He has published the book *Scientific Writing for Impact Factors*, which includes a new tool – the Micro-Article – to identify the novelty of research results. He is founder and chief editor of *Environmental Chemistry Letters*, *Environmental Chemistry for a Sustainable World* and *Sustainable Agriculture Reviews*, published by Springer Nature. He got the Analytical Chemistry Prize by the French Chemical Society, the Grand Prize of the Universities of Nancy and Metz, a Journal Citation Award by the Essential Indicators, a Springer Nature Award for editorial excellence, and he is top 2% world scientist in Environmental Sciences. In sports, he is XTerra World Vice-Champion and ITU Cross Triathlon Bronze Medal in his age category.

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Chapter 1
The Perpetual Battle of Bacteria and Phages

Kul Bhushan and Sameer S. Bhagyawant

Abstract The interaction between bacteria and phages is one of the most important microbial interactions in biological systems. Such interactions influence the other components of the ecosystems because the winner of the competition between bacteria and virus will cause adverse effects on other organisms coming in contact. Bacteria adapt to changes in the natural environment by developing mechanisms to tide over the new physical condition. This chapter reviews the complex interactions between the bacteria and phages. Bacteria are changing their strategies to resist phage infection by a number of defense mechanisms. These defense strategies include restriction-modification system, abortive infection system, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (CRISPR/Cas) system and bacteriophage exclusion system. Bacteria counteract phages using strategies such as blocking the adsorption of phages to the cell wall, blocking phage DNA injection into the bacterial cell and adopting variations in bacterial cell in response to phage attack. Phages, in response, have evolved counterstrategies to circumvent these anti-phage barriers, resulting in a co-evolutionary arm-race. Both try to override each other by exerting selective pressure.

Keywords Abortive infection · Bacteria · Bacteriophage exclusion · CRISPR/Cas system · DNA · Phages · R-M system · Arm race · Resistance · Virions

Abbreviations

Abi	Abortive infection
Acr protein	anti-CRISPR proteins
BREX	Bacteriophage exclusion

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CRISPR	Clustered regularly interspaced short palindromic repeat
Omp	Outer membrane protein
Pgl	Phage growth limitation

1.1 Introduction

Bacteriophages are the most abundant and widely distributed biological agent in the biosphere. One hundred year ago, Richard Twort (bacteriologist) at University of London and Felix d'Herelle (microbiologist) at Pasteur Institute in Paris, independently discovered bacteriophages in 1915 and 1917 (Twort 1915; d'Herelle 1917). The term bateriophage (bacteria eater) was coined by d'Herelle in 1917. During his research, d'Herelle identified the phages in the feces of patient suffering from dysentery (d'Herelle 1917). He found that culture of some dysentery bacteria dissolved by the addition of a bacteria-free filtrate. "Phage" term is derived from the Greek word "devour". The total numbers of phage particles are probably more than 10^{31} on the planet and exceeding their bacterial host by at least ten-fold (Thomas et al. 2011; Williamson et al. 2013) The International Committee of Taxonomy of Virus divide phages mainly on the basis of genome, host range and replication system (Ackermann 2007). Proliferation of bacteriophage depends upon their bacterial host survival and generally consists of three steps:

(A) **Phage adhesion on to host cell surface:** phage and bacteria interact to each other through random Brownian motion. The rate of adsorption depends upon the medium, correct orientation of receptors and the physiological state of the cell when the interaction takes place (Delbruk 1940). The phage particles undergo a chance collision on the bacterial surface, then adsorb at specific receptor site through tail fibres. Once the phage attaches to the bacterial cell surface, it penetrates the cell wall using specific enzymes and injects its genetic material (DNA or RNA) into the cytoplasm. Few proteins are also injected along with DNA into bacterial cell, for example, gp2, which protects the DNA from exonucleolytic degradation.

(B) **Expression of viral genes**: after penetration of the genetic material, phage starts synthesis of new virions using host cellular machinery.

(C) **Release of virions**: finally packaging of new genome into the capsids and progeny virions is released into the extracellular environment form lysed host. Bacteriophages are broadly divided into two types: virulent (lytic) and temperate, depending on their life style. Lytic phages involve the production of new viral progeny by take over the host transcription and translation machinery. After enough virions have been produced, they lyse the host cell and release the newly assembled virions into the environment, where they can infect new bacteria (Young 2013). Temperate phages have the ability to integrate their genetic material into the bacterial genome and persist in a quiescent state (Feiner et al. 2015). Prophages are replicated together along with the host chromosomes during host cell replication and they can change the characteristics of their bacterial host by interrupting their genes. Most of the prophage genes are repressed or remain un-functional due to deleterious mutations, resulting in a defective prophage so they can lose their ability to excise from the host genome, called cryptic prophage (Wang and Wood 2016). Sometimes, the phages neither integrates into the host genomes nor enters into lytic cycle, known as pseudo lysogeny state, induced by cell starvation conditions (Ripp and Miler 1997) which may be beneficial for bacterium perspective, for example by increasing resilience under stressful condition (Lawrence et al. 2001). Some bacterial genomes contain approximately 10–20% of bacteriophage genes causing genetic variability in many bacteria and play an important role in bacterial evolution (Rice et al. 2009). Additionally, phages have a significant impact on bacterial turnover, an estimated 10^{23} infection occurring per second in the oceans (Suttle 2007).

Bacteria are incessantly challenged by phages and have developed a formidable arsenal of numerous strategies to combat bacteriophage infection such as block phage adsorption, preventing phage DNA entry, restriction-modification system (Tock and Dryden 2005), abortive infection system (Chopin et al. 2005) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (CRISPR/Cas) (Labrie et al. 2010). In contrast, phages rapidly co-evolve to circumvent these barriers by phage variation, chemical modification of nucleotide against host restriction enzymes, incorporation of unusual bases, internal proteins that protect the DNA against host endonuclease and by producing anti-CRISPR proteins, resulting in a constant molecular arm race. Therefore, in this perpetual evolutionary arm race both are taking on each other to stay on the top but phages always find a way to evolve, persist and abound.

1.2 Bacterial Anti-phage Strategies

1.2.1 Blocking of Adsorption

The initial stage of phage infection is to recognize a specific receptor on bacterial surface such as lipopolysaccharide, membrane proteins and polysaccharides. Lipopolysaccharide consists of toxic lipid A, a core polysaccharide and O-antigen side chain. Phage attachment to the host cell is a highly specific process and recognition of a complementary receptor on cell surface is the most intricate events. Bacteria can hide or mask their receptor to avoid phage attachment. Bacteria have evolved many strategies to prevent phage adsorption such as masking of phage receptor, production of extracellular matrix to occlude receptors and the production of competitive inhibitors (Labrie et al. 2010). For example; *E. coli* use outer membrane protein (Omp) A, which is involved in bacterial conjugation process serve as receptor for many T-even-like *E. coli* phages. The TraT protein (surface exposed protein) encoded by conjugative plasmid modifies the conformation of OmpA. TraT directly interact with the OmpA protein and mask its surface exposed part that is essential for binding of phage to infect the cells (Seed 2015). This suggests that TraT protein play an important role in surface exclusion by blocking the interaction between OmpA and other phages (Sukupolvi and O'Connor 1990) that inhibit phage attachment (Riede and Eschbach 1986) and protein A mask the phage receptor for *Staphylococcus aureus* (Nordstorm and Forsgren 1974) which reduce phage adsorption.

Bacteriophage lambda uses bacterial maltose pore also called maltoporin, LamB, as specific receptor for binding. The phage attaches to the LamB receptor via its J chain, located at the tail end. Bacteria escape it by inducing mutation in the *malT* gene (regulator of LamB expression) which reduces phage adsorption (Meyer et al. 2012). In turn, bacteriophge lambda induces mutation in its *J* gene, which enables to infect *E. coli* through new receptor OmpF. J chain gets easily attached to OmpF receptor because of formation of a trimeric porin structure like LamB (Gurnev et al. 2006). Small molecules that are present in bacterial environment can also occlude at the active site of receptors and interfere with the phage attachment. For example, in *E. coli*, Ferric hydraxamate uptake protein A (FhuA,79 kDa) is a high affinity iron transporter protein, which also transport antibiotics such as albomycin and rifamycin CGP4832, and serve as a receptor for coliphages such as T7 and UC-1 (Braun 2009) and is also a potent receptor for anti-microbial peptide MccJ25 (microcin J25). MccJ25 acts as a competitive inhibitor for FhuA receptor on *E. coli*. It binds competitively to FhuA in 2:1 ratio and blocks the interaction between coliphages and FhuA receptors. It is produced under nutrients depletion condition by the *E. coli* AY25 strain and rescue the bacterium from coliphage infection (Destoumieux-Garzon et al. 2005).

Another strategy for inhibition of phage attachment is the production of extracellular matrix such as capsule and other exopolysaccharides which act as physical barrier and restrict phage adsorption. For example, some lactococcal species increase the amount of lipid in the cell wall which produces a galactose and rhamnose containing layer which in turn shield the phage receptors and avoid infection (Garvey et al. 1995). Exopolysaccharides such as alginates and hyaluronic acid are mainly produced by *Pseudomonas* and *Azotobacter* species. Hyaluronic acid composed of a linear repetition of N-acetyl glucosamine and D-glucuronic acid, constitute bacterial capsule (Forde and Fitzgerald 2003) impart resistance to phage attachment. Capsule is widely distributed among bacterial species and provide protective coat against host immunity (Scholl et al. 2005).

Phages overcome these exopolysaccharides defense by producing polysaccharide degrading enzymes such as hydrolases and lyases. Hydrolases degrade peptidoglycan layer or O-side chain of lipopolysaccharides by breaking the glucosyl-oxygen bond in the glycosidic linkage and it act hydrolytically. The lyases cleave the linkage between monosaccharide and the C4 of uronic acid (Sutherland 1995). Several hyaluronate lyases have been identified such as HylP1, HylP2, HylP3 and

SEQ2045 in phages H4489A mainly found in *S. pyogene* and *S. equi* (Baker et al. 2002). These are present in the tail fibres that degrades hyaluronic acid layer and reduce the capsule viscosity and thereby, help the phages to easily access the hidden receptors (Hynes et al. 1995). Some T7-related phage also acquires exopolysaccharide-degrading enzymes through horizontal gene transfer in tail spikes which also help in unmasking the receptors (Cornelissen et al. 2012). Furthermore, PT-6 phage of *P. aeruginosa* produce alginate lyase enzyme which degrade host alginate through beta-elimination of glycosidic bond and reduce the viscosity of the exopolysaccharide (Glonti et al. 2010).

1.2.2 Blocking of Phage DNA Injection

Superinfection exclusion systems are generally encoded by temperate phages that prevent the secondary infection with the same or closely related phages through cell surface modification known as phage warfare. Some superinfection exclusion system consists of membrane-anchored or membrane associated proteins that act as molecular decoy which are responsible for phage-phage interaction rather than phage-host interaction. Most of the *Pseudomonas aeruginosa* phages mediated superinfection exclusion by modification of the Type IV pilus and O-antigen (Bondy-Denomy et al. 2016). In *Pseudomonas* phage (D3112); phage-encoded protein, twitching-inhibiting protein, inhibit bacterial twitching mobility through interaction with Type IV pilus assembly and in turn inhibit bacterial ATPase, and protect from phage MP22 (Chung et al. 2014). Several superinfection exclusion systems have been identified in gram-positive bacteria, for example, Tuc_{2009} from lactococcal strain UC509 phage is a well characterized superinfection exclusion system (Mahony et al. 2008). Sie_{2009} is a membrane protein that provides phage resistance against a number of phages of 936 species (McGrath et al. 2002). Similarly, the temperate *Streptococcus thermophilus* phage TP-J34 produces lipoproteins ($\text{Ltp}_{\text{TP-J34}}$) that interact with other phages via tape measure protein.

The tape measure proteins play an important role in tail assembly and facilitate DNA transit to the cell (Bebeacua et al. 2013). An $\text{Ltp}_{\text{TP-J34}}$ protein targets phage measure protein and prevents DNA infection into the host cell (Sun et al. 2006). This superinfection exclusion system is highly effective against phage P008 but not against the c2 and P335. The temperate phage HK97 of *E. coli* produce a protein that provide resistance to infection by phage HK97 and HK75 by blocking their life cycle at DNA entry step (Cumby et al. 2012). Similarly, in the Sie proteins immunity and spackle are encoded by coliphage T4. Immunity changes the conformation of the phage DNA injection site and block DNA injection directly or indirectly (Lu and Henning 1989), and spackle inhibit the activity of T4 lysozyme, a component of phage tail. Therefore, it inhibits the degradation of the peptidoglycan layer (murein layer) (Lu and Henning 1994; Labrie et al. 2010).

1.2.3 Phase Variations of Bacteria

Bacteria usually face unpredictable changes frequently in their natural environment. For survival and propagation in diverse conditions bacteria can modify their cellular components through the regulation of complex gene expression network which are subject to reversible or temporal expression (a term called phase variation) or by evolving phenotypic heterogeneity. For example, two-component system (BvgAS) was identified in *Bordetella* species having two phase variants (Merkel et al. 1998). The BvgS is a 135 kDa transmembrane protein (sensor kinase) of *Bordetella* species that sense various environmental stimuli and activate the regulatory BvgA by phosphorylation. BvgA is a 23 kDa cytoplasmic protein (response regulator) that directly activates some of the virulence genes. *Bordetella* species therefore varies between Bvg^+ phase and Bvg^- phase. The Bvg^+ phase express high BvgAS activity, virulence and colonization factors which are not expressed by Bvg^- phase (Decker et al. 2012). The bacteriophage BPP-I (*Bordetella* plus trophic phage) preferentially infect the bacteria in Bvg^+ phase because it specifically expresses the adhesion protein pertactin (Prn, Outer membrane protein) which serves as phage receptors and are not expressed in the Bvg^- phase (Fig. 1.1a) (Liu et al. 2002). BPP phage can evolve variants that recognize different surface receptors and infect Bvg^- phase cells. Therefore, the infection efficiency of the BPP-I is 10^6-fold higher for Bvg^+ cell than Bvg^- cells.

Some phages infect *Bordetella* species by mutating the major tropism determinants (*mtd*) gene. *Mtd* gene encode protein that is responsible for host recognition, but the interaction between wild and prn molecules enhances the rate BPP-I infection (Dai et al. 2010).

Streptomyces coelicolor A3(2) is naturally resistant to temperate phage ϕC31 and homo-immune relatives. The resistance was later found to be due to a novel phage defense system called Pgl (phage growth limitation) system conferred by a Russian group (Chinenova et al. 1982). *Streptomyces lividans* (lack Pgl system naturally), a closely related strain of *Streptomyces coelicolor* A3(2) produce ϕC31 phage and has the ability to infect a wild type Pgl^+ bacterial strain which produce ineffective phage progeny phage but in subsequent round of infection progeny become severely attenuated. On the other side, these progenies can efficiently infect a Pgl^- host and give normally phage burst. The model proposed by Chinenova and colleagues postulates that while the first phase of infection Pgl^+ strain modifies the progeny phage, and this activate a mechanism which restrict them during the second round of infection (Fig. 1.1b). Pgl system is composed of four genes, located in two closely related operons, pglWX and pglYZ (Laity et al. 1993; Sumby and Smith 2002). PglW contains three predicted motifs; nuclease related domain, a tyrosine kinase domain and a Hanks-like serine/threonine protein kinase domain, PglX encode a DNA methyltransferase, PglY contain Walker A or Walker B motifs and PglZ contains a predicted alkaline phosphatase fold (Hoskisson et al. 2015). PglX fulfill the proposed hypothesis of Chinenova that it modifies the phage progeny through DNA methyltransferase (reverse of restrction-modification system).

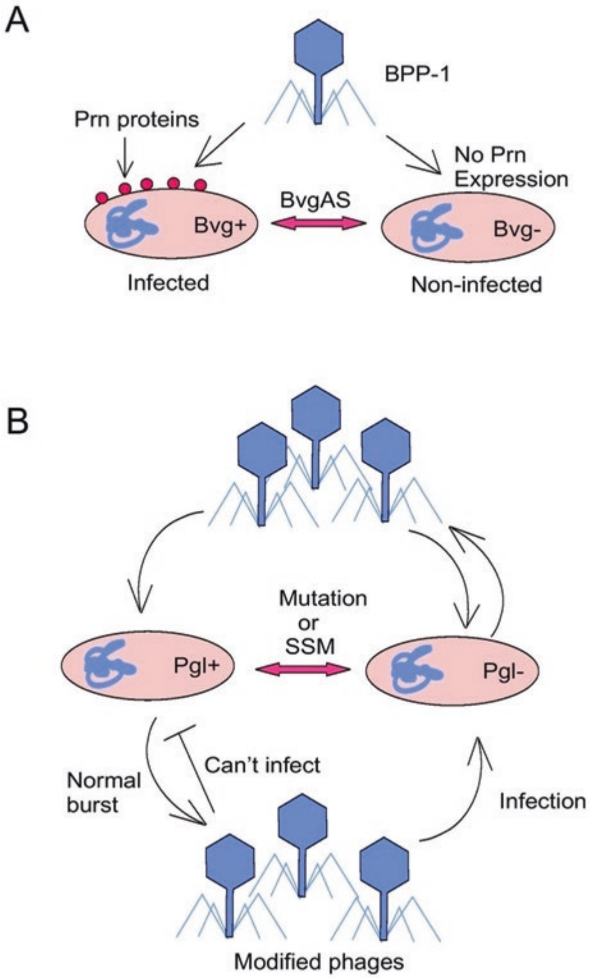

Fig. 1.1 Phase variation in bacteria. (**a**) Pgl system in *Streptomyces coelicolor* enables protection against the temperate bacteriophages by first normal lytic cycle. But the progeny are modified by Pgl methyltransferase, so that they cannot further infect the Pgl$^+$ cells but can infect Pgl$^-$ cells. This system switches between two phases through slipped-strand mispairing (SSM) or mutation in *PglX* gene. (**b**) *Bordetella* species also undergoes phase variation by a regulatory system BvgAS. Bvg$^+$ phase express pertactin (Prn) that is recognized by Bordetella Plus trophic Phage-1 (BPP-1) and infect but Bvg$^-$ cells does not express pertactin so they prevent BPP-1 infection

The *PglX* gene contains a long tract of poly-guanine bases. This tract expands and contract during replication through mutation and slip-strand mispairing which leads to the truncated or frameshift protein. This causes variation in the poly-guanine tract of PglX system (Sumby and Smith 2003). Furthermore, *S. coelicolor* encodes a paralogue of PglX, called PglS, which interact with the other Pgl protein by competing with PglX resulting in a weak Pgl$^-$ phenotype (Laity et al. 1993).

1.3 Restriction-Modification System

In 1978, Werber Arber, Daniel Nathan and Hemilton Smith were awarded Noble prize for their discovery of restriction enzymes and their potential applications in gene cloning and in molecular biology. The advent of restriction enzyme as a part of restriction-modification system and their ability to cleave the invading DNA changed the dimension of this never-ending battle. Restriction-modification system was first discovered by Luria and Bertani in 1952 and 1953, respectively (Luria and Human 1952; Bertani and Weigle 1953). During their research they observed that phage lambda grows poorly on *E. coli* K-22 strain. Restriction-modification system consists of two opposing activities: a restriction endonuclease and methyltransferase and work as van-guard against the entry of foreign DNA.

Restriction endonuclease recognizes and cleave foreign DNA sequence at specific sites by hydrolyzing the phosphodiester backbone on both strands, and methyltransferase add methyl group to same sequence which protect it from lethal effects of endonuclease (Fig. 1.2). methyltransferase catalyze the transfer of activated methyl group from S-adenosyl-L-methionine to cytosine or adenine (Dryden 1999) which leads to the base flipping (involve rotation of target adenine or cytosine). Restriction-modification systems are classified into four categories (Table 1.1) on the basis of subunit composition, cofactor requirement, cleavage position, sequence recognition and substrate specificity (Roberts et al. 2003).

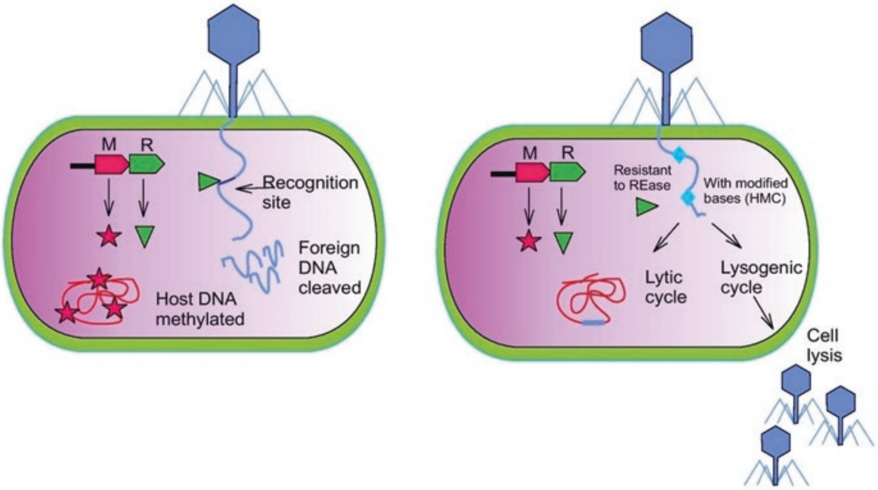

Fig. 1.2 Restriction-modification system. The Methyltransferase (MTase) normally recognizes a palindromic site and methylate at both strands of self-DNA. The restriction endonuclease (REase) recognizes the foreign unmethylated DNA and cleaves it into harmless fragments. On the other hand, some phages incorporate unusual bases such as 5-hydroxymetylcytosine or change the pattern of its recognition site which prevents phages from the action of R-M system

Table 1.1 Comparison between different types of restriction-modification systems

	Restriction-modification system type I	Restriction-modification system type II	Restriction-modification system type III
Feature			
Composition	A single enzyme with three subunits, R (endonuclease) M (methylase) S (specificity) R_2M_2S.	Two separate enzymes either to cut or modify the recognition sequence. R and M proteins (R_2 and M). R and M do not function as multi-subunit.	A single enzyme with subunits for recognition and cleavage R, M (RM).
Recognition site	Asymmetric, bipartite, may be degenerate.	Symmetric may be bipartite.	Asymmetric, require two recognition sites in opposite direction.
DNA cleavage	Cleavage occurs far from recognition site.	Cleavage occurs within recognition site.	Cleavage site is 24–26 nt from recognition site
Co-factors	Mg2+, ATP, S-adenosyl-L-methionine.	Mg2+ (S-adenosyl-L-methionine for methylation only)	Mg2+, ATP is allosteric effector.

Note: *R* Restriction, *M* modification, *S* specificity

The antiviral efficiency of a restriction-modification system depends on several factors such as the relative activity of restriction endonuclease and methyltransferase, the number of recognition sequences in phage genome, presence of modified base in phage DNA and the kinetics of phage replication. Among these, only type II restriction enzymes are widely used for genetic engineering due to their target specificity. Phages have evolved numerous mechanisms to defend themselves from diverse Restriction-modification system a different way such as incorporation of modified bases into their genome, stimulation of the host methyltransferase and by alteration in their restriction recognition sites (Stern and Sorek 2011).

The phage T4 genome contains the base 5-hydroxymethylcytosine in place of cytosine, found in the host DNA, which make it resistant to many restriction enzymes. Bacteria circumvent by producing some unique restriction endonucleases e.g., McrA, McrBC and Prr from *E. coli* which cleave foreign DNA at specific site (Stewart et al. 2000). Phages further modify these unusual bases by glycosylation, which protect it against the action of these enzymes and challenge the bacteria. To counterattack the phage, some *E. coli* strain such as CT596 contains a two-component system (glucose-modified restriction) GmrS(36 kDa)/GmrD (27 kDa) that are encoded by a cryptic prophage. This system target and cleavage glycosylated-HMC modified DNA and block its infection while has no impact on un-glycosylated DNA (Bair and Black 2007). In yet another twist, some T4-like phages have a gene encoding internal protein1 (IP1). The mature IP1 called IP1* transferred into the host cell along with T4 DNA, hinder the GmrS/GmrD complex through direct protein-protein interaction and block its restriction activity (Rifat et al. 2008).

Phage13 genome encodes an enzyme, S-adenosyl-L-methionine hydrolyses, that destroy intracellular S-adenosyl-L-methionine, which is important co-factor for restriction-modification type II enzyme activity and improve the chances of phage survival. In addition, coliphage T7 possesses the 0.3 gene encoding overcome classical restriction protein which are synthesized in the virus infected cells, and are potent inhibitor of type I restriction-modification system. Overcome classical restriction proteins are acidic in nature with negative charge on their surface and this charge is complementary to the charge on binding site for type I restriction endonuclease.

These proteins imitate the B-form of DNA of exact shape and size and prevent phage DNA from binding to $R_2M_2S_1$ complex (Walkinshaw et al. 2002), and thus allows the phage to propagate. Due to their small size, they do not have any restriction site for restriction endonuclease, but restriction endonuclease has higher binding affinity for overcome classical restriction enzyme than target DNA (Atanasiu et al. 2001). Similarly, many conjugation plasmids and transposons express alleviation of restriction of DNA (ard) proteins which mostly consist of Asp and Glu residues. This protein appears to mimic 42-bp stretches of B-form DNA and impede the restriction-modification I system (McMahon et al. 2009; Wilkins 2002). Finally, phage P3 encodes defense against restriction A and B (DarA and DarB) proteins found in phage head which are 69 kDa and 251 kDa, respectively. These are structural proteins that are co-injected with infected DNA into the host cell and bind to phage DNA; thus, occlude the restriction sites, so phage can easily escape from type I restriction-modification system and propagate (Iida et al. 1987).

1.4 Abortive Infection

Abortive infection systems, also called phage exclusion, are a bacterial (host) mechanism which prevents phage multiplication at various steps to eliminate the production of virus progeny. Abi-mediated resistance leads to death (suicide) of the infected bacterial cell and protects adjacent bacterial populations from predators, and thus prevents further infection. In most Abi systems phages are trapped inside the cells. An infected cell undergoes cellular suicide (show altruistic behavior) after infection by phages – akin to prokaryotic apoptosis.

1.4.1 Abi System in L. lactis

Currently, twenty-three abortive infection systems (designated AbiA to AbiZ) isolated from *Lactococcus lactis*, most of which are plasmid encoded (Chopin et al. 2005). Majority of the abortive infection systems are encoded by single gene; but often, consist of two or three. AbiD1 is the well characterized Abi system of *L. lactis* active on 936 phages and c2 phages, and its expression is induced by phage bIL66 infection (Bidenko et al. 2002). The middle regions of gene of bIL66 phage are transcribed from phage activated promoter. Open reading frame (ORF)-1 bind to the

AbiD1 and triggers protein translation (Bidenko et al. 2009). Moreover, AbiD1 blocks expression of ORF3 which prevents phage maturation and packing and result in abortion of phage multiplication (Bidenko et al. 1998). In turn, phage rise this challenge by inducing mutation in ORF1, so it does not activate AbiD1, stop its expression, and then, phages escape easily from this system.

AbiA is active against mainly *lactococcal* phage species by inhibiting DNA replication. This Abi system is encoded by conjugation plasmid (pRT2030) from which it's expressed constitutively (Hill et al. 1990). Unlike AbiA system, AbiB is chromosomal encoded Abi system (Cluzel et al. 1991). AbiC contain a putative transmembrane helix which restricts production of major capsid protein. AbiP is a membrane-anchored protein which is only effective against 936 phages. It halts DNA replication after 10 min of phage infection (Domnigues et al. 2004). Another Abi system, such as AbiG and AbiU, both are two component systems and block RNA transcription of 936 phages and c2 phages (Dai et al. 2001). AbiQ in *L. lactis* can alter the early expression of the corresponding phage mRNA and strongly inhibit the replication of phage P008, phage bIL170 and phage c2 (Samson et al. 2013). AbiV can interact with the bacteriophage protein SaV that infects bacteria and prevent phage protein synthesis (Haaber et al. 2010). A newly characterized AbiZ system located on the pTR2030 plasmids which function by premature killing of the infected cell and is effective only against P335 phage (Durmaz and Klaenhammer 2007).

1.4.2 Abi System in E. coli

Abortive infection Rex System is the best characterized and a typical example of Abi system in *E. coli*. Rex is a two-component (RexA and RexB) system present in phage lambda lysogenic *E. coli* strain. RexA (31 kDa) resides in the cytoplasm and RexB (16 kDa) is a membrane-anchored protein containing four transmembrane spanning domains (Parma et al. 1992). RexA is an intracellular sensor which is activated during phage infection by the production of phage protein-DNA complex (an intermediate of replication and recombination) that activates membrane anchored RexB protein (Snyder 1995). RexB form an ion channel that depolarizes the cytoplasmic membrane, allowing passage of monovalent cations (Snyder 1995). This reduces membrane potential causing reduction of cellular ATP levels, decrease the synthesis of macromolecule and stop cell multiplication which leads to bacterial death. The efficacy of this system depends on its expression level and also affected by RexA/RexB ratio.

Two other Abi systems are Lit and PrrC both repress DNA translation machinery to achieve coliphage abortion by similar mechanism. Both systems consist of a constitutively expressed inactive protein which is activated upon phage infection by a small phage encoded peptide. *Lit* gene is present in a defective e14 prophage of *E. coli* K12 (Kao and Snyder 1988). This gene encodes a protease which contains a zinc metalloprotease domain that block translation elongation factor – TU and abort phage infection (Yu and Snyder 1994). Protease activity of Lit gene is activated by Gol peptide (29 amino acids), a major head protein (Dy et al. 2014) which is expressed late in the lytic cycle of phage T4. *PrrC* gene is located inside a type I restriction-modification cassette containing three other genes prrA, prrB and prrD (Labrie et al. 2010). PrrC is an endonuclease that is activated by a small (26 amino acids) phage T4 encoded peptide Stp (suppressor of the three-prime phosphatase) which cleaves $tRNA^{Lys}$ in the anticodon loop and stop phage infection. PrrC activity is inhibited by EcoprrI (prrD-encoded restriction enzyme). Stp change the conformation of EcoprrI-prrC complex by binding and release of the activated prrC which cause phage abortion. Mutation in these phage polypeptides (Gol and Stp) suppress Abi mechanism and rescue the infecting phage.

Toxin-antitoxin systems are extensively spread in bacteria consist of two components: a stable toxin and a labile antitoxin function in phage defense via Abi. Toxin-antitoxin modules are divided into six (I-VI) classes (Fig. 1.3) based on their genetic structure, regulation and mode of action and binding partner (Page and Peti 2016). Toxins are always proteins whereas antitoxins could be non-coding RNAs or proteins. In class III Toxin-antitoxin system toxIN is the best studied example. This system is bicistronic: *toxI* gene encoding an antitoxin and *toxN* gene encoding the toxin, both are transcribed from a common promoter (Schuster and Bertram 2013). ToxI, an antitoxin RNA molecule neutralizes their cognate toxins through the formation of RNA-protein complex. Antitoxins generally have small half-life (0.5 min.) as compared to toxin (20 min.), so it does rapidly degrade (Fineran et al. 2009). In response to a particular stress (phage infection), antitoxins are degraded rapidly by one of the housekeeping bacterial proteases, so toxins are free to exert their toxic effects such as inhibition of cellular process (replication and translation) to inhibit growth and inducing bacteriostasis.

A similar system has been identified in *E. coli* for type I *hok* (host killing)/*sok* (suppressor of killing) system that inhibit phage T4 infection by post-segregational killing (Pecota and Wood 1996) and for type II *mazEF* system (protein-protein system) that protect against phage P1 infection (Hazan and Engelberg-Kulka 2004). It has been recently shown that AbiQ system function as protein-RNA toxin-antitoxin system.

1.5 The Clustered Regularly Interspaced Short Palindromic Repeats System

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a widely used defense system by bacteria to prevent bacteriophage infection through DNA cleavage. CRISPR was first identified in *E. coli* genome K12 as an unusual structure (Ishino et al. 1987) and later found in the 40% bacterial and 90% archeal genomes (Horvath and Barrangou 2010). The CRISPR/Cas system is composed of two main components: the Cas protein, which work as a scissor and cleave the DNA, and a gRNA which shepherd the Cas nuclease at specific target site (Westra et al. 2014; Barrangou and Marraffini 2014). CRISPR loci generally consist of short stretches of

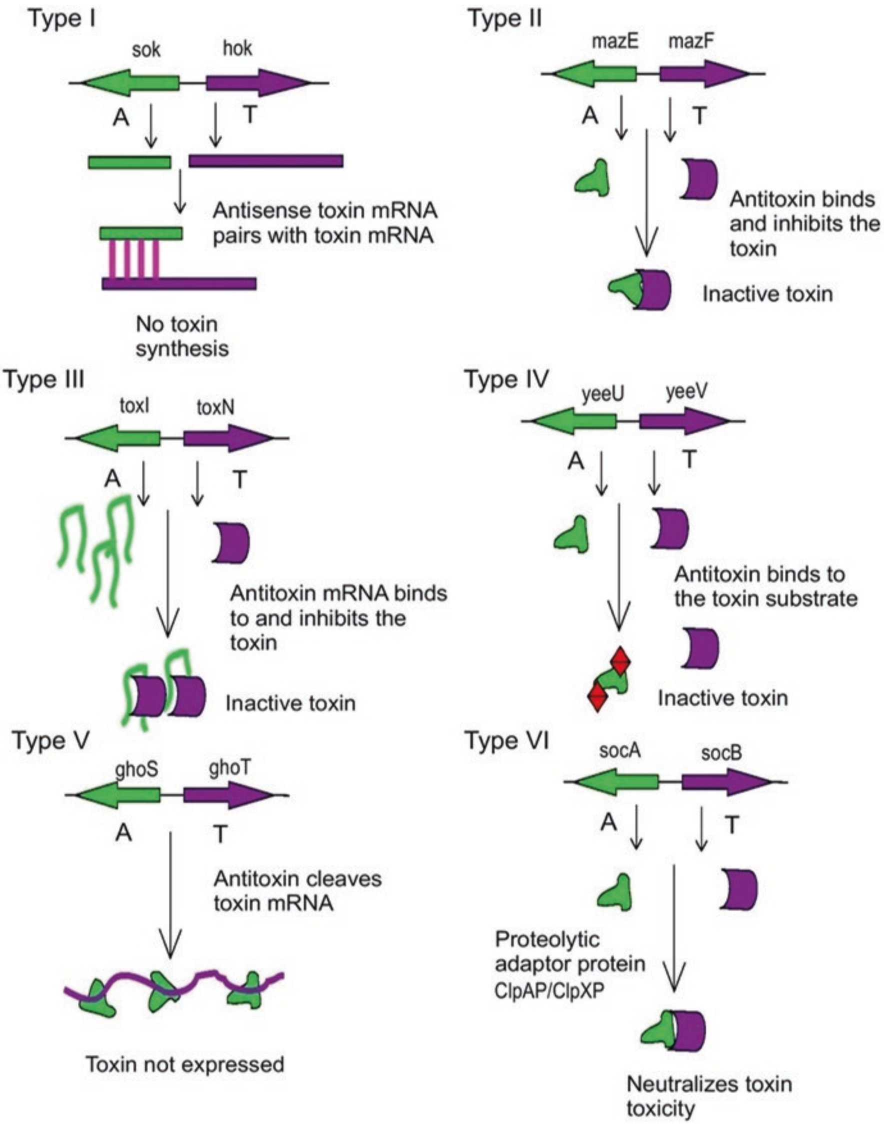

Fig. 1.3 Different type of toxin-antitoxin systems. Type I (hok/sok): The antisense RNA binds to the complementary sequence within the toxin mRNA. This leads to the formation of double-stranded RNA, which inhibits the translation of toxin mRNA. Type II (mazF/mazE): The antitoxin and toxin are proteins that combine and formed a complex under non-stress condition, which neutralizes the toxin activity (Rocker and Maisonneuve 2016). Type III (toxN/toxI): Antitoxin is a small RNA that combines with toxin and form a TA complex which neutralizes the toxin. Type IV (yeeV/yeeU): The antitoxin interacts with the substrate of toxin and leaves the toxin in inactive form. Type V (ghot/ghoS): Antitoxin act as a sequence-specific endoribonuclease which cleave the toxin mRNA but resulting toxin are not expressed. Type VI (socB/socA): Toxin is an unstable protein so it's constitutively degraded by ClpAP/ClpXP protease. The antitoxin neutralizes toxin by acting as an adaptor protein which promote the degradation of toxin by protease (Aakre et al. 2013)

non-repetitive DNA (21-48 bp), separated by non-repeating DNA sequences called spacer (26–72 bp). Spacers are primarily derived from invading phage, plasmid or transposon DNA. CRISPR associated (*Cas*) genes are located adjacent to the CRISPR sequence. CRISPR system have been divided into three main types (type I, II and III) based on the *Cas* genes they possess and their mode of action, with a further division into several subtypes (Makarova et al. 2015). CRISPR mediated immunity function in three different stages: (a) adaptation: first recognition of protospacer sequence and then incorporate into CRISPR array by Cas1/Cas2 complex, (b) CRISPR locus is transcribed into a mixture of small crRNA and then processed into mature crRNA. Each crRNA contains a full spacer and partial repeat form a CRISPR/Cas complex with a set of cas proteins and finally (c) CRISPR interference: identification of foreign DNA by sequence complementarity to the crRNA-Cas complex which bind and destroy targeted invading DNA (Nunez et al. 2014; van der Oost et al. 2014).

In turn, phages evade CRISPR system by inducing point mutation in PAM (protospacer-adjacent motif) region and genome rearrangement. In response to the CRISPR war, phages produce proteins which inactivate the CRISPR/Cas system called anti-CRISPR proteins (acr) (Chaudhary et al. 2018). The acr proteins were first identified by Davidson group at University of Toronto in *Pseudomonas aeruginosa* (Bondy-Denomy et al. 2013). These are small proteins (50–150 amino acids) closely related to each other show sequence similarity. Acr proteins inactivate the CRISPR system by direct interaction with different proteins of CRISPR surveillance complex by distinct modes of action (Bondy-Denomy et al. 2015).

In type I-F system, CRRISPR array are transcribed and processed into mature crRNA by Csy4 endonuclease which remains bound to its 3′ end. This Csy-crRNA duplex interacts with different Csy proteins and formed a Csy surveillance complex (Haurwitz et al. 2010). The acr proteins, acrF1 and acrF2 bound directly to the distinct subunits of CRISPR/Csy complex. The acrF2 block the binding of complementary DNA to the 5′ end of crRNA, leads to the inactivation of the CRISPR system (Chaudhary et al. 2018). It has been showed that acrF1 attached to the Csy backbone which inhibit target binding (Maxwell 2016). Unlike acrF1 and acrF2, acrF3 directly interact with Cas3 helicase-nuclease and prevent the binding of target DNA molecule (Fig. 1.4).

A recent study by Rauch and colleagues showed that the most prevalent type II CRISPR system is also blocked by two acr proteins (acrIIA2 and acrIIA4). These proteins block the CRISPR system by directly binding to the Cas9/gRNA complex (Rauch et al. 2017). Once again, phages overcome this bacterial defense system by producing acr proteins and upgrade their attack arsenal and show the indomitable traits.

1.6 Bacteriophage Exclusion

A novel phage resistance mechanism called bacteriophage exclusion (BREX), which confers partial or complete resistance against phages was first identified in *Bacillus cereus* and then further tested in *Bacillus subtilis*. BREX systems are found

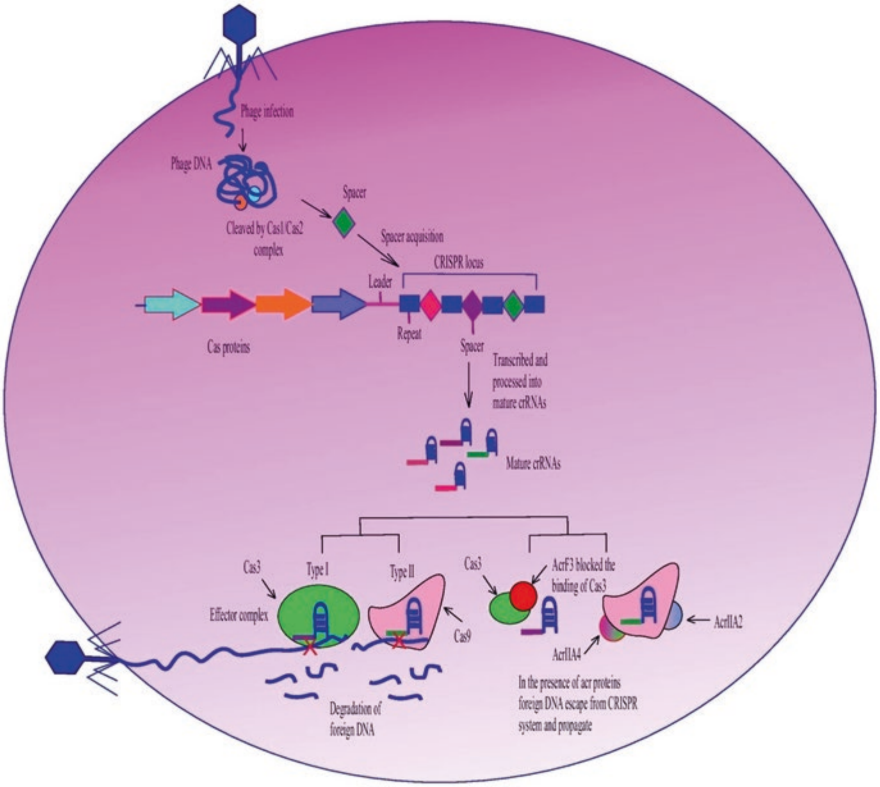

Fig. 1.4 CRISPR/Cas system in bacteria. CRISPR/Cas functions as an adaptive immune system in bacteria. CRISPR locus contains a series of repeats which are separated by spacers. These spacers are derived from the DNA of viruses they prey on bacteria and serve as memory of past exposure (bacteria save them in their genetic code). When the same or related virus attack again the CRISPR repeat-spacer array is transcribed and processed into mature crRNAs and form an effector complex with other proteins such as Cas9, Cas3 etc. This effector complex scans the viral DNA to seek the complementary sequence of the spacer and cleave it's both strands and degrade. In turn, some phages produced anti-CRISPR proteins (AcrF3, AcrIIA2, AcrIIA4 etc). The AcrF3 interact directly to the Cas3 nuclease and prevent its recruitment to the effector complex while AcrIIA2 and AcrIIA4 directly binds to Cas9 and block its cleavage activity by shielding RuvC active site (Dong et al. 2017), so phages escape from the CRISPR system and infect the bacteria

in approximately 10% of bacterial and archael genome and protect them against virulent and temperate phage infection (Goldfarb et al. 2015). BREX is a cassette of six genes which encode alkaline phosphatase, methyltransferase, RNA-binding protein, an unknown protein, an ATPase-domain protein and a protease (Fig. 1.5). Sorek and colleagues uncovered the mode of action of this novel system. They showed that BREX system repressed phage DNA replication (Goldfarb et al. 2015). Although the function of the BREX resembles restriction-modification system, the methylation of bacterial genome in BREX occurs at the non-palindromic hexameric

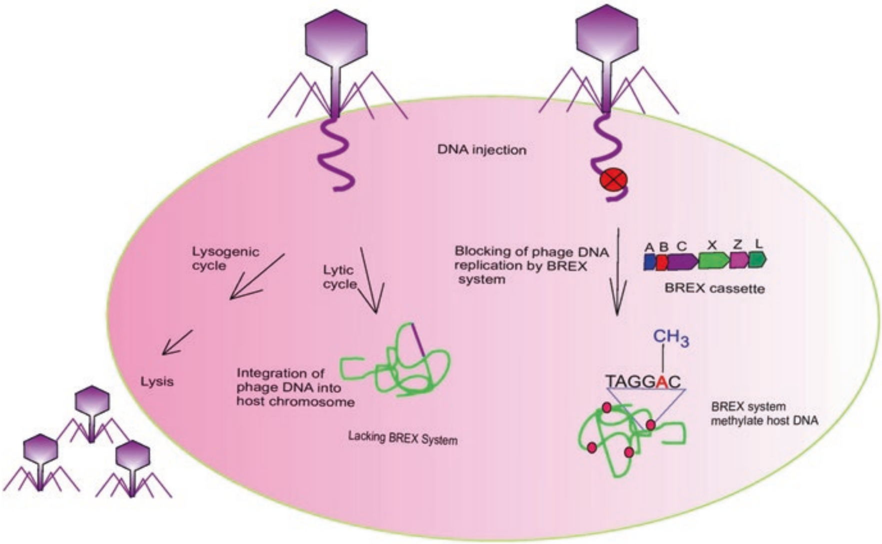

Fig. 1.5 Bacteriophage exclusion (BREX) system. BREX is composed of a cassette containing six genes (BrxA-B-C-X-Z-L). Bacterial cells that possess BREX system blocked the replication of phage DNA and modify the host DNA by methylation so prevent phage propagation. But the host cells which lack this system are successfully infected by phages

(TAGGAG) motif (Chaudhary 2018). In addition, BREX does not cleave or degrade phage DNA, suggesting that it employs an alternative mode of action compared to restriction-modification and Abi system. But some phages circumvent BREX barrier by evolved some anti-BREX mechanisms as the repercussions of their incessant never-ending battle (Barrangou and van der Oost 2015).

1.7 Conclusion

Bacteria and phages are in an incessant arm race since the dawn of life. The perpetual battle of bacteria and phages evolved many layers of bacterial defense systems (restriction- modification system, Abi system, CRISPR/Cas system and bacteriophage exclusion system) to resist phage attack. These defense systems play a fundamental role in conferring protection against phages. In response, phages can overcome these defense systems and evolved mechanisms to target resistant bacteria. Such as, recently discovered CRISPR/Cas system has been widely used as an important tool against phages and plasmids. But some phages produce acr proteins, which block the formation of surveillance complex and prevent the action of Cas nucleases and shut down the CRISPR system. In this co-evolutionary arm race, both have developed diverse strategies to gain upper hand in this never-ending strife. Only the future tells us who the winner is because many phage resistance mechanisms remain to be revealed. The battle is not all doom and gloom for bacteria they are fighting tooth and nail to win this game. Ahead, the odyssey of battle is strewn with full of mystery.

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Chapter 2
Orchestration of the Plant Microbiome for Enhanced Agriculture

Shubha Rani Sharma and Rajani Sharma

Abstract The fast rising human population calls for new methods to enhance agricultural production because classical methods such as the excessive use of mineral fertilizers are not sustainable. For instance, recent advances in microbial ecology reveal that many microbial properties can be tuned to favor plant health with positive effects on nutrients availability, root growth enhancement, toxin neutralization, providing resistance to various diseases, enduring stresses such as heat, flood, and drought. Here we review microbial strategies that favor plant health, with focus on the plant-associated microbiome and the use of the soil microbiome for management. Rhizosphere properties influence crop yields through production of exopolysaccharide for soil aggregation, alleviation of crop stress, enhancement of nutrient uptake by soil microbes, and efficient nitrogen mineralization and phosphate solubilization. Plant-microbe interactions increase colonization of plant-beneficial bacteria, which promotes enhanced root growth and phytoremediation.

Keywords Microbiome · Agricultural · Rhizosphere · Phytoremediation · Stress · Bioavailability · Nif genes · Biofertilizers · Mutualism · Phytohormones · Voodoo juice

2.1 Introduction

Science has revolutionized the living standards of the common people. This sounds good that with advanced formulas and technologies death rate has decreased. On the other hand, it is quite alarming as this has resulted in an increase in the consumption

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rate in each field. Increased population has directly influenced the farming trying to enhance it beyond limits. Scientists have tried to formulate the techniques which can increase the crop yield in limited space and time. The major step taken was the use of synthetic fertilizers having all the constituents needed for the high crop yield (Tilman et al. 2002). As the natural manures were not sufficient to meet the demands of the huge agricultural land masses and were slow in their action so we had to take refuge of the synthetic fertilizers.

Synthetic fertilizers were introduced to fulfill the needs of macro and micronutrients for high crop yield as well as chemical pesticides have over-powered the pests and pathogens but the prominent and exhaustive use of both the chemicals actually disturbed the ecological balance (Bhanti and Taneja 2007). On one hand, these chemicals increased the crop yield to great heights in order to quench the demands of the food for the population while on the other hand; it degrades the soil quality (Chowdhury et al. 2008). Some serious steps need to be taken which can increase the crop yield without disturbing ecological balance and the soil physiology (Sharma and Singhvi 2017). Need is the mother of all inventions, this proverb proved right in this case. Severe needs to increase crop production and curb the problem of soil pollution led to the discovery of microbial exploitation to increase the crop yield. Plants parts are known to be associated with multifarious microbiome just like the gut of human beings.

The microbial communities which reside in various plant parts have their own significance. These may be involved in the uptake of nutrients, defense against pathogens and combating abiotic stress (Abhilash et al. 2012). It has been an established fact that, just like animals, plants are not self-sufficient but are highly colonized by an abundance of microbes. Microbes are known to play essential roles in agricultural ecosystems (Gopal and Gupta 2016). The agriculturists have well known that the healthy soils are the foundation for healthy plant life as well as high crop yield. Healthy soil is characterized by sufficient moisture, organic matter and other physical soil attributes (Doran and Safley 1997). In this chapter, we will discuss the role of microbes in enhancing crop yield targeting all the above-mentioned issues.

The excessive use of chemical fertilizer disturbs the soil physiology and frequently leads to accumulation of heavy metals, nitrate and increases the salinity (Pogrzeba et al. 2018; Lominadze and Nakashidze 2016). Increase in salinity also influences the pH level of the soil (Dong et al. 2012). The effect is not limited to soil only; rather it also pollutes the water bodies. The rushing of rainwater to the water bodies may lead to eutrophication (Savci 2012). Uses of biofertilizers have come up with most of the above-mentioned drawbacks. A comparative study between chemical and biofertilizer has been shown in Fig. 2.1.

Actually, the use of earthworms is in practice but microbiomes are preferred for sustainable agriculture. This is due to the emission of carbon dioxide and nitrous oxide by earthworm (Giannopoulos et al. 2010). These two are the main greenhouse gases. On the other hand, the maintenance of earthworm is not cost effective. This directly affects the price index of the crops. For this reason, microbes are targeted to enhance soil fertility and have a positive association with yield improvement.

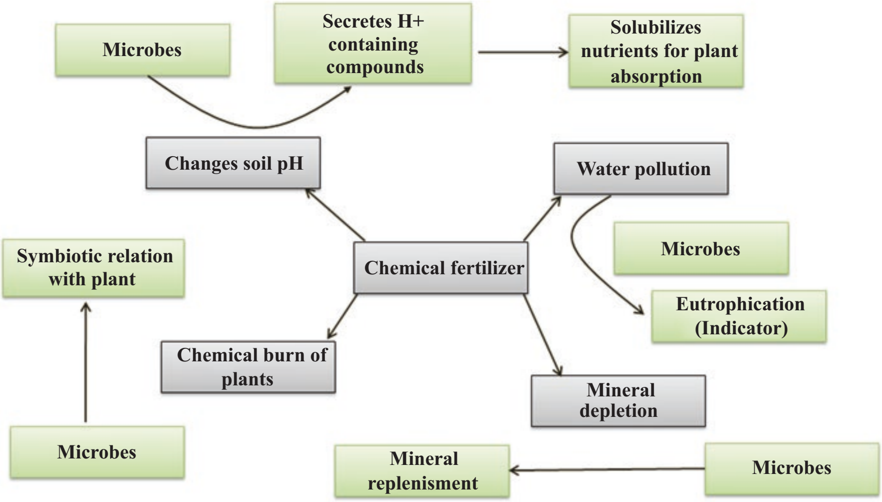

Fig. 2.1 Comparative study of chemical fertilizer and biofertilizer. Chemical fertilizer has adverse effects on soil texture. Biofertilizer meets the entire requirement without disturbing the nature of the soil. Microbes lead to mineral replenishment and increase the bioavailability of unsolubilized minerals

Microbes show symbiotic relation with the plants and may be present as epiphytes or as endophytes. Plant roots create a good rhizosphere by secreting ion and mucilage rich exudates which play an important role in plant microbe interaction Plants are specific for the bacterial colony. This specificity is regulated by the chemical composition of the exudates secreted by roots. Plants root exudates lead to physiological changes in plants that allow the growth of beneficial microbes in its rhizosphere. Microbes support plants by secreting the growth promoting hormones like auxins and cytokinins. Many of the microbes improve the yield by enhancing nutrient uptake, controlling infection; make the plant resistant to biotic and abiotic stresses.

2.2 Plant-Associated Microbiome

The diversity of soil microbes are the most important components of the different factors needed for enhancement of crop production. Among all the soil microbiome, bacteria, fungi, and archaea are the spotlights of the microbiology research. About one million taxa among the 10^9 microbes per g of soil have been documented. The soil is inhabited with millions of types of culturable as well as unculturable microbes (Blackwood et al. 2006). The population of microbes from the rhizosphere flee from the immune responses of the plant and make their way into the plant tissues and reside there as endophytes (Mercado-Blanco and Prieto 2012). These endophytes prove beneficial for the plant in a variety of ways like they assist the plant in defense against pathogens and produce secondary metabolites (Hallmann 2001). Metagenomics of the rhizosphere soil reveals a bizarre of microbes, which reside in the soil around the plant. Also, the molecular analysis of the endophytes exposes the variety of the microbes which reside inside the plants.

The endophyte colonizing the plant tissues includes mycorrhizal fungi, rhizobia, some pathogens and these assist in the absorption of nutrients from soil or atmosphere. These microbes prove to be a boon for plants as they perform a lot of functions that assist the plants eg. they act as decomposers of organic substances, also promote plant growth with the help of rhizobacteria which are designated as plant growth promoting rhizobacteria and are also witnessed to act as antagonists to plant pathogens (Beneduzi et al. 2012). They also help in nutrient cycling by fixing atmospheric nitrogen, mobilization of the phosphate present in the rhizosphere so that these nutrients can be utilized by the plants to the maximum (Sharma et al. 2013).

The nitrogen-fixing bacteria are the versatile group of bacteria which are also designated as ‘rhizobia’. They are able to fix nitrogen in legume plants by the residing inside the root nodules of the plants in a symbiotic relationship. The microorganisms belonging to the actinomycetes of the genus *Frankia* of the family Frankiaceae form N_2-fixing nodules on the root (Santi et al. 2013). None of the eukaryotic enzymes can split the triple bond of N_2. Thus, the prokaryotic species are exploited for the reduction of N_2 to NH_3 for nitrogen fixation and this reaction is catalyzed by the enzyme nitrogenase present in the microbes residing in root nodules of plants.

The symbiotic arbuscular mycorrhizal fungi are known to establish mycorrhizal associations with the roots of most angiosperms (Igiehon and Babalola 2017). The formation of arbuscular mycorrhizal formation is a kind of adaptation, in which the plants on association with the arbuscular mycorrhiza are endowed with increased power for nutrient absorption. The additional functions that arbuscular mycorrhiza endows plant include resistance to stresses like pathogen assault, drought, salinity, heavy metals, organic pollutants. It also aids in the development of good soil constitution by aggregate formation (Jeffries et al. 2003). Thus, we can say that arbuscular mycorrhiza has a key role to play in agricultural advancement (Jeffries and Barea 2012). Basically, we harness the microbial benefits by two different approaches, either we apply a different combination of the microbial inoculants or we manipulate the natural microbial flora and fauna to enhance agriculture.

Pseudomonas species is designated as protective bacteria which repress root-fungus disease (Gaffney et al. 1994). An antibiotic produced by *Streptomyces diastatochromogenes* restrains the infection of potato scab disease-causing bacteria (Sarwar et al. 2018). The novel bacteria *Bacillus thuringiensis* protects the plants from fungus gnat. The spores of *B. thuringiensis,* when consumed by fungus gnat larvae, germinate in the gut of the insect and produce the crystal protein known as cry protein. These bacterial inoculants are now marketed in the pelleted form (Kergunteuil et al. 2016).

2.3 Utilization of the Soil Microbiome by Proper Management Techniques

The theoretical and experimental models are very minutely studied and different strategies are designed in order to get the maximum output. One of the wonderful strategies chalked out was to maneuver the exudation produced by root so that we could select the beneficial combination of the microbes for the plant growth and enhancement (Berendsen et al. 2012). A method of plant-microbe co-adaptation is actually followed where the existing pathogens activities are controlled to get the benefit out of it. When a plant is luxuriously thriving in an environment, it develops various relations with various microbes to produce a very rich soil environment so that it happily grows there, but once the same plant is transferred to another environment, it needs to struggle in the new habitat. If we manipulate the new habitat with the help of microbiome present in the previous habitat soil, the plant does not need to fight adverse situation and will thrive better.

The plant or the crop will bracket together with an un-adapted microbe environment, which does not restrict pathogen and thus will become vulnerable to different kinds of diseases. Thus, manipulating the plant environment to attract favorable microbes in its rhizosphere for introducing specific functions like nitrogen fixation, P-mobilization, biocontrol of diseases would prove beneficial for further agricultural developments (Lareen et al. 2016). The main endeavor is to renovate a nominal rhizobiome which will be capable of providing maximum advantage to crops at minimum photosynthetic expenditure (Pérez Jaramillo et al. 2016).

2.4 Bacterial Mechanisms of Plant Growth Promotion

The role of prebiotics is very nicely studied in the context of the microbes in the soil which act as food for them. When we provide prebiotics, it acts as precursors for rooting compounds and allows the bacteria to assimilate the biochemicals and change them into natural rooting compounds which are instrumental in stimulating the growth of the plant. Different types of microbes have different strategies and pathways for processing their foods. Some of the bacteria have pathways exclusively for releasing Plant Growth Promoting biochemicals like auxin-based compounds that are instrumental in inducing and promoting root growth (Costacurta and Vanderleyden 1995). When bacteria combination is provided with appropriate food and nutrition as well as soil extracts, a specialized microbiome can be created which promotes plant growth as well as profuse root development. The following microbial functions can be tapped for the enhancement of agriculture:

1. Bioavailability of the recalcitrant nutrients to the plants by the help of beneficial bacteria to convert them into easily accessible forms (Igiehon and Babalola 2018).

2. Beneficial bacteria for plants produce chemicals and hormones that stimulate growth (Olanrewaju et al. 2017).
3. Beneficial bacteria help prevent infections from pathogens by coating the root surfaces and triggering systemic disease resistance (Kant et al. 2015).
4. Beneficial bacteria for plants help filter out heavy metals and other contaminants from the soil (Khan et al. 2018).
5. The beneficial bacteria act as fertilizer once they die, by releasing helpful nutrients that are absorbed by the plant's roots (Jacoby et al. 2017).

2.5 Rhizosphere Contribution to Crop Yields

The soil is a very versatile habitat for the microbes. The microbes present in the soil can be tapped for the increased production of crops (Andrén et al. 1999). Microbes have a bizarre of applications in the soil and are found to be associated with plants in a variety of ways (Jacoby et al. 2017). They may be present as epiphytes as well as endophytes. The Plant roots prove to build a good rhizosphere. Rhizosphere is the region of soil which is enriched with the exudates produced by roots which actually attracts and communicates with the microbes. Thus, the rhizosphere forms the home of diverse microbes. The microbes present in the rhizosphere prove to improve the crop yield by nutrient uptake enhancement, combating infection and producing resistance in the plant against different types of stresses (Hartmann et al. 2008). Thus, the microbiome present in the plant rhizosphere plays a key role to shoot up crop productivity. It has been witnessed that the microbes play an important role in the biogeochemical cycles and provide the plants, a healthy and improved life.

2.5.1 Exopolysaccharides to Promote Soil Aggregation

Exopolysaccharide is a high molecular weight carbohydrate which is instrumental in promoting soil aggregation. Aggregated soil also provides a high concentration of nutrients and favors proper growth of the plant. Bacteria and fungi both have the property to secrete polysaccharides which adheres the soil particles together. Additionally, extra-radical mycelium of fungi can also enforce the soil particles to come together (Fig. 2.2). Exopolysaccharide secreting microorganisms aggregate soil even in stress condition. It has been seen that exopolysaccharide secretion increases during the stress condition. Increased concentration brings about thermal and osmotic regulation, essential for the survival of bacteria. In 2000, an experiment was conducted on sunflower (*Helianthus annuus* L.) using a strain YAS34 as an inoculum under water stress condition. The bacterial strain not only brought aggregation of soil rather also increased the yield. The inoculum increases the uptake of nitrogen from the soil in the stress condition (Alami et al. 2000).

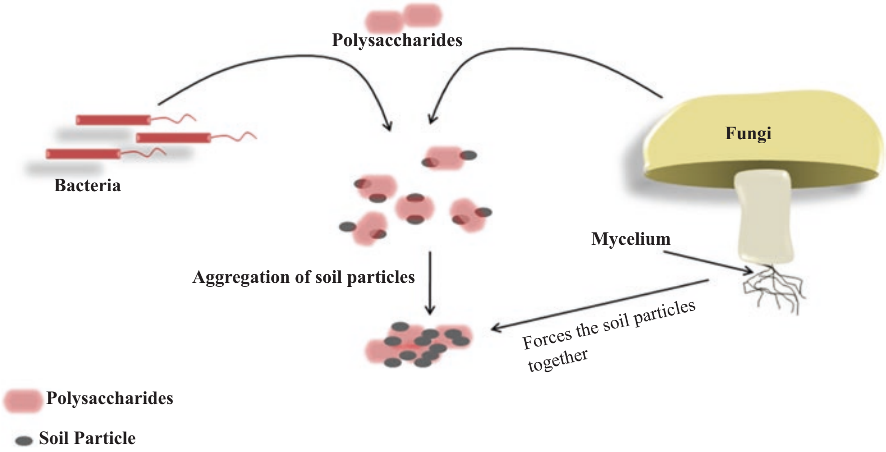

Fig. 2.2 Bacteria and fungi secrete polysaccharides. Polysaccharides adheres the soil particles together. Fungi force the soil particles together by their extra-radical mycelium

A similar study in Chickpea under high salinity condition proved the significance of exopolysaccharide in improving the yield. Under high salinity condition, chickpea was inoculated with salt-tolerant strains *Halomonas variabilis* HT1 and *Planococcus rifietoensis* RT4. Both the strain increased the secretion of exopolysaccharide which helps them to adapt under the stress condition. The increased secretion of exopolysaccharide enhances the aggregation rate of soil (Qurashi and Sabri 2012).

2.5.2 Improving Soil Microbes for Stress Alleviation in Crops

The agricultural scenario always faces a plethora of biotic as well as abiotic stress conditions like stress due to excess salinity, drought, nutrient deficiency, infection, pathogenic diseases and many more. Rhizosphere microbiome benefit plants in many ways. First and foremost, they help the plants to counter the biotic and abiotic stresses (Table 2.1) and boost the productivity of plant as well as their health (Bowen and Rovira 1999). One should have a sound knowledge of the plant-microbe interactions so as to exploit the microbial benefit to the maximum. Researchers have nicely exploited the interactions between *Arabidopsis thaliana* and its rhizobiome (Lundberg et al. 2012). The root exudates initiate and enhance the interactions among the microbiome. The Earth Microbiome Project investigations and results provide us with 2290 plant-associated and 4279 soil microbe data from various collection sites (Gilbert et al. 2014) which overcome the stress.

Studies reveal that the soil properties and the type of land-use greatly influence the soil microbiomes (Dignam et al. 2018). These microbes may be either saprophytic or symbiotic like rhizobia which form nodules as well as like the arbuscular

Table 2.1 Soil microbes for various stress alleviation in the host plants

Abiotic stress	Host plants	Microbes involved
Cold stress	Maize plants	*Pseudomonas* sp. DSMZ 13134, *B. amyloliquefaciens* subsp. *plantarum*, *Bacillus* strains
	Tomato	*Arthrobacter*, *Flavobacterium*, *Flavimonas*, *Pedobacter,* and *Pseudomonas*
	Wheat (*Triticum aestivum* L).	*Methylobacterium phyllosphaerae* strain IARI-HHS2–67
	Methylobacterium phyllosphaerae strain IARI-HHS2–67	*Burkholderia phytofirmans*
Heat stress	Wheat (*Triticum* spp.)	*Pseudomonas putida* strain AKMP7
	Sorghum	*Pseudomonas* sp. strain AKM-P6
Salinity	*Zea mays*	*Rhizobium*
	Tomato	*Pseudomonas fluorescens*, *P. aeruginosa*, and *P. stutzeri*
	Rice	*Pseudomonas pseudoalcaligenes*
	Wheat	*Dietzia natronolimnaea*
	Lens esculenta Var. Masoor-93	*Staphylococcus saprophyticus* ST1 and *Oceanobacillus profundus* Pmt2
Water stress resistance	Tomato	*Achromobacter piechaudii* ARV8
	Green gram	*P. fluorescens*

mycorrhizal fungi. Depending upon the host genotype and physiology (e.g., root exudates and metabolites) as well as environmental factors, the microbiota varies (Lundberg et al. 2012). Bacteria like *Azotobacter chroococcum* or *Bacillus megaterium* have been used for the first time in the 1950s to improve growth as well as yield of the crops (Brown 1974). Rhizobia not only fix the soil nitrogen rather it grabs atmospheric nitrogen also (Teintze et al. 1981).

The scientists and researchers are constantly involved in the discovery of ways and means to combat these stresses. To the utter astonishment of the scientists a very interesting fact was discovered that when the plant undergoes certain kind of stress, the interaction of the plant crop and the associated microbiota is altered in the rhizosphere. The beautiful fact is that the alteration of the microbial population due to the stresses is for benefit of the plant species. It has been shown that the environmental stresses on plants induce the production and activation of phytohormones (Rejeb et al. 2014). These phytohormones actually induce better plant growth as well as manipulation and alteration of soil microbes which is beneficial for the plant in all aspects.

A deep insight into the mechanism of the interaction of the phytohormones with plant and the microbiome can provide us with the advantages and disadvantages of the process. This understanding can equip us with the strategy to manipulate the environmental conditions and the microbiome so that it becomes beneficial for the plant. We can design biotechnological strategies with the help of the knowledge so that plant adaptation mechanisms can be optimized and the microbes involved in stress alleviation in crops can be utilized. Plant stress like ‘drought’ is considered as one of the most important matter of concern on agricultural output. The food security is at stake due to unfavorable alterations in the climate. Major research is being targeted on emerging novel strategies to produce crops which are more pliable so that they can endure any kind and any degree of stress. Nowadays root-associated microbiomes with chemical induction are being used which confers plant tolerance to abiotic stresses like drought.

The role of arbuscular mycorrhizal fungi and chemical agents to develop plant tolerance to drought has been highly investigated and still, it is in its adolescent stage (Khan et al. 2018). It was concluded that with an increase in nitrate and potassium ion fluxes in *Pinus pinaster,* the roots were inhabited by the ectomycorrhizal fungus *Rhizopogon roseolus* (Gobert and Plassard 2007). mRNA of several nitrate, phosphate and manganese transporter genes in *Medicago truncatula* roots colonized by *Glamos intraradices* and *Glamos mosseae* were found abundant when the plant was subjected to drought (Gomez et al. 2009). It was demonstrated that arbuscular mycorrhiza symbiosis modulates the root hydraulic properties and thus increases tolerance to drought, cold and salinity stresses (Sharipova et al. 2016). The regulation of PIP2 protein amount and phosphorylation state actually influences the root hydraulic properties (Li et al. 2011). It is possible that each *PIP* gene would be having different function and regulated by arbuscular mycorrhiza symbiosis in stress condition. Investigations have confirmed that the arbuscular mycorrhiza symbiosis can shield host plants against the unfavorable effects of drought stress (Ruiz-Sánchez et al. 2010; Volpe et al. 2018). The microbes of certain plants which help in combating stress are enumerated in Table 2.1. These microbes enhance the crop yield by surpassing abiotic stress.

The beneficial plant-microbe interactions have been researched and explored to define strategies to enhance agricultural yields. The rhizobia have been exploited for their biological nitrogen fixation capability, which launches a symbiotic relation with legumes and crop which contribute to soil fertility (Zahran 1999; Ahemad and Kibret 2014). Internal colonization of the plants by arbuscular mycorrhizal fungi with the help of arbuscular and vesicular part of the hyphae aids in the plant nutrition accomplishment (Berruti et al. 2016). Another more specific symbiotic defensive mutualism between Pooideae grasses and endophytic fungi of the *Epichloe* have been investigated to reveal its importance in the enhancement of crop production (Clay 1988). When some specific microbes are inoculated, we can foretell which plant microbiome interaction will be beneficial and necessary for the enhancement of crop production by just investigating the effects observed.

The composition of the phytomicrobiome is influenced by a variety of factors like the plant genotype, type of pesticide and fertilizers application. In order to enhance the diversity of microbiome constituents, higher plant diversity is used in agriculture like crop rotation technique with the legumes. Other strategies to increase agricultural productivity are the use of different soil combination, fertilizers and pesticides, which will not produce any adverse effects in the population of the microbiome. Critical dosage has a great effect on the microbiome activities as overdosing may produce adverse effects on the microbial population in the soil milieu. In the present scenario when different strategies are being employed for the increased production of the crops, the industries are commercializing the soil microbiome by permutation and combination of the microbes to produce the best combination to enhance the production by natural means (Lupatini et al. 2017). There is a vital requirement to develop processes and application techniques to better recognize the interaction between plants and microorganisms in the soil ecosystem.

2.5.3 Enhancing Nutrient Uptake by Plant Using the Microbiome

Bacteria, as well as fungus, contribute to the bioavailability of nutrients to the plants. The increment of bioavailability is through nitrogen fixation and mobilization of key nutrient (Nitrogen, Phosphorous and Iron) (Rashid et al. 2016). Chemical fertilizer has been formulated to increase the yield. Chemicals affect the soil physiology as well as the environment, so biofertilizers are always preferred. Symbiotic relation of microbes with plants not only avail the nutrients to the plants but also reinstate the fertility of degraded soil. Nitrogen is the major constituent of biomolecules such as protein and nucleic acid. All organism including plants requires nitrogen. Nitrogen present in the environment but it cannot be used directly by the plants. They need to be fixed with the help of microbes. Bacteria directly involve in nitrogen fixation while fungi provide a positive environment to the bacteria. Bacteria shelter inside mycelium and remain protected from oxygen. Bacteria has *nif* gene which helps in the fixation of atmospheric nitrogen to ammonia (Fischer 1994). Table 2.2 shows the list of bacteria involved in nitrogen fixation. Nitrogen not only as a major constituent of biomolecules but itself also regulates the biosynthesis of phytohormones. Phytohormones are regulated through nitrogen signaling. This states that plant growth and development are regulated by nutrients availability.

Not only nitrogen but P, K and Fe are also solubilized by bacterial as well as fungal activity. Microbes extrude proton and some lower molecular weight organic ions like succinic, citric, gluconic, oxalic acid and α-keto-gluconic acid which lowers soil pH (Marra et al. 2015). With the lowering of pH, the extruded ions got exchanged with P by the process of ligand exchange. Fungi solubilize phosphorous by siderophores. A similar release of an organic acid by bacteria solubilizes potassium by the process of acidolysis, chelation and ligand exchange. Fungi solubilize potassium through mycelia transport of citrate, malate and oxalate. Phosphorus like nitrogen is the structural component of nucleic acid along with phospholipids. Being part of nucleotide triphosphate, the phosphate bond store high energy in it. The soil is rich in the inorganic form of phosphorous like as $Ca_3(PO_4)_2$. Bacterial isolates belonging to genera *Enterobacter, Pantoea and Klebsiella* can solubilize $Ca_3(PO_4)_2$ (Delétoile et al. 2008). These genera of bacteria also show nitrogen-fixing ability.

Table 2.2 Bacterial microbiome for enhanced nitrogen mineralization

S. No.	Bacteria	Host Plant	Reference
1	*Gluconacetobacter*	Sugarcane	Bertalan et al. (2009)
2	*Herbaspirillum*	Rice	Elbeltagy et al. (2001)
3	*Bradyrhizobium, Mesorhizobium, Rhizobium*	*Acacia*	Teixeira and Rodríguez-Echeverría (2016)
4	*Micromonospora, Rhizobium meliloti*	Alfa-alfa	Martínez-Hidalgo et al. (2015)
5	*Rhizobium phaseoli* and *Solanum americanum*	Common bean seed	Mora et al. (2014)
6	*Burkholderia*	Chickpea	Abi-Ghanem et al. (2012) and Wadhwa et al. (2017)
7	*Bradyrhizobium lupini*	Lupin	Schulze et al. (2006)
8	*Allorhizobium, Azorhizobium, Bradyrhizobium Mesorhizobium, rhizobium and Sinorhizobium*	Mesquite	Sprent et al. (2013)
9	*Rhizoctonia solani* and *Fusarium oxysporum*	Lentil	El-Hersh et al. (2011)

Fe is an essential micronutrient which is involved in all the major physiological process in plants. It is also involved in chloroplasts synthesis. On earth, it is predominantly present in an insoluble form ferric (Fe^{3+}). Bacteria convert it into soluble form through the production of low molecular weight protein siderophore. During anaerobic or flooded condition, it is reduced to Fe^{2+} which is the toxic form of iron. This toxicity is controlled by bacterial inoculation like neutrophilic lithotrophs. Such bacteria utilize Fe^{2+} as an electron donor for their metabolism (Nguyen et al. 2015). Microbes which utilizeFe^{2+} from biotite excretes some chemicals like oxalic and tartaric acid. The release of such acid mobilizes potassium from minerals like biotite.

The microbes like bacteria as well as fungus are instrumental in extending their helping hand in making the nutrients bioavailable to the plants. The term bioavailability means the degree and rate at which the nutrients enter the plant system from the soil or atmosphere. Though the nutrients like nitrogen, phosphorous and iron may be present in the milieu, still it is not 100% available to the plant for various reasons. The reasons may pertain to solubility, complex formation, etc. Now if we employ certain strategies in order to solubilise these nutrients so that it is available for the plants to absorb them actually adds to the bioavailability. The recent and magnificent technology that is the best employed is the use of manipulated microbiome.

Though the different chemical fertilizers have been formulated to increase the yield of different types of crops yet we always try to employ certain natural phenomenon so that the extensive chemicals used in the formulation do not add to the soil pollution as well as poison the resources. The biofertilizers have always been a better option over the chemical fertilizers. Vermicompost is one of the biofertilizers which has been applied to the field to increase production. This method successfully uses earthworms to produce natural manures but its high maintenance cost has forced the farmers to prefer the orchestration of the microbiome to enhance the agriculture. This method exploits soil microbes to solubilize soil nutrients and thus facilitates increased nutrient absorption by the plants. The ubiquitous bacteria and fungi not only help the plants to utilize the nutrients but also replenish the fertility of degraded soil. Bacteria and fungi help each other in order to profit the plant crops. The fungi create a positive environment for the bacteria involved in the nitrogen fixation. The fungi protect the bacteria from oxidation by providing them refuge in its mycelium.

Organic ions like succinic, citric, gluconic, oxalic acid and α-keto-gluconic acid as well as protons are released in the soil which makes the soil acidic. This helps in solubilization of phosphorus. The siderophores produced by the bacteria and fungi are used in the phosphorus as well as potassium solubilization. Nitrogen-fixing symbiotic bacteria enhance the absorbance of micro and macronutrients for plant uptake and also lower the salt and water stress. Both the symbiotic as well as free-living bacteria augment the growth of plant crops by Nitrogen fixation. These special microbes are also instrumental in producing antimicrobials and phytohormones like produce auxins, cytokinins and gibberellins so as to endow the plant crops with the property of resistance to diseases. The endophytes like fungi extrude mucilaginous extracellular polysaccharides which are chiefly accountable for the complex aggregates, which actually increases soil porosity and aeration. This mechanism of soil aggregation is shown in Fig. 2.2. Some of the microbes like *Bradyrhizobium japonicum* and *Nocardioides sp*. are involved in the oxidation of the mica enriched with Fe^{2+} (Masuda et al. 2017). Increased production of citric acid, tartaric, oxalic, succinic acid, and keto-gluconic acid can be achieved by inoculation of *B. edaphicus* NBT strains (Hu et al. 2006).

2.5.4 Microbiome for Efficient Nitrogen Fixation

Nitrogen is one of the major limiting elements for crop growth. Plants cannot utilize the free atmospheric nitrogen, so the nitrogen has to be fixed. **Nitrogen fixation** can be defined as any natural or industrial procedure that causes free atmospheric nitrogen (N_2), to chemically combine with other elements to form more-reactive nitrogen compounds which can be very well utilized by the plants. For time immemorial, the farmers have been using a profuse number of chemical fertilizers to promote crop production. This is the case with the developed nations that have surplus money to account for the cost of fertilizers, but on the other hand in the still progressing countries inorganic fertilizers are often not available and small-holder farmers suffer the resultant poor yields.

Natural and cost-effective alternatives have been probed in the countries where the use of microbiome present in the soil is exploited for nitrogen fixation. A lot of research has been performed in the area of nitrogen fixation by the microbes. The investigations revealed that bacteria and archaea have the capability to fix atmospheric nitrogen to ammonia, which can be readily utilized by the plants. Thus, the nitrogen uptake by the plants can be improved by either introducing nitrogen-fixing bacteria into the crops or adding nitrogenase enzyme to the crop soil environment for nitrogen fixation. The mechanism of nitrogen fixation is shown in Fig. 2.3. The scientists have come up with biotechnological solutions to solve the problem of nitrogen availability.

Plants, specifically legumes, are found to promote facilitate colonization of nitrogen-fixing rhizobacteria in their root nodules to create a suitable oxygen-limited environment for nitrogen fixation (Mus et al. 2016). As flavonoids are released, the rhizobial bacteria trigger the production of the Nod factor that starts the plant processes essential for the symbiotic association (Abdel-Lateif et al. 2012). Whereas legumes fix nitrogen by the technique of nitrogen-fixing symbiosis, the special association of mycorrhiza is omnipresent within the whole plant kingdom and not restricted to only legumes (Mus et al. 2016; Santi et al. 2013).

Companion planting is the method that was used by the Native American farmers for nitrogen fixation when the use of rhizobial inoculants was not known. In this technique, different plants were grown together to augment each other's health (Cunningham 1998). Hereby planting leguminous crops like corn, squash, and beans, the Americans residents utilized the ability of legumes to attract nitrogen-fixing bacteria. Nowadays harnessing the beneficial properties of more Plant Growth Promoting Bacteria such as *B. thuringiensis* are being put to use (Macdonald and Singh 2013).

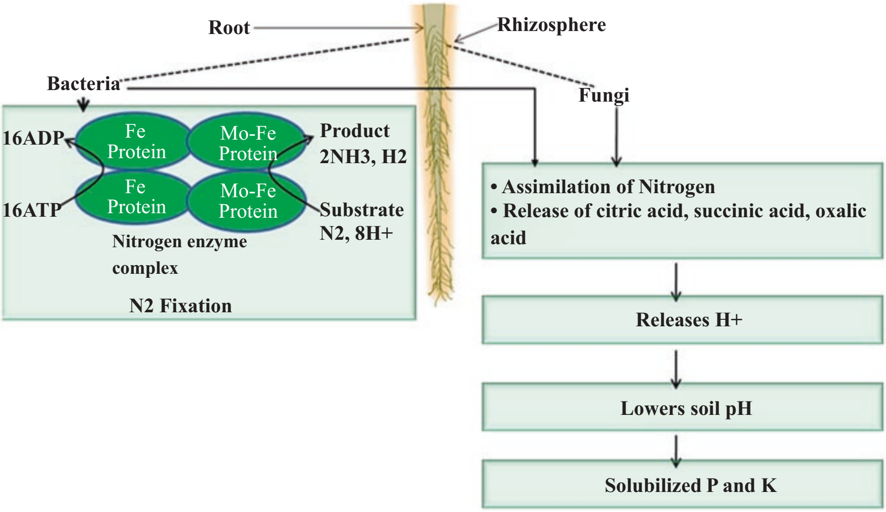

Fig. 2.3 Bioavailability of nutrients by bacteria and fungi. Bacteria have tetramer units of the nitrogen enzyme complex. This complex is involved in fixation of atmospheric nitrogen with the involvement of 16 molecules of ATP. Fungi are not directly involved in nitrogen fixation but it favors bacterial environment for nitrogen fixation. Both fungi and bacteria release certain acidic compounds in soil which solubilizes phosphorous and potassium to be consumed by plants

2.6 Plant–Microbiome Interactions to Improve Phytoremediation

Phytoremediation refers to a promising technology that utilizes synergistic interaction of living plants and microbe to cleanse the contaminated soils. There are a number of different types of phytoremediation techniques. One of them being rhizosphere biodegradation, in which, the plant roots secrete bio-chemicals and make nutrients available to the microbes. The microbes get attracted by the exudates and form aggregates in the rhizosphere. They in turn work for the biological biodegradation of the contaminants present in the soil.

The other is phytostabilization where the biochemicals produced by the plant roots just immobilize the contaminants and thus they are removed from the soil replenishing the fertility and freshness of the soil (Lone et al. 2008). The next to be mentioned is phytoaccumulation which also known as phytoextraction where the plant roots absorb the pollutants besides the nutrients and water. The contaminants accumulate in the plant parts rather than being destroyed. This process is more applicable for wastes containing metals. One common example is plants capable of absorbing water-soluble metals like ‘lead’. The aerial shoots of these plants which store the metals like lead are then exploited to recover the metal by the process of smelting (Sheoran et al. 2011). Phyto-volatilization needs a special mention because here the plants absorb the aqueous organic pollutants and then release them into the atmosphere in gaseous form through their leaves. The phyto-degradation process employs plants to metabolize and demolish the pollutants in the tissues of the plant itself.

The phytomicrobiome like bacteria and Arbuscular Mycorrhizal fungi are exploited for their capabilities of phytoremediation of the soils profoundly contaminated with radionuclides, xenobiotics and heavy metals. The associated microbes actually use different types of mechanisms to get rid of the pollutants. These mechanisms comprise of a number of functions like plant growth enhancement, nutrient supply, assembly of Fe-binding siderophores, plant hormones production, ACC-deaminase activity enhancement, etc. Bio-augmentation of plant-associated microbiome has also been exploited for phytoremediation. Bioaugmentation is a technique where a pregrown microbial culture is added to boost microbial populations which aid in speeding up the removal of contaminants saving time and cost.

The Arbuscular Mycorrhizal fungi have different strategies to check the toxic metal ions to enter into the intracellular environment (Millar and Bennett 2016). This is done through the process of metal immobilization, extracellular metal sequestration as well as the chelation of metallic ions. They are also equipped with strategies to resist the oxidative stress produced by heavy metals. Heavy metals can be permanently confiscated by Glomalin-related soil proteins which are produced by Arbuscular Mycorrhizal fungi (Yang et al. 2017), leading to the stabilization of heavy metals. This property of heavy metals stabilization or removal is shown by Arbuscular Mycorrhizal fungi due to the virtue of heavy metals transporters present in them. Heavy metal-adapted rhizobacteria and Arbuscular Mycorrhizal fungi interact with each other and due to co-inoculation resulted in the accumulation of huge amounts of heavy metals and also enhanced plant growth, growth hormone production, and enzymatic behavior. One more strategy to remediate heavy metal contaminated soils was to inoculate Arbuscular Mycorrhizal fungi with plant growth-promoting rhizobacteria, in the agro waste residue which resulted in altered bacterial population and improved phytoextraction. Once we focus on improving the condition of the soil, bio-availability and easy access of contaminants, exploitation of soil and plant-associated microbes for better plant growth, we can achieve the best model for phytoremediation.

2.7 Disease-Induced Assemblage of Plant-Beneficial Bacterial Consortia

After the epidemic of a disease, it has been witnessed that a community of disease protective microbiome is developed which proves to be resistant to the disease in the rhizosphere. This can be considered as the development or establishment of the immune system of the plant. Stimulation of the host plant immune system by the protective rhizosphere microbes, present in rhizosphere produces the induced systemic resistance (Yuan et al. 2018). It has been demonstrated that three bacterial species colonized the rhizosphere post infection of downy mildew pathogen *Hyaloperonospora arabidopsidis* on *Arabidopsis thaliana* (Cabral et al. 2011). It was found that all the three bacteria in synergism with each other were able to form biofilm *in vitro* and resulted in resistance against downy mildew and promoted growth of the plant. From the above finding, it was concluded that the plants can regulate the type microbial community in the soil of the root region. This regulated growth of some specific microbiota confers disease resistance and promote plant growth and develops an environment in the plant crops potentiating the plants to grow luxuriously without the fear of some dreaded disease. The negative soil-feedback technique involves the interplay of the pathogens, promoting the plant biodiversity which is now equipped with the property of disease resistance and thus a better variety of plant is obtained.

When crops like wheat and sugar beet are continuously cultivated in an area, it can induce soil suppressiveness to disease, which is caused by special microbiota that can inhibit the growth as well as the activity of the pathogens present in the soil (Mazzola 2002). This observable fact is due to the manufacture of antimicrobials produced by the existing microbes that specifically inhibit the growth of the pathogen. The phytohormones salicylic acid and jasmonic acid are important modulators of the rhizosphere microbiome assembly of *Arabidopsis thaliana* (Kniskern et al. 2007). Jasmonic Acid is normally effective against necrotrophic pathogens while Salicylic Acid is effective against pathogens which prove to be biotrophic. Both the phytohormones are seen to be produced in response to infection by certain pathogens, so they actually influence the diversity of the microbial population.

2.8 Microbes to Enhance Root Growth

A very healthy as well as luxuriant root system confers plant a strong and healthy living. A good root mass is a requirement for a high agricultural yield. Either it is for breeding or making a clone, a root mass on seedlings is large is always desired. The question is that why do need such a profusely growing root. We need them for proper uptake of nutrients, prevention of diseases and disease resistance. There are various biotic and abiotic factors which regulate growth in plants. Phytohormones play a vital role in the regulation of growth. Auxin, cytokinin, ethylene and gibberellins control the plant growth positively while abscisic acid controls cell growth.

Apart from the plant, few of the microbes are also involved in the biosynthesis of phytohormones. More than 80% of bacteria present in rhizosphere show the production of auxin (Spaepen and Vanderleyden 2011). *Agrobacterium spp., Pseudomonas spp., Azospirillum spp.* are well-known bacteria involved in auxin biosynthesis (Glick 2012; Somers et al. 2005). *Agrobacterium* has Indole Acetic Acid gene in its T-DNA. It controls both the steps of IAA synthesis (Zhao 2010). One is the conversion of tryptophan to IAM while another is hydrolysis of IAM to IAA. Tryptophan independent pathway is also there for auxin synthesis (Woodward and Bartel 2005). Though there is no solid evidence of the presence of such genes in plants but in *A. brasilense, a* tryptophan independent pathway has been observed (Prinsen et al. 1993).

Though synthetic hormones have been used for this purpose time immemorial but the after effects and the high cost involved cannot be denied. Synthetic rooting products have also been used as regulators of plant growth to induce rooting in plants. But these products when probed into were found to be potent carcinogens. So, the hunt for a natural product was the main aim of agriculturists. After much of investigation, the researchers have found that the microbiome of the soil surrounding the plants can be very well used for inducing roots in the plants. The interplay of the microbes present in the soil can be studied and the mechanism of root induction can be tapped for inducing roots in the plants so that the plants are equipped with better nutrition absorption capacity and better grip in the soil.

Cultured Biologix, LLC, Colorado, United States, has come up with rooting products those utilize beneficial microbes to augment luxuriant growth of roots. The mechanism that they have adopted may be by shielding the plant from pathogens or extruding natural biochemicals which is instrumental as rooting agents. These produce signals that encourage the plant to generate larger and faster roots. This product is then paired with particular plant extracts obtained from soybeans and Aloe vera which are organic in nature and produce stimulants which signal for profuse rooting.

A specific product of Cultured Biologix, Dr. Root is a redimix of mycorrhizae and bacteria which cooperate with each other to produce endomycorrhizae and promote healthy root growth. In this magnificent product, spores of the bacteria and mycorrhizae are combined with a growth-inducing biochemical present in the cottonseed meal to provide enhanced sticking and coverage of the root system. It is also enriched with seaweed and humic acids which accounts for stimulation of microbial growth, and extracts of Aloe vera which is a rich source of organic compounds (Goudarzi et al. 2015). Voodoo Juice is considered to be ultra-premium, a patented combination of beneficial bacteria demonstrated to enhance root mass and crop yield. The bacterial combination in Voodoo Juice is shown in Table 2.3. The composition is used as biofertilizer.

The four different strains of bacteria in the Voodoo Juice work in synergism so as to develop a fine cannabis root hairs system that enhance the absorption of water and nutrients, inhabit the vascular systems of the plants, boosting nutrient transport into and within the crop, manufacture plant growth factors that enhance growth, solubilize nutrients and phosphates so that they become easily available for the plants. The best part of this juice is that it can be applied at all the year round in the life cycle of the plant. It works best during the first 2 weeks of both grow and bloom phases. The advantages of using Voodoo juice are immense like maximal-development of roots on the seedlings, transplants, and clones, roots have enhanced branching, root density, root mass, increased efficiency of nutrient intake which saves time and money and maximize growth and flowering. The favorable microbes break down old root material to provide nutrition to the plants. The microbes provide aeration so as to make way for the extra oxygen into the roots. They also sequester the atmospheric nitrogen and help make phosphorus more accessible to the plants (Gougoulias et al. 2014).

Tarantula, a product of Company called Advanced Nutrients comprises of a combination of about 11 strains of advantageous bacteria that boost lateral root development, produce natural growth cofactors and defend against harmful bacteria in the root zone. When Tarantula and Voodoo Juice are combined together, they provide a multifaceted variety of advantageous bacteria that maintain more vigorous root development increasing the transport of necessary nutrients into the plants and help fight plant disease caused by harmful pathogens and destructive bacteria strains. Some of the commercial bacterial mix for plant growth enhancement is given in Table 2.4.

Table 2.3 Synthetic fertilizer (Voodoo juice) containing bacterial colonies

Voodoo juice: Bacterial composition	Colony-forming unit/ml
Bacillus subtilis	10,000,000 cfu/ml
Bacillus megaterium	5,000,000 cfu/ml
Bacillus amyloliquefaciens	250,000 cfu/ml
Bacillus pumilus	5,000,000 cfu/ml
Bacillus licheniformis	10,000,000 cfu/ml
Bacillus mycoides	50,000 cfu/ml
Paenibacillus azotofixans	100,000 cfu/ml
Bacillus laterosporus	250,000 cfu/ml
Bacillus macerans	25,000 cfu/ml
Bacillus polymyxa	5,000,000 cfu/ml
Bacillus cereus	250,000 cfu/ml
Paenibacillus polymyxa	500,000 cfu/ml

Table 2.4 Commercially available advanced fertilizers and their function

Advanced fertilizers	Function
Tarantula	1. Shorter stems, more flowers, and have increased branching in plants
Voodoo juice	1. Maximum-development of roots on seedlings, transplants and clones with roots have enhanced branching, root density, root mass. 2. Enhanced efficiency of nutrient intake, saving time and money. 3. As well as maximize growth and flowering.
Nirvana	Stimulate root function, cell replication, and flower.
Piranha (fungi based)	1. Beneficial fungi maximize root absorption of oxygen, nutrients and water, also maximizes root growth for optimum yields. 2. Helps plant roots gain surface mass as well as maximizes production of floral essential oils.
Sensizyme organic	1. Strengthens the roots of the crops and keeps them clean, white and bright boosting the amount of bioavailable nutrition in your root zone, giving them more fuel to reach their full genetic potential as well as provides a broad spectrum of enzymes to more closely replicate the most fertile natural soil conditions.

2.9 Metabolic Potential of Endophytic Bacteria

Endophytic bacteria are directly involved in enhancing the metabolic potential of plants. However, endophytic bacteria also indirectly assist in the metabolism in plants. The bacterial endophytes robustly affect the performance, growth and stress tolerance of plants. *Actinobacterium*, *Pseudonocardia sp*. strain YIM 63111 are few endophytic bacteria which enhance the production of the anti-malarial compound artemisinin in its host plant *Artemisia annua* (Li et al. 2012). The endophytes induce secondary metabolite production in aromatic and medicinal plants. The endophytes sometimes not only enhance the secondary metabolite production but produced them along with the plants in combination.

One of the examples may be quoted where the flavor of strawberries is due to the furanoids which produce a typical fragrance. This unique feature is due to the plant-associated methylobacteria which actually influence the quality and quantity of the flavor (Verginer et al. 2010). The bilateral biosynthesis of the polyamine pavettamine of South African Rubiaceae in association with nodulating plants is one of the wonderful features (Brader et al. 2014). Nodulating plants devoid of bacteria do not produce pavettamine. The mangrove tree Kandelia *candel* having endophytic *Streptomyces* sp. HKI0595, produce multicyclic indolosesquiterpenes (Xu et al. 2014) while the endophytic actinomycete *Streptosporangium oxazolinicum* K07-0450^T associated with orchids produce antitrypanosomal alkaloids spoxazomicins A-C. The metabolites produced by the plant which associated with bacteria in roots and the rhizosphere is generally in low quantity. Future research in this area pertains to the enhancement of these metabolites. Direct analysis of metabolites in situ has been achieved for antibiotic lipopeptides from several *Bacillus subtilis* and for pyrrolnitrin, 2, 4-diacetylphloroglucinol and phenazine-1-carboxylic acid from *Pseudomonas fluorescens* strains (Thomashow et al. 1990).

2.10 Conclusion

The microbial population is one of the magnificent gifts from God which actually have a plethora of advantages for mankind. As there are two faces of the same coin these microbes may also be harmful as much, they prove to be beneficial. It is upon mankind as to how we want to utilize them. The microbes are adorned with so special features that on one side they can be used as bio-weapons and on the other hand they can serve as antibiotics to fight the different infections. Now we need to decide where we are going to use them. From the above review, we can well witness the bizarre of advantages the microbes bestow in the field of agriculture. Agriculture remains the backbone of primary sector and may guarantee nutritional security for the mankind. So, we need to expedite the technologies to enhance agriculture to get the maximum yield. There are numerous microorganisms which play a very crucial role in the life of plants in different ways. Each type of microbe is unique in its kind. Now if exploit these microbes in different permutation and combination we can really orchestrate them to produce maximum yield in the agricultural scenario.

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Chapter 3
Plant Growth Promoting Rhizobacteria to Mitigate Biotic and Abiotic Stress in Plants

Shikha Gupta and Sangeeta Pandey

Abstract Plant growth promoting rhizobacteria are microbes that promote plant growth and alleviate plant stress such as salinity, flooding, heavy metals, drought, cold, soil compaction, mechanical impedance, and nutrient deficiency. This chapter reviews mechanisms that alleviate plant stress using microorganisms. Mechanisms include the production of phytohormones such as auxin, of volatiles such as hydrogen cyanide and ammonia, of osmolytes such as proline and sugars, of exopolysaccharides, and the activation of antioxidant defence systems in plants. Under stress, the ethylene concentration increases in plants, leading to senescence and plant death. Here, plant growth promoting rhizobacteria favors the activity of 1-aminocyclopropane-1-carboxylate deaminase, which indirectly reduce ethylene concentration.

Keywords Induced systemic resistance · Rhizobacteria · Stress tolerance · Siderophore · Indole acetic acid · Phosphate solubilization · Salinity stress · Drought stress

3.1 Introduction

The population has been increasing at an accelerated rate, projected to reach ten billion in next coming 50 years. This rising population will simultaneously become a burden on existing, natural resources and increase global food requirements. Apart from, rising population and increasing food demands, climate variation due to global warming has also presented challenges for sustainable development of agriculture (Timmusk et al. 2017).

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The crop plants in the agricultural farm, encounter a plethora of environmental stress, both abiotic and biotic stress which directly or indirectly has drastically declined crop yield, soil health and fertility status by depleting rhizomicrobiome. Major abiotic stresses that affect the development of agriculture sector is drought, salinity, soil pH, soil sodification, desertification, waterlogging or flooding conditions, temperature fluctuations, nutrient deficiency, and heavy metal stress (Bray et al. 2000; Lawlor 2011). In addition to these stressed conditions, plants are also confronted to many pathogenic bacterial, viral, fungal and insect attacks, constituting biotic stressors, lead to many plant diseases. These diseases pose a major threat to growth and yield of field crops (Pandey et al. 2017). These constraining factors, encompassing both abiotic and biotic stress, adversely affect morphological, physiological, and biochemical parameters of the crop plant.

Moreover, in response to external stress environment plants generates reactive oxygen species, accumulates plant growth regulators such as stress ethylene, salt ions such as Na+, Cl-, reduce the chlorophyll content and photosynthesis. This further trigger secondary stress such as oxidative stress, nutrient deficiency, promote root curling, epinastic movements in leaves, ion and metal toxicity which collectively results in growth and biomass reduction and ultimately leads to plant death (Glick et al. 2007; Nadeem et al. 2014).

In order to obviate the negative impact of the physical environment stressors and plant-pathogen interaction on the crop yield, the farmers of especially low-income countries where agriculture is economically important occupation, rely on the intensive use of chemicals-based fertilizers, pesticides, insecticides which forms a major part of modern cultivation practices (Rahman and Zhang 2018). The indiscriminate and constant doses of synthetic agrochemicals can result in deterioration of soil sustainability (Good and Beatty 2011). Hence, it is necessary to develop new and sustainable agricultural practices with long term approach that could enhance crop yield without compromising with natural environment as well as human health.

3.2 Major Abiotic Stresses Limiting Agricultural Production

Under field conditions, crops plants exposed to unpredictable extreme fluctuations of climate, resultant of global warming and environment destructive activities of humans, collectively termed as abiotic stresses. Drought, salinity, flooding or waterlogging conditions, high and low temperatures and presence of metal contaminants constituted as some of the major abnormalities of physical environment that adversely affects crop growth and development, sometimes becomes fatal and thereof declines the agricultural yield (Lobell and Gourdji 2012).

3.2.1 Drought

Drought is a serious water deficit conditions occurred due to prolonged decline of water availability to plants and excessive loss of water (evapotranspiration). This results from indefinite period of inappropriate and erratic rainfall, loss of water holding capacity of soil as well as high temperature and carbon dioxide levels in the atmosphere (Wilhite 2000; Fahad et al. 2015). The foremost negative impact of drought stress on the plants is the impaired seed germination and seedling growth. The low moisture content in the soil will alters the root architecture and thus, affects the mobilization and uptake of various nutrients by plants for instance, nitrate, sulfate, calcium, magnesium, silicon. Therefore, reduction in the availability of nitrate (the primary nitrogen source of plants) will consequently lower the enzymatic activity of nitrate reductase and associated nitrate assimilation. Drought triggers stomatal closure to minimize leaf transcription and water loss, alters leaf water potential ultimately decrease the rate of photosynthesis, stomatal conductance and electron transport process resulting in significant crop loss (Caravaca et al. 2005).

Drought considerably increases the stress ethylene levels, restricting the development of sustainable agriculture. It triggers the generation of free radicals such as superoxide, hydrogen peroxide, hydroxyl radicals which promote lipid peroxidation, degradation of macromolecules such as proteins, lipids, nucleic acids and induces oxidative stress in plants for instance, generation of free radicals such as superoxide, hydrogen peroxide, hydroxyl radicals which induces lipid peroxidation, degradation of macromolecules such as proteins, lipids, nucleic acids, oxidative stress in plants (Ali et al. 2014; Saleem et al. 2018). It augments early leaf senescence, abscission, and reduction in leaf area, root and shoots growth under water deficit stress (Chaves et al. 2003; Attila Tátrai et al. 2016).

3.2.2 Salinity

In today’s agricultural system, due to less precipitation, non-availability of fresh water and use of seawater for irrigation and drainage, irrational application of chemical fertilizers has turned most of the arable land into barren, saline and unproductive (Egamberdiyeva et al. 2007; Ondrasek et al. 2009). It has been reported that the excessive salt concentration in the rhizosphere has inhibitory effects on the early growth stages of seedling development and thus reduces the seed germination rate and vegetative growth of various crops (Akbarimoghaddam et al. 2011; Shrivastava and Kumar 2015).

Apart from accentuating ethylene and its direct precursor 1-aminocyclopropane-1-carboxylate levels (Glick et al. 2007), soil salinity also induces ion toxicity, osmotic stress, oxidative stress, and nutritional imbalance in plants. There is a considerable reduction in K+ and P availability to plant tissues and excessive accumulation of Na+ which results in the low K+/Na+ ratio which affects the physiological processes of plants (Negrão et al. 2017; Rahneshan et al. 2018). In addition to this, the salinity stress conditions have detrimental effects on stomatal conductance, chlorophyll content and therefore, disrupt the photosynthetic machinery and food reservoir of plants which in turn affects plant growth and hinders the development of sustainable agriculture (Parida and Das 2005).

3.2.3 Flooding

Flooding is recognized as the most frequent abiotic stress that has many detrimental effects on plant physiology, morphology, anatomy, and growth. Under flooding conditions, the roots and soil are completely saturated with water, therefore, plants mainly suffer from hypoxia (O_2 deficiency) and later anoxic (complete absence of O_2) conditions which stimulate anaerobic fermentation pathway producing a small amount of ATP thus lowering the overall energy profile of plant tissues.

The prolonged exposure of plants to flooding can result in accumulation of phytotoxins such as sulphides, soluble Fe and Mn, acetaldehyde, acetic and formic acid, ethanol, lactic acid (by-products of anaerobic respiration). The excess water also affects plant water potential creating osmotic stress that ends up in stem and leaf wilting (Salazar-Parra et al. 2015; Luan et al. 2018). Like in any other stress condition, the ethylene levels increased in the plants. This is due to elevated levels of enzyme ACC synthase in the roots system, however, ACC oxidase, last enzyme of ethylene biosynthetic pathway stops in the anoxic conditions. So, ACC is transported to shoots via the transpiration stream (aerobic environment) where ACC oxidase catalyse the conversion of ACC into ethylene (Grichko and Glick 2001).

The ethylene mediates morphological and anatomical changes, for instance, the formation of gaseous spaces (aerenchyma), adventitious roots for better diffusion of gases between roots (anoxic environment) and shoots (aerobic environment), causing epinastic growth in any part which help the plants to withstand the stress. The excess ethylene production accompanied with the nutrient deficiency in flooded plants cause further reduction in leaf growth, early leaf senescence, chlorosis, root tissue necrosis and also triggers initiation of stomatal closure declining the rate of leaf transpiration and photosynthesis, gradual loss of carbohydrates in plants (Ali and Kim 2018; Sasidharan et al. 2018).

3.2.4 Metal Contaminants

Metal toxicity is one of the most severe environmental problems today. Heavy metals such as Co, Cu, Fe, Mn, Mo, Ni, V, and Zn are necessary for normal plant growth and development as micronutrients but their unwanted and excessive presence in the soil is detrimental to plant health. The presence of heavy metals in soil is attributed to some natural activities and human activities such as mining and smelting of metals, burning of fossil fuels, application of fertilizers and pesticides in agriculture, production of batteries and other metal products in industries, sewage sludge, and municipal waste disposal. Excessive accumulation of heavy metallic ions in the environment are absorbed by roots and translocated to shoots, disrupting normal metabolism and reduced growth of plants (Gonnelli et al. 2002; Seregin and Kozhevnikova 2006; Rasool et al. 2013).

Apart from this, the elevated level of heavy metals in soil negatively influenced biodiversity of the rhizospheric microbiome, reduces the fertility resulted in the loss of agronomic yield (Rout and Das 2009). The increased accumulation of metal ions in the rhizosphere has inhibitory effects on the physiological parameters of plants such as root length, plant height, biomass weight etc., and decreased enzymatic activity of amylase, protease, ribonuclease etc. involved in the seed germination process thus, facilitate the retardation in growth and development of crop plants (Sethy and Ghosh 2013).

Moreover, they exchange with essential nutrient of plants thus depleting soil nutrient reservoir and their availability to plants. Heavy metals interfere with photosynthetic machinery, distorting thylakoid membranes of the chloroplast, interrupting light reactions, reducing the efficiency of enzymes of the Calvin cycle (Sheoran et al. 1990; Parmar et al. 2013). In their pioneering work Vassilev et al. (2004); Iakimova et al. (2008); Rodríguez-Serrano et al. (2009) reported that plants growing on the soil polluted with metal contaminants showed an increased level of stress hormone ethylene. All these factors result in the reduction in growth, yield, and performance of crop which ultimately lead to plant death, pose threats to the sustainable agriculture and food security.

3.2.5 Extreme Temperatures

The Plants required optimal surroundings temperature to carry out biochemical, physiological and metabolic processes for sustainable growth and development of crop plants.

The Extreme temperatures stress in terms of heat (high) stress and cold (low) or chilling stress damage the plant morphologically i.e. reduced biomass weight, shoot length, primary root length, relative growth rate, discoloration of fruits, floral sterility, scorched leaves and other plant organs or physiologically i.e. decline rate of seed germination and emergence, chlorophyll content, damage cellular membrane and disrupt the normal functioning of biochemical processes of plants such as photosynthesis, oxidative respiration, nutrient and water uptake potential thus limiting the agronomic yield. The induction of Oxidative stress due to excessive generation of reactive oxygen species would be the major manifestation of temperature stress on plants which eventually leads to their death (Hatfield and Prueger 2015).

3.3 Biotic Stress

Various plant diseases including cankers, damping off, downy mildew, bacterial wilts, Mosaic leaf, caused by wide range of pathogens such as bacteria, fungi, root-knot nematodes, viruses, pests, and insects result in substantial loss of crop production and agronomic yield. The conventional agricultural practices with the application of synthetic pesticides, herbicides, insecticides and the development of pathogens resistant plant varieties are frequently used in today's agricultural system. The ensuing environmental degradation and increasing cost pose restriction in usage of synthetic chemicals in the field. Moreover, the development of resistant varieties is less encouraged due to the time-consuming breeding techniques as well as certain ethical and social concerns associated with genetically modified crops (Lugtenberg and Kamilova 2009). They further concluded that the plant growth promoting bacteria outcompete pathogens for ecological niche and nutrients (roots exudates).

3.4 Plant Growth Promoting Rhizobacteria

The researchers of agricultural biotechnology and microbiology have focused towards safe and eco-friendly biological solutions, of which exploiting beneficial soil microbial community; more specifically rhizobacterial-plant interactions have gained paramount importance in sustainable agriculture applications and bio-safety programmes.

The rhizosphere is the plant-root junction (Hartmann et al. 2008), densely colonized by numerous types of microorganism including bacteria, fungi, protozoa, algae, nematodes (root microbiome). These rhizospheric microbes are influenced by physiochemical properties of soil and largely, by the plant roots secretions such as amino acids, organic acids, enzymes, and sugars, known as roots exudates through chemotaxis (Helliwell et al. 2017).

Among these rhizospheric microorganisms, bacteria present near or on the root surface possesses some plant growth and tolerance stimulating characteristics referred to as plant growth promoting rhizobacteria (Kloepper and Schroth 1978). There are numerous plant growth promoting bacteria belonging to genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* which have proved to be the alternative of chemical fertilizers to enhance crop production under stress conditions and improve the soil health and fertility (Saharan and Nehra 2011).

The population of plant growth promoting rhizobacteria was categorized into 2 groups – extracellular plant growth promoting bacteria and intracellular plant growth promoting bacteria (Gray and Smith 2005). As the name implies, extracellular plant growth promoting bacteria densely colonizes the rhizosphere, rhizoplane (on the surface of roots of host plants) or void spaces between cortical spaces of roots cells (inside the root tissues) (Glick 1995). They positively influence the growth and development of a plant by mediating nutrient solubilization, disease resistance and production of growth-stimulating biomolecules (Verma et al. 2015). While intracellular plant growth promoting bacteria are basically endophytic plant growth promoting rhizobacteria that form a close association with the internal tissues of roots of host plant and remain confined to special root structures such as nodules. These bacterial strains promote plant growth mainly by enhancing growth limiting nutrient nitrogen pool, easy accessibility to the plants by symbiotic biological nitrogen fixation process, thereof reducing the dependency on synthetic nitrogen fertilizers (Verma et al. 2015).

The plant growth promoting bacteria were also classified on another ground i.e., based on their mechanistic action performed for plant growth promotion. The plant growth promoting rhizobacteria can either be- growth promoting bacteria (involve in nutrient acquisition, growth hormone production, degradation of metals contaminants from soil) or disease suppressing bacteria (involve in repression of plant pathogenesis) or stress tolerating bacteria (supports the plants growth under adverse environmental conditions) (Bashan and Holguin 1998). The plant growth promoting bacterial strains of rhizospheric microbiota stimulate forefront defensive response and enhance tolerance to a wide range of biotic and abiotic stresses. The application of plant growth promoting rhizobacteria in the agricultural field is the better, environmentally sound alternative to conventional farming practices and plant breeding technique in achieving the sustainable agriculture approach (de Souza et al. 2015).

These bacteria alleviate the negative effects of abiotic stress and promote plant growth under such extreme condition by mediating direct plant growth promoting mechanism which mainly focuses in revitalizing the nutrient reservoir and in enhancing the tolerance potential of plants against harsh climatic conditions. This includes the production of acetic acid, gibberellin, cytokinin, iron chelating siderophores, biofilm formation, production of stress triggered ethylene deaminating enzyme (Kumari et al. 2016).

In addition to this, the potential of plant growth promoting rhizobacteria in suppressing the inhibitory effects of biotic stresses has been postulated in the several literatures which described the indirect plant growth promoting mechanism to boosts the plant defensive immune system and make them resistant against a plethora of plant-borne pathogenic bacteria, fungi, nematodes, pests, insects. This mainly includes (1) production of antibiotic compounds such as 2,4-diacetylphloroglucinol, phenazine, pyoluteorin, and pyrrolnitrin; (2) establishment of iron depleting environment for phytopathogens by promoting iron sequestering siderophores; (3) Production of lytic enzymes such as chitinases, glucanases against the cell wall of pathogenic fungi; (4) Competitively inhibiting pathogens for infection site or space for colonization (Beneduzi et al. 2012) (Fig. 3.1).

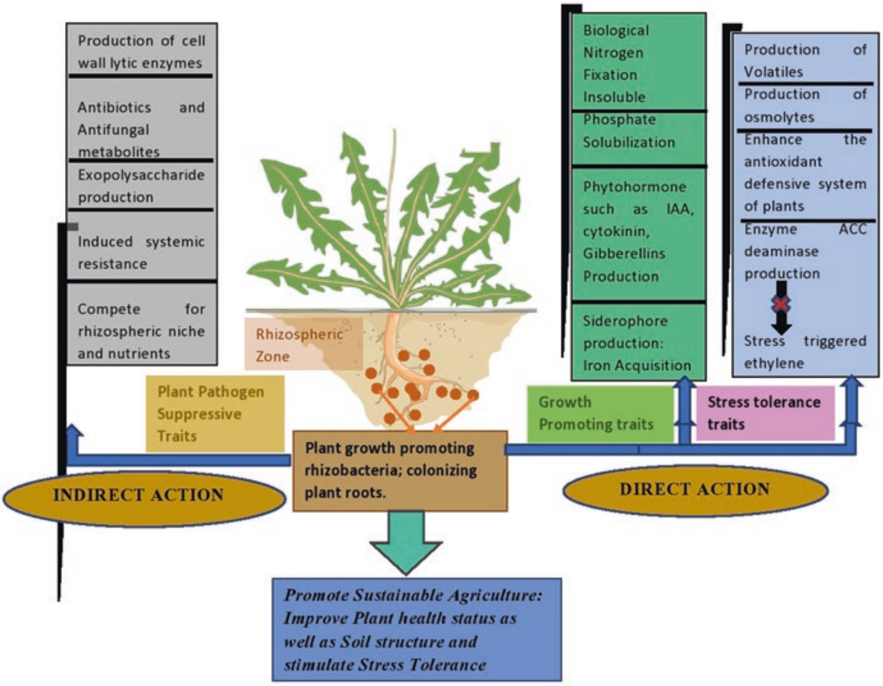

Fig. 3.1 Direct and indirect mechanisms of plant growth promoting rhizobacteria to promote the development of sustainable agriculture. *IAA* Indole acetic acid, *ACC* 1-aminocyclopropane-1-carboxylate

3.5 Stress Tolerance Mechanisms of Plant Growth Promoting Rhizobacteria

The use of plant associated growth promoting rhizobacteria has garnered significant importance as a better, environmentally safe and cost-effective alternative in ameliorating the negative impact of synthetic agrochemicals, enhancing the plant tolerance to stressful environmental conditions and suppressing the plant diseases; thereby promotes sustainable agricultural production without compromising with the environment and human health (Table 3.1).

3.5.1 Production of Phytohormones

The plant growth regulators or phytohormones are the chemical messengers which play a strategic role in different physiological processes of the plant. Plant growth promoting rhizobacteria modulate endogenous levels of plant growth regulators by producing various phytohormones such as auxin or indole acetic acid, abscisic acid,

Table 3.1 Growth promotion by plant growth promoting rhizobacteria under abiotic and biotic stressed conditions

PGPR strain	Host plant	Stress factor	Effect on host plant	Reference
Stenotrophomonas maltophilia	Wheat	Salinity and *Fusarium graminearum*	Increased plant growth in terms of growth parameters and enhanced the level of defensive and antioxidative enzymes.	Singh and Jha (2017)
Arthrobacter protophormiae (SA3) and *Dietzia natronolimnaea* (STR1)	Wheat	Salinity	Enhance photosynthetic efficiency. Increase phytohormone level such as indole acetic acid and decrease abscisic acid and ACC levels	Barnawal et al. (2017)
Bacillus subtilis (LDR2)	Wheat	Drought		
Rhizobia	Mungbean (*Vigna radiata* L)	High temperature and drought	Increase biomass and nodulation efficiency	Mondal et al. (2017)
Bacillus megaterium and *Pantoea agglomerans*	*Vigna radiata*	High Aluminium levels and drought	Improved plant growth and reduced Aluminium uptake in plants	Zhang et al. (2019)
Jeotgalicoccus huakuii	Tomato, Okra and Maize plants	Salinity	Enhanced the levels of defensive enzymes, chlorophyll, and osmoprotectants levels	Misra et al. (2019)
Pseudomonas veronii KJ	*Sesamum indicum* L.	Flooding	Increased leaf chlorophyll fluorescence signals, chlorophyll content, root and shoot length, and fresh and dry biomass of stressed plants	Ali et al. (2018)
Azospirillum brasilense Az39	Rice (*Oryza sativa* L.) cv. El Paso	Osmotic stress	Production of polyamines to mitigate osmotic stress and to enhance growth of rice plants.	Cassán et al. (2009)
Azospirillum brasilense	*Arabidopsis thaliana*	Drought	Production of phytohormone mainly abscisic acid was enhanced, reduced the water loss, augmented plant growth, increased proline levels, leaf water content.	Cohen et al. (2015)
Pseudomonas putida UW4 and *Gigaspora rosea BEG9*	Cucumber	Salinity	Augmented plant growth, enhanced root architecture and improved photosynthetic activity.	Gamalero et al. (2010)
Rahnella aquatilis B16C, Pseudomonas yamanorum B12 and Pseudomonas fluorescens B8P	*Faba bean*	*F. solani* root rot	Production of antibiotics such as 2,4-diacetylphloroglucinol, pyrrolnitrin, phenazine	Bahroun et al. (2018)

(continued)

Table 3.1 (continued)

PGPR strain	Host plant	Stress factor	Effect on host plant	Reference
Streptomyces spp.	Chickpea	*Sclerotium rolfsii*	Enhancement of defense related enzymes such as polyphenol oxidase, peroxidase, phenylalanine ammonia-lyase	Singh and Gaur (2017)
Bacillus amyloliquefaciens Ba13	Tomato	Tomato yellow leaf curl virus disease	Enhanced the gene expression of pathogenesis related-proteins, defensive enzymes and improve rhizosphere microbiome	Guo et al. (2019)
Bacillus velezensis OEE1	*Olea europaea* L.	*Phytophthora ramorum*, *Phytophthora cryptogea*, *Phytophthora plurivora* and *Fusarium solani*	Production of hydrolytic enzymes and secondary metabolites-protease, chitinase, glucanases, bacillibactin, bacillaene, surfactin, fengycin and subtilin	Cheffi et al. (2019)

cytokinin, gibberellins which alters the root morphology and enabled plants to withstand the harsh environmental conditions that limit their growth (Fahad et al. 2015).

Auxin being highly characterized and most active hormone regulates various processes in plants such as root initiation and development, shoot elongation, apical dominance, phototropism etc. It has also been reported that inoculation of indole acetic acid synthesizing rhizobacteria in the agricultural field has improved the root morphology, growth, biomass, nutrient (higher P, K uptake) of drought-stressed rice plants, apparently enhancing stress tolerance in plants. There are several bacterial species belonging to *Pseudomonas*, *Bacillus*, *Azotobacter* etc. which have been reported to produce indole acetic acid in the presence of tryptophan as a substrate. *Azospirillum brasilense* was shown to confer tolerance to water deficit in wheat plants, thus improving water potential with the consequent increase in root growth as well as proline accumulation in leaves and roots. It has also been documented that indole acetic acid producing bacteria stimulate plants to exudate carbohydrates more which indirectly involved in phosphorous mobilization thus result in the better nutrient status of the bacteria (Upreti and Sharma 2016).

Abscisic acid, an isoprenoid, plays an important role in osmoregulation under drought and flooding stress. It has been reported that Arabidopsis plants inoculated with *A. brasilense* has elevated levels of Abscisic acid in flooding stress which initiate stomatal closure and control hydrogen peroxide (H_2O_2) by increasing activity of antioxidant enzymes thus enhancing anoxic tolerance in plants (Cohen et al. 2015). Under water deficit surroundings, rhizobacteria *Phyllobacterium brassicacearum* conferred osmotic stress tolerance in inoculated Arabidopsis plants by decreasing turgor pressure and eventually stomatal closure, which is associated with elevated Abscisic acid levels, preventing intracellular water loss (Verma et al. 2016).

Cytokinin is another class of phytohormones which help in increasing plant growth under abiotic stress. It mainly involved in various plant growth and developmental processes. Several studies reported that inoculation of seedlings with cytokinin producing plant growth promoting rhizobacteria generally delayed leaf senescence and increased plant tolerance to salt (Upreti and Sharma 2016).

Gibberellic acid generally controls leaf expansion, seed germination, fruit development, stem elongation and thus play an important role in alleviating the negative effect of abiotic stress. Kang et al. (2014) have reported that Gibberellic acid secreting rhizobacteria *P. putida* H-2–3 has enhanced the growth of Soybean plant under drought conditions.

3.5.2 Production of Volatile Compounds

The plant growth promoting rhizobacteria produces a diverse range of secondary organic compounds called volatiles such as acetoin and 2,3-butanediol which facilitate growth, pathogen resistance and abiotic stress tolerance in plants. They are the promising candidates in mediating salt stress tolerance by enhancing excess Na^+ export, accumulating proline, choline and other osmoprotectants which maintain osmotic balance, activates the activity of many antioxidant enzymes such as peroxidase, dismutase etc. thus preventing the plant from oxidative stress and stabilize other cellular structures.

Increased production of exopolysaccharide, biofilm component, by certain plant growth promoting rhizobacteria strains such as Pseudomonas aeruginosa resulted in induced resistance against desiccation and drought stress. It has also been reported that certain strains of plant growth promoting rhizobacteria such as induce resistance to drought by producing an exopolysaccharide (biofilm formation). There are several other volatiles such as hydrogen cyanide, ammonia which induces stress tolerance by inhibiting the growth of pathogens (Liu and Zhang 2015).

3.5.3 Production of Osmolytes

Osmolytes or osmoprotectants are non-toxic organic compounds such as proline, sugars, polyamines, betaines, quaternary ammonium compounds, polyhydric alcohols, and other amino acids and water stress proteins like dehydrins produced by plants in response to stress. There are certain plant growth promoting rhizobacteria strains which produced osmolytes and alter the levels of plant-produced osmolytes level to protect cellular membrane which maintains water potential under stress conditions.

Vurukonda et al. (2016) described that inoculation of rhizobacteria *Pseudomonas putida* and *A. lipoferum* improved water potential of a maize plant by secreting proline, free amino acids and soluble sugars. Trehalose, another osmoprotectant, is a non-reducing sugar molecule which enhanced the plant growth under abiotic stress conditions. It has been reported that inoculation of nodule plants with plant growth promoting rhizobacteria strains *Rhizobium etli* and *A. brasilense* have overexpressed the trehalose-6-phosphate synthase gene responsible for trehalose metabolism which in turn allowed plants to adapt to abiotic stress and increase agronomic yield (Paul et al. 2008).

More recent evidence has been reported by Vurukonda et al. (2016) that some plant growth promoting rhizobacteria strains such as *B. subtilis*, *Pseudomonas fluorescens*, *Raoultella planticola* have increased the content of choline, a water-soluble nutrient precursor of Glycine betaine (GB) which resulted in enhanced leaf water potential, improved biomass, decreased water loss thus protecting osmotically stressed maize and Arabidopsis plants.

Similarly, there are some polyamines which are also considered as potential candidates to mitigate the negative effects of osmotic stress in plants. Cassán et al. (2009) have shown the prominent role of cadaverine producing plant growth promoting rhizobacteria *A. brasilense* in osmotically stressed rice seedlings. Moreover, inoculation of plant growth promoting bacteria in the agricultural field reduced electrolyte leakage and increased sugars, proline contents which impart tolerance to cold temperature or cold acclimation (Chakraborty et al. 2015).

3.5.4 Production of Exopolysaccharides

Exopolysaccharide production is considered as one of the indirect mechanisms by which plant growth promoting rhizobacteria protect plants from unfavorable climatic conditions such as saline soils, temperature variation, water stress, pathogen attack. There have been several authors who reported that exopolysaccharide producing rhizobacteria such as *Rhizobium phaseoli*, *Rhizobium leguminosarum*, *Proteus penneri*, *Pseudomonas aeruginosa*, *Alcaligenes faecalis* when inoculated in the stressed agricultural soils have provided microenvironment to maintain the plant water potential, soil moisture content, increase the root growth and biomass and thus ensured plant and seedlings survival under stress conditions (Arora et al. 2013).

It has also been reported by Paul and Lade (2014) that exopolysaccharide-producing bacteria developed soil sheaths around the roots of salt-stressed wheat seedlings which in turn caused the reduced inflow of sodium ions and imparts salt tolerance. The plant growth promoting rhizobacteria producing exopolysaccharide on application apart from their role in nutrient acquisition and water potential maintenance, also promote protection from desiccation as well from biotic challenges (Gupta et al. 2015).

3.5.5 Antioxidant Defence

The reactive oxygen species including superoxide, anion radicals, hydrogen peroxide, hydroxyl radicals, singlet oxygen, alkoxy radicals are produced when plants are subjected to inhospitable climatic conditions which in turn triggered oxidative destruction, degrading proteins, lipids, deoxyribonucleic acid leading to impairment of normal functioning of plant cells. The plants have the antioxidant system to curb the negative effects of oxidative stress by stabilizing reactive oxygen species which includes superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase, cysteine, glutathione, and ascorbic acid.

The soil bacteria have the potential to modify the level and activities of the antioxidant enzymatic system to alleviate the harsh effects of oxidative stress induced by reactive oxygen species conferring stress tolerance in plants. Several studies have provided evidence for plant growth promoting rhizobacteria application in enhancing stress tolerance in plants, for instance, Sandhya et al. (2010) has concluded the reduced levels of antioxidant enzymes catalase, ascorbate peroxidase in maize seedlings bacterized with *Pseudomonas* spp. as compared to uninoculated seedlings under drought stress. Heidari and Golpayegani (2012) have shown the effectiveness of plant growth promoting bacteria on basil plants by increasing the activity of glutathione reductase and ascorbate peroxidase under drought stress.

3.5.6 Production of 1-Aminocyclopropane-1-Carboxylate Deaminase

There is an increase in biosynthesis of ethylene in plants called “stress ethylene” in response to various biotic and abiotic stresses. It is key phytohormone regulating the physiology of plants such as seed germination, root hair development, roots elongation, leaf and petal abscission, fruit ripening, stem elongation but its excessive secretion resulted in root curling and shortening, reduced shoot growth and eventually lead to plant death under the stress conditions (Gamalero and Glick 2015).

The plant growth promoting rhizobacteria with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity has been reported in numerous species of genera Agrobacterium genomovars and *Azospirillum lipoferum*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Methylobacterium fujisawaense*, *Pseudomonas*, *Ralstonia solanacearum*, *Rhizobium*, *Rhodococcus* and *Sinorhizobium meliloti* and *Variovorax paradoxus* (Glick 2014; Gontia et al. 2014).

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase containing rhizobacteria in association with plants rhizospheric zone hydrolyzed ACC, excreted from plants roots, into α-ketoglutarate and ammonia, thus inhibited the stress ethylene production and reduced its negative effects (Raghuwanshi and Prasad 2018). In the experiment conducted by Gamalero et al. (2010) increase in root and shoot biomass was reported when plant growth promoting bacteria *Pseudomonas putida* interacted with cucumber plant under salt stress condition. Similarly, bacterization of tomato and pepper seedlings with plant growth promoting rhizobacteria *Achromobacter piechaudii* resulted into reduced ethylene production and increased the fresh and dry weight of biomass (Heidari and Golpayegani 2012).

Besides this, rhizobial strains with ACC deaminase activity are found to facilitate nodulation (nitrogen-fixing nodules) and increase the nitrogen content of plant which is restricted because of high ethylene concentration under stress (Nascimento et al. 2016). Chen et al. (2013) have reported that inoculation with *Variovorax paradoxus* increased nodulation, seed yield and seed nitrogen content of stress affected peas plant. The experiment conducted by Sharma et al. (2013) have shown that efficiency of ACC deaminase producing *Bacillus*, *Pseudomonas* and *Mesorhizobium ciceri* in mitigating the effects of stress by increasing proline concentration, root and shoot length, fresh weight of seedlings and improving seed germination in plants.

3.5.7 Phytoremediation and Tolerance to Metal Toxicity

Phytoremediation is the technology to clean heavy metal polluted soils with the help of plants with potential to stabilize, extract and degrade pollutants but toxic levels of heavy metals suppress plants growth which affects their phytoremediation potential. Therefore, soil bacteria are employed which assist plants in phytoremediate metal contaminants more efficiently and enhance tolerance to metal stress. It has been reported that iron deficiency in plants growing in heavy metal contaminated soil resulted in chlorosis because low iron content cause reduced chlorophyll pigment and impaired chloroplasts. Therefore, in this regard, many rhizobacteria could release metal chelating secondary metabolites such as iron chelating siderophore that sequesters ferric ions from surroundings and provides iron nutrition to plants protecting them from becoming chlorotic in the presence of heavy metals including nickel, lead, and zinc.

These metal chelating siderophores have been reported to scavenge the stress-induced free radicals and thus preventing plans from oxidative damage and promote plant growth (Chibuike and Obiora 2014; Ojuederie and Babalola 2017). The plant growth promoting rhizobacteria *Klebsiella mobilis* inoculation in Barley plants grown on cadmium contaminated soil resulted in higher yields and decreased cadmium content in grains (Dimkpa et al. 2009). It was observed that soil bacteria have metal binding properties that make plants enable to uptake metals such as cadmium. The chromium tolerance was enhanced in *Brassica juncea* with inoculation with indole acetic acid and siderophore-producing bacteria without altering chromium uptake (Rajkumar and Freitas 2008).

3.5.8 Production of Antibiotics

The plant growth-promoting rhizobacteria colonizes and provide the rhizospheric zone of host's plant with a diverse range of low molecular weight antagonistic molecules called antibiotics such as phenazines, phloroglucinols, pyrrolnitrin, pyoluteorin, hydrogen cyanide and cyclic lipopeptides that inhibit the growth and metabolism of phytopathogens (Glick et al. 2007). It has been reported that phenazines (heterocyclic pigments) and phloroglucinols (2,4-diacetylphloroglucinol are extensively produced and studied antibiotics produced by plant growth promoting bacterium fluorescent *Pseudomonas* strains that significantly destroy cellular membrane and vegetative reproduction of soil-borne pathogens such as *Pythium* spp., *Rhizoctonia solani* (Chin-A-Woeng et al. 2009).

Another secondary, volatile metabolite known as hydrogen cyanide (HCN) is produced by various strains of plant growth promoting rhizobacteria belonging to genus *Pseudomonas*, *Bacillus*, *Streptomyces* which has the potential to biologically control weeds and suppress the pathogenesis of *Thielaviopsis basicola*, causative agent of black root rot tobacco plant disease (Kamei et al. 2014). Another class of antibiotics is produced by plant growth promoting rhizobacteria called bacteriocins that possess narrow killing spectrum, suppress the growth of related strains (Tariq et al. 2017).

3.5.9 Production of Antifungal Metabolites Such as Hydrolytic Enzymes

The plant growth promoting rhizobacteria for example, *Bacillus subtilis*, *Serratia marcescens*, *S. plymuthica*, *Pseudomonas cepacia*, *Bacillus megaterium*, *Pseudomonas aeruginosa* has the potential to produce another category of antimicrobial (or antifungal) metabolites with enzymatic activity such as chitinases, dehydrogenase, β-glucanase, lipases, phosphatases, proteases that have substantial negative effect on the cell wall components such as cellulose, hemicellulose, chitin and thereof provides biotic stress tolerance in plants by degrading of the cellular structure and integrity as well as inhibiting the mycelial proliferation of pathogenic fungi (Gupta et al. 2015).

3.5.10 Enhancement of Plant Defensive Response by Induced Systemic Resistance

Plants possess a basic level of innate immunity against root and foliar pathogen attack which can be positively triggered in response to appropriate stimuli. This state of amplified defensive potential of plants is known as induced resistance, expressed upon subsequent infectious pathogenic challenge. The induced resistance falls into two major category- acquired systemic resistance and induced systemic resistance based on stimulator that induces the resistance and mode of their action (Choudhary et al. 2008; Ramamoorthy et al. 2001; Jain et al. 2014). The plant growth promoting rhizobacteria strain *Pseudomonas fluorescens* suppress the mycelial proliferation in rice plants by mediating induced systemic resistance. Similarly, other strains of plant growth promoting rhizobacteria belonging to genera *Pseudomonas*, *Bacillus* has been reported to inhibit the growth of pathogens such as *F. oxysporum*, *R. solani*, *Colletotrichum lindemuthianum* by activating the expression of defense-related genes and establishing induced systemic resistance (Labuschagne et al. 2010).

3.5.11 Siderophore Production for Acquisition of Iron

Iron, micronutrient is essential for normal growth and development of plants and microorganism because it played an important role in various physiological and biochemical processes such as photosynthesis, the formation of macromolecules such as hemoglobin, chlorophyll, cytochrome; oxidative respiration, heme formation, and ATP synthesis. Being present in plenty amount (Fe^{+3}), yet it is unavailable to plants due to its low solubility in soil suspension, therefore the limited concentration of iron is present especially in calcareous soils. The plant growth promoting rhizobacteria restore the iron nutrient reservoir by secreting low molecular weight iron chelators known as siderophores which sequester iron from the surrounding environment and form complexes with ferric ion. In this way, siderophore producing plant growth promoting rhizobacteria provide iron nutrition to plants in iron-limiting conditions and simultaneously suppressing the pathogenic growth by establishing the iron deficient environment for pathogens. The beneficial role of siderophore producing plant growth promoting rhizobacteria in providing iron nutrition and preventing metal toxicity has been postulated in the literature (Sharma and Johri 2003; Gupta and Gopal 2008; Ghavami et al. 2017).

3.6 Conclusion

Root colonizing bacteria exerting a positive influence on plants via various direct or indirect methods have been defined as plant growth-promoting rhizobacteria. Keeping in view the above discussion, significant progress has been made in understanding the mechanism of plant growth promotion and alleviation of adverse effects of stress by plant growth promoting rhizobacteria, however, their application under field conditions is still in infancy. The demonstration of positive interaction of plants under laboratory and greenhouse conditions is mostly not in agreement with the results under practical conditions. Because of these and other challenges in the screening, formulation, and application of plant growth-promoting rhizobacteria (PGPR) still have to go a long way and fulfil their promise of achieving the full potential of sustainable agriculture.

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Chapter 4
Ecology and Mechanisms of Plant Growth Promoting Rhizobacteria

Amit Verma, Shulbhi Verma, Mahender Singh, Harish Mudila, and Jitendra Kumar Saini

Abstract The rapid increase in population and climate change is calling for sustainable methods to improve food production such as soil microbial management. Here we review the ecology and mechanisms of action of plant growth-promoting rhizobacteria. Rhizosphere comprises both symbiotic and non-symbiotic microorganisms that influence plant growth positively by their effect on mineral nutrient uptake and bioavailability. Plant growth-promoting rhizobacteria facilitate resource acquisition and modulate phytohormone levels. Indirect mechanisms include production of antibiotics, lytic enzymes and siderophores, competition to harmful organisms, regulation of ethylene production, and induced systemic resistance. Plant growth-promoting substances suppress plant pathogens through competition for nutrients and space. Application of plant growth-promoting rhizobacterial increases crop yields. Numerous plant growth-promoting rhizobacteria are already marketed and are actually replacing mineral fertilizers and pesticides.

Keywords Siderophore · Induced systemic resistance · Phytohormones · Rhizosphere · Rhizobacteria · Plant growth · Crop yield · Soil microbiology

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Abbreviations

ACC	1-amino cyclopropane-1-carboxylic acid
DAPG	2,4 diacetyl phloroglucinol
IAA	Indole Acetic Acid
PGPR	Plant Growth-Promoting Rhizobacteria

4.1 Introduction

Free-living soil microorganism and rhizosphere bacteria that are beneficial to plants, are referred as plant growth-promoting rhizobacteria (PGPR). Plant inoculation with about 2–5% of rhizobacteria in a soil containing competitive microflora, is sufficient to sustain the plant growth and development to fulfil the requirement to some extent (Kusum et al. 2019; Dweipayan et al. 2016). PGPR terminology was first proposed by Kloepper in the 70s. According to the original definition, rhizobacteria include free-living bacteria, except nitrogen-fixing rhizobia and *Frankia*. Hence, growth stimulation which results from the biological nitrogen fixation by rhizobia or *Frankia* cannot be considered as a PGPR mechanism of action and are thus excluded from PGPRs. Plant growth-promoting rhizobacteria have the ability to colonize rhizosphere aggressively, that benefits plant and inhibit minor pathogens (Antoun and Prévost 2005). Plant growth-promoting rhizobacteria includes various types of bacterial groups including symbiotic ones viz., *Rhizobium, Bradyrhizobium, Actinomycetes* and free-living bacteria of genus *Acetobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas*. Many of the enlisted bacteria affect the plant metabolism either in the rhizospheric region or sometimes invade the plant tissue.

PGPRs have been classified based upon their activities as biofertilizers which increases the nutrient bioavailability to plant (Sushanto et al. 2017), phytostimulators which releases different phytohormone like chemicals resulting in plant growth promotion (Kloepper 2003), biocontrol agents which release a wide variety of antibiotics and antifungal compounds imparting plant resistance against biotic stress, sometimes also referred to as biocontrol plant growth-promoting bacteria (Bashan and Holguin 1997) and rhizo-remediators which promotes the plant growth by the removal of different organic pollutants present in the rhizosphere.

Plant growth-promoting rhizobacteria are sometimes classified into two groups, symbiotic and free-living based on their relationship with plants (Khan 2005), and they are studied as extracellular and intracellular PGPR (Gray and Smith 2005) based upon their occurrence in plant i.e., extracellular PGPR exist in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex, whereas intracellular PGPR exists inside the root cells. Due to their plant growth promotion effects, these beneficial microorganisms are termed as yield-increasing bacteria (Sayyed et al. 2010); plant health-promoting rhizobacteria, or nodule-promoting rhizobacteria as per their mechanism of action on the plant metabolism (Burr and Caesar 1984; Vessey 2003). Plant growth-promoting rhizobacteria can be present in proximity to the plant roots at different levels i.e., near roots of the plant, in the rhizoplane, within the root tissue, or in the case of symbionts as specialized structures known as nodules (Gray and Smith 2005). PGPR helps in water retention in the soil which can be combat drought to some extent (Wenjuan et al. 2018).

Apart from the issue of high cost, many of the agrochemicals used for fungal and bacterial disease control are hazardous to the living system along with humans and persist for a longer time resulting in bioaccumulation in natural ecosystems. Production of chemical fertilizers is also one of the major factors causing depletion of non-renewable resources. Fuels like petroleum oil and natural gas utilized in the production of fertilizers and other agrochemicals pose environmental hazards to human and other living community. Due to their biological nature, PGPR approach is environmentally safer and sustainable.

4.2 Applications and Mechanisms

Plant growth-promoting rhizobacterial action involves diverse mechanisms and generally, more than one mechanism is responsible for plant development. A summary of the effects of plant growth-promoting rhizobacteria on different crops under laboratory and field conditions are presented in Table 4.1.

4.2.1 Direct Mechanisms

4.2.1.1 Facilitating Resource Acquisition

Plant growth-promoting rhizobacteria may provide plants with lacking resources or nutrients like fixed nitrogen, iron, and phosphorus etc. (Munees and Mulugeta 2014). Nitrogen is the building block of the living cells and essential for growth, development and enhances productivity. Nitrogen facilitates from the atmosphere to the living system through microorganisms that resides in the soil. Nitrogen is fixed in the living system through symbiotic and non-symbiotic bacteria. A popular example of symbiotic nitrogen-fixing bacteria from family *Rhizobiaceae* that is associated with leguminous plants and *Frankia*, a soil actinomycete that do symbiosis in non-leguminous plants such as *Gymnostoma* (family Casuarinaceae), *Myrica* (family Myricaceae), *Comptonia* (family Myricaceae), *Casuarina cunninghamiana*, *Purshia tridentata*, *Allocasuarina decaisneana*, *Alnus*, *Hippophae*, *Coriaria*.

Rhizobia also establish symbiosis in relation with non-legume plant such as *Parasponia*. Their symbiosis results in the nodulation for bacteria and those bacteria assist in providing atmospheric nitrogen to plants (Raklami et al. 2019). In contrast, non-symbiotic microorganisms may be free-living, associative or endophytic in

Table 4.1 The effects of plant growth promoting rhizobacteria and their mechanisms of action

PGPR	Crop system	Trial setup	Soil modification	Effects on plant	Mechanism of action	References
Acinetobacter calcoaceticus SE370	*Cucumis sativus*	*Pot trial*	–	Increase in biomass and chlorophyll contents, water potential and decrease in electrolytic leakage	Stress adaptation imparted through: 1. Reduced antioxidant enzyme activity of catalase, peroxidase, polyphenol oxidase. 2. Decreased polyphenol content. 3. Decreased abscisic acid and increased salicylic acid and gibberellin levels.	Kang et al. (2014)
Acinetobacter sp.	Bean and wheat	*Field trial*	Improvement in the amount of soil organic matter	Increased plant growth, leaf production, shoots, and root system	PGPR influenced efficient colonization of bean and wheat plant roots by the mycorrhizal consortium	Raklami et al. (2019)
Bacillus amyloliquefaciens	Rice	*Pot trial*	–	Increased stress adaptation against various abiotic stresses	Elevated phytohormone induced stress tolerance	Tiwari et al. (2017)
Bacillus cereus	*Sunflower*	*Pot trial*	Enhancement in the accumulation of Cu, Co, Fe, and Zn	Increased tolerance towards drought and heavy metal stress	Increase in the translocation and accumulation of micronutrients	Khan et al. (2018)
Bacillus megaterium	*Phaseolus vulgaris*	*Pot trial*	Increased phosphorus availability	Increased nodulation, shoot weight and root weight	Increased N2 fixation by legume-*Rhizobium* symbiotic association	Korir et al. (2017)
Bacillus sp. B55	*Nicotiana attenuata*	*Petri dish*	–	Increased germination and seedling growth	Emission of volatile organic compounds	Meldau et al. (2013)
Bacillus sp.	Durum wheat	*Field trial*	Increased mycorrhizal colonization	Increased biomass, elevated N and P content	Increased phosphate transferase gene expression	Saia et al. (2015)
Bacillus sp.	*Spartina maritima*	*Pot trial*	–	Increased belowground biomass	Decreased antioxidant enzyme activity along with slower respiration rate	Mesa-Marín et al. (2018)

Burkholderia cepacia SE4	*Cucumis sativus*	*Pot trial*	–	Increase in biomass and chlorophyll contents, water potential and decrease in electrolytic leakage	Stress adaptation imparted through: 1. Reduced activity of catalase, peroxidase, polyphenol oxidase 2. Decreased total polyphenol 3. Decreased abscisic acid and increased salicylic acid and gibberellin levels	Kang et al. (2014)
Burkholderia phytofirmans	*Arabidopsis thaliana*	*Pot trial*	Increased root colonization	Improved plant tolerance towards lower temperature stress	Strengthening of the cell wall in mesophyll	Su et al. (2015)
Burkholderia sp.	*Schizolobium parahyba*	*Field trial*	Increased mycorrhizal association and increased nutrient supply	Increased biomass and wood yield	Improved nutrient acquisition	Cely et al. (2016)
Enterobacter cloacae and *Bacillus drentensis*	Mung bean	*Field trial*	–	Improvement in stomatal conductance, transpiration, relative water content, chlorophyll content, plant height, leaf area, dry biomass, seed yield, and salt tolerance	Mitigation of the adverse effects of salinity due to the additive effect of PGPR and Si foliar spray on various physiological factors	Mahmood et al. (2016)
Enterobacter hormaechei	Foxtail millet	*Pot trial*	–	Improved drought tolerance and increased seed germination and seedling growth	ACC deaminase activity and exopolysaccharides production which enhance drought tolerance	Niu et al. (2018)
Paenibacillus polymyxa	*Phaseolus vulgaris* L.	*Pot trial*	Increased phosphorus availability	Increased nodulation, shoot weight and root weight	Increased N2 fixation by legume-*Rhizobium* symbiotic association	Korir et al. (2017)
Paenibacillus polymyxa NSY50	Cucumber seedlings	*Pot trial*	–	Increased defence against pathogenic attack by *Fusarium*	Elevated defence related to protein metabolism	Du et al. (2016)

(continued)

Table 4.1 (continued)

PGPR	Crop system	Trial setup	Soil modification	Effects on plant	Mechanism of action	References
Planomicrobium chinense	Sunflower	*Pot trial*	Enhancement in the accumulation of Cu, Co, Fe, and Zn	Increased tolerance towards drought and heavy metal stress	Increase in the translocation and accumulation of micronutrients	Khan et al. (2018)
Promicromonospora sp. SE188	*Cucumis sativus*	*Pot trial*	–	Increase in biomass and chlorophyll contents, water potential and decrease in electrolytic leakage	Stress adaptation imparted through: Reduced catalase, peroxidase and polyphenol oxidase activity, decreased total polyphenol content. Decreased abscisic acid and increased salicylic acid and gibberellin levels	Kang et al. (2014)
Pseudomonas fluorescens, P. migulae	Foxtail millet	*Pot trial*	–	Improved drought tolerance; increase in seed germination and seedling growth	ACC deaminase activity and exopolysaccharide production enhance drought tolerance	Niu et al. (2018)
Pseudomonas sp.	Maize	*Pot trial*	Increase in Pseudomonas population	Better plant growth and development.	Increased root colonization by Pseudomonads	Vacheron et al. (2016)
Rahnella aquatilis	Bean and wheat	*Field trial*	Improved amount of total organic matter and carbon content	Increased plant growth, leaf production, shoots, and root system	PGPR influenced efficient colonization of bean and wheat plant roots by the mycorrhizal consortium	Raklami et al. (2019)
Rhizobium sp.	*Schizolobium parahyba*	*Field trial*	Increased mycorrhizal association resulting in increased nutrient supply	Increased biomass and wood yield.	Improved nutrient acquisition	Cely et al. (2016)

terms of nitrogen fixation; cyanobacteria (*Anabaena, Nostoc*, *Azospirillum, Azotobacter, Azoarcus*) are the prevalent example of non-symbiotic microorganisms. They provide lesser nitrogen to plants in contrast to symbiotic microorganism all nitrogen fixation capacity depends on the requirement of the plant. Nitrogenase enzyme plays important role in nitrogen fixation based on metal cofactor. This enzyme is classified into three categories; (i) Mo- nitrogenase (ii) V- nitrogenase (iii) Fe- nitrogenase and *nif* gene responsible for nitrogen fixation (Raklami et al. 2019).

Most of the soil phosphorus is insoluble (inorganic apatite or as organic inositol phosphate (soil phytate, phosphomonoesters, or phosphodiester) and therefore unavailable for plant growth. Phosphorous fertilizer is also immobilized immediately after application and becomes unavailable to plants. An important trait of plant growth-promoting rhizobacterial action is to convert the phosphorus into available form for the plant by phosphate-solubilizing bacteria, which is carried out by a variety of different phosphatase enzyme synthesis (Sivasakthi et al. 2015). Bacteria such as *Azotobacter, Bacillus, Enterobacter, Flavobacterium, Rhizobium* are categorized under phosphate solubilizing bacteria. Plants always need phosphorus for nutrition. Now-a-days biotechnology approaches are used for efficient use of P from soil to plants involving PGPR bacterial inoculation (Elizabeth et al. 2017). Due to providing nutrition to plants, phosphate solubilizing rhizobacteria are used as an alternative to chemical fertilizers for development and production in *Triticum aestivum* var. Galaxy 2013 (Batool and Iqbal 2019).

Aerobic soils contain iron in the ferric form which is not readily accessible by plants. Bacteria considered under plant growth-promoting rhizobacteria secrete siderophores which are soluble in water, produce iron chelators that assist the soil ferric ions to get accessible by plants. Therefore, PGPR-mediated iron sequestration is one of the important to survive with such a limited supply of iron. It is mediated through low-molecular-mass siderophores (~400–1500 Da) that bind with Fe^{+3} and attach to bacterial membrane receptors to form Fe-siderophore complex, thereby microorganisms facilitate the reduction of iron and compensate the iron nutrition in the plants.

In some non-Gramineae plants rhizosphere needs acidification for efficient utilization of iron. Generally, iron in soil is present in ferric form. Siderophores increases the solubility of iron through proton extrusion; because it lowers the soil pH and this process enhances the trans-plasma membrane redox activity for ferric to ferrous reduction via ferric chelate reductase transport ferrous form into root cell via iron regulator transporter1 (IRT1) and in some plant such as Gramineae they secrete mugineic phytosiderophore via efflux transporter of mugineic acid (TOM1) present in the plant for solubilizing ferric ion in the rhizosphere and transport into the plant via yellow stripe 1 transporter with the help of Fe(III) phytosiderophore complex (Cely et al. 2016). In this way, iron availability to plants is in under circumference of redox potential and pH of the soil and siderophores secreting microbes are quite helpful in the absorption of iron; apart from the uptake of iron they also assist in tackling and controlling the stress conditions imposed on plants by high heavy metal content present in soil (Saif and Khan 2018). Generally, bacteria produce few kinds of siderophore such as hydroxamate, catecholate, salicylate, carboxylate; and their amount gets changed according to the PGPR bacteria.

The leguminous plants need more iron-containing protein for nodulation in comparison to non-nodulating plants. It has been observed that Fe deficiency in soil can induce signals for phenolic exudation which alters the rhizosphere microbial environment (Jin et al. 2014). Under the PGPR section, *Escherichia coli, Salmonella, Klebsiella pneumoniae, Vibrio cholerae, Vibrio anguillarum, Aeromonas, Aerobacter aerogenes, Enterobacter, Yersinia and Mycobacterium* species, *Geobacter sulfurreducens,* are very popular. The ferrous amount converted from Ferric present in soil can be measured from Ferrozine assay and determine its concentration from UV spectrophotometer. Further, scanning electron microscopy, energy disperse spectroscopy, Fourier transformed infrared spectroscopy, and X-ray diffraction are used for analysis. Malachite green is used for the cross-check of iron content and quantification (Polgari et al. 2019; Kooli et al. 2018; Kannahi and Senbagam 2014).

4.2.1.2 Modulating Phytohormone Levels

Phytohormones play significant roles in plants related to cell division, cell growth, cell development, vascular bundle development, and are ultimately involved in plant growth. Apart from these attributes, they have the ability to cope in adaption in the changing environmental conditions including reduction of stress conditions (Dilfuza et al. 2017). Endogenous phytohormones of the plant are variable in relation to the environmental conditions and are adjusted accordingly in order to tackle the negative effects of environmental stress. Soil bacteria are reported for phytohormone production in several reports especially Plant growth Promoting bacteria and can produce either cytokinins or gibberellins or both or auxins, such as indole acetic acid viz. within the acellular strains of *Acinetobacter* sp., *Rhizobium* sp., *Mycobacterium, Pseudomonas sp., Bacillus sp., Rahnella, Arthrobacter, Klebsiella,* and *Paenibacillus polymyxa* (Verma et al. 2016). Moreover, plant growth promotion by auxin, cytokinin, gibberellins, and other phytohormones is involved in different activity related to plants growth and development.

Phytohormone affects differently in different parts of plants such as auxin is involved in the plant cell division, cell development, cell elongation, tissue differentiation, apical dominance, root growth formation and assist in the germination. Indole 3 acetic acid (IAA) is well-known auxins produced by many PGPRs. IAA effects on branching number, weight, size, enhance the surface area in the soil which increase the nutrition exchange, re-differentiate root tissue from stem tissue, and overall IAA support plant growth (Gouda et al. 2018). Different PGPRs have different pathways for the synthesis of IAA such as *Rhizobium, Bradyrhizobium, Azospirillum* synthesize via indole 3 pyruvic acid pathways; some pathogenic bacteria *Agrobacterium tumefaciens, Pseudomonas syringe, Pantoea agglomerans, Rhizobium, Bradyrhizobium* and *Erwinia herbicola* synthesize IAA via Indole 3 acetamide pathways; and *Bacillus subtilis*, *B. licheniformis*, *B. megaterium*, etc. produce IAA via. tryptamine pathway.

Indole acetic acid is produced in *Azospirillum brasilense* by the independent pathway of L-tryptophan. Cytokinins involved in the shoot development, cellular proliferation, differentiation activity, influence physiological processes, involved in chlorophyll production, enhanced root hair production, promotion of seed germination and assist in grain filling stage. Plant growth-promoting strains of bacteria isolated from barley, canola, bean, and Arabidopsis plants produce cytokinins. The bacteria belong to the genera, *Pseudomonas Azospirillum*, *Bacillus, Proteus*, *Klebsiella, Escherichia*, *Xanthomonas*, *Rhizobium, Bradyrhizobium* etc. Zeatin and Kinetin are abundant cytokinins widely produced by plant growth-promoting rhizobacteria.

Abscisic acid plays important role in adaption and growth regulation. Gibberellic acid shows the importance in their shoot growth, seed germination, stem elongation, flowering, and fruit setting and assist in maintaining the metabolism of plants. Among *Bacillus* sp., two strains show prominent production of gibberellic acid; *B. pumilus* and *B. licheniformis*. Besides, *Azospirillum* sp. and *Rhizobium meliloti*, *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae* are also popular PGPRs (Gouda et al. 2018). Ethylene hormone is also quite essential for growth and development. PGPRs microbes involved in ethylene regulation possess the enzyme 1-aminocyclopropane 1-carboxylate deaminase enzyme which protects plants from adverse environmental situations, and also reduces senescence and facilitates nodulation (Muhammad et al. 2007). *Pseudomonas putida* UW4, *Azospirillum lipoferum* 4B, *Rhizobium leguminosarum* bv *viciae* 128C53K, *Agrobacterium* spp., *Achromobacter* spp., *Burkholderia* spp., *Ralstonia* spp., *Enterobacter* spp. *Mesorhizobium*, *Phyllobacterium brassicacearum* STM196 isolated from *Arabidopsis thaliana* seedlings are responsible for regulation of ethylene production.

Several microbes produce groups of phytohormone that aid plant growth and development such as *Pseudomonas putida, Enterobacter asburiae, Pantoea agglomerans, Rhodospirillum rubrum, Pseudomonas fluorescens, P. aeruginosa, Paenibacillus polymyxa, Stenotrophomonas maltophilia, Mesorhizobium ciceri, Klebsiella oxytoca, Bacillus subtilis, Azotobacter chroococcum and Rhizobium leguminosarum* stimulate the auxins, gibberellins, kinetin and ethylene production (Dilfuza et al. 2017).

4.2.2 Indirect Mechanisms

Indirect action of plant growth-promoting rhizobacteria is related to its ability to act against phytopathogenic species and thus promote the plant growth and development by providing safe uncompetitive surrounding to the plants. Understanding the indirect mechanism of plant growth-promoting rhizobacterial action may add to the exploration of the best biocontrol agents replacing the commercial chemical pesticides and other agro-additives used presently for the control of plant diseases.

4.2.2.1 Antibiotics and Lytic Enzymes

Antibiotic synthesis by plant growth promoting bacteria is an important trait that prevents the proliferation of plant pathogens (Beneduzi et al. 2012). Some of these biocontrol PGPR strains have been commercialized. To prevent antibiotic resistance development, some biocontrol strains synthesizing hydrogen cyanide as well as one or more antibiotics have been utilized. Some biocontrol bacteria produce lytic enzymes that can lyse portion of the cell wall of many pathogenic microorganisms. Plant growth promoting bacteria which are able to synthesize one or more of these antibiotic is found to have biocontrol activity against a range of pathogenic microorganism including pyrrolnitrin and 2,4 DAPG antibiotics from *P. fluorescens* BL915 for *Pythium*; Phenazine antibiotic from *Pseudomonas* for *Fusarium oxysporum;* Circulin, polymyxin and colistin antibiotics from *Bacillus* sp. for gram-negative, gram-positive bacteria and pathogenic fungi; and Fngycin and iturin antibiotics from *Bacillus subtilis*. Apart from these other well-known antibiotics are Oomycin A, Viscosinamide, Butyrolactones, Kanosamine, Pseudomonic acid, Azomycin produced by PGPRs (Tariq et al. 2017).

4.2.2.2 Siderophores

Siderophores are low molecular weight organic compounds consisting of hydroxamates (composed of a hydroxyl group closely associated with a nitrogenous portion of the molecules) which have a high affinity for multivalent iron chelators that transport iron into bacterial cells. Siderophores produced by Plant growth promoting bacteria acquire iron in sufficient quantity, and thus, can prevent some phytopathogens growth and thereby limiting their ability to proliferate (Beneduzi et al. 2013). It is one of the effective mechanisms of biological control by plant growth promoting bacteria which are able to produce siderophores that forms a complex with iron and deprive pathogenic microorganism and lead them for starvation by causing non-availability of iron. The concentration of siderophore in the soil is approximately 10^{-30} M. Siderophore-producing bacteria usually encountered are *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* which release pyochelin and pyoverdine type of siderophores like, Fe–ferrioxamine for oat, Fe–pyoverdine for Arabidopsis, Fe–erobactin for soybean and oat, Fe–rhizoferrin for tomato, barley, and corn and *Bacillus subtilis* GB03 form *Arabidopsis* (Nadeem et al. 2015; Chaparro et al. 2012; Sayyed et al. 2013; Jin et al. 2014).

4.2.2.3 Competition

Disease incidence and severity depends upon the competition between pathogens and nonpathogenic plant growth promoting bacterial species. PGPR microorganism assists in controlling pathogen growth by producing a limiting factor for the pathogenic microorganism in the nutrition and their fundamental niche. Similarly, it is observed that the leaf bacterium *Sphingomonas* sp. produces a shield to plants from the bacterial pathogen *Pseudomonas syringae* and *Bacillus* sp. shows the competition for nutrition to *Botrytis cinerea* (Innerebner et al. 2011; Kundan et al. 2015).

4.2.2.4 Ethylene

The presence of phytopathogens forms a typical stress response resulting in ethylene production in plants which exacerbates the effects of the stress on the plant (Muhammad et al. 2007). Thus, regulation of plant's ethylene is quite responsive to cope up the plants from biotic and abiotic stresses. It assists the plants in PGPR colonization and elimination of phytopathogen and root formation by regulating auxin transport within root tip zone of the root. Gene coding ethylene synthesis enzymes such as ACC synthase, ACC oxidase, ACC deaminase containing plant growth-promoting rhizobacteria can easily mediate this effect and maintain the homeostasis of the hormone.

4.2.2.5 Induced Systemic Resistance

Plant growth-promoting rhizobacteria and pathogenic microorganism induce the phenomenon of induced systemic resistance and systemic acquired resistance, respectively, in the plants; which trigger against a broad spectrum of a plant pathogen. There is a slight difference in the induction of the plant immune system through PGPR microorganisms and pathogenic microorganisms. PGPR-related induced systemic resistance involves primarily jasmonic acid and ethylene phytohormone in the defense process while systemic acquired resistance related to plant-pathogen involves salicylic acid which induced the pathogenesis-related proteins. Therefore, induced systemic resistance and systemic acquired resistance regulate different signaling pathways for defense in plants. These phytohormones are involved in the host plant's defense stimulation which results in tackling responses against a range of pathogens.

However, both induced systemic resistance and systemic acquired resistance are related to defense activity but induced systemic resistance defense is faster, higher, and more effective than systemic acquired resistance defense. Several enzymes activated for defense in induced systemic resistance is chitinase, β-1, 3-glucanase, phenylalanine ammonia lyase, polyphenol oxidase, peroxidase, lipoxygenase, Superoxide Dismutase, Catalase, and Ascorbate peroxidase along with some proteinase inhibitors (Annapurna et al. 2013; Romera et al. 2019).

4.3 Ecology of Interactions of Plant Growth-Promoting Rhizobacteria

Understanding ecological aspects of plant growth-promoting rhizobacterial interactions are of prime importance to achieve sustainable agriculture in the present climatic scenario. Rhizosphere ecology is very complex involving physical and chemical interactions of different PGPRs, plants, and other organisms which overall affects the soil fertility and crop yield which is of main interest to humankind for understanding the plant growth-promoting rhizobacterial ecology. Thus, there is a need to understand such synergistic PGPR interactions under phyto-microbiome through holistic studies which include PGPR symbionts, soil microbiota, and other components constituting this ecosystem (Hol et al. 2013).

4.3.1 Symbiotic Organisms

Rhizobium and Leguminosae symbiotic association are one of the crucial sources of nitrogen supplementation in the soil. However, this association is influenced by various abiotic and biotic factors in the rhizosphere. It has been observed through various studies that several mutualistic species benefit the plants much in comparison to a single mutualistic relationship (Hol et al. 2013). Such observations are also seen in the case of successful nodulation in leguminous plants by different *Rhizobium* sp. Several plant growth-promoting rhizobacteria are observed to increase the nodulation efficiency of *Rhizobium* strains, and thus, nitrogen fixation (Subramaniam et al. 2015). Apart from the nodulation, at biochemical levels, plant growth-promoting rhizobacteria had been observed to increase the nitrogenase activity which in turn increases crop yield.

The nodulation incrementation, however, varies on the *Rhizobium* and plant growth-promoting rhizobacterial strains used for the study. In addition to PGPR effect on nodulation, it was observed that such a system also helps in tackling abiotic and biotic stresses. In a report, plant growth-promoting rhizobacterial inoculation helped in escaping low-temperature stress in Chickpea (Nascimento et al. 2016). Similarly, the synergistic relationship of rhizobium, PGPR, and phosphate solubilizing bacteria helps in better development of legume crops increasing production, nitrogenase activity, mineral uptake, shoot and root development, chlorophyll content and fresh and dry weight. However, effect of PGPR varies according to inoculation modes of PGPR and rhizobia controlling infection, nodulation and nitrogen fixation (Tajini et al. 2012).

Similar to the rhizobial symbiotic associations, mycorrhizal symbiotic associations influence the water and nutrient absorption in plant roots and thus help in plant growth and development along with protection against many plant diseases. Arbuscular Mycorrhiza fungi are seen to be associated with 80% of plant species where they act as the inter-connections between soil and root in the form of spores, hyphae, propagules, or extraradical hyphae (Carolina et al. 2000). Mycorrhizal association starts with intraradical hyphae growing along with the cortical cells and arbuscules formation establishing root colonization and this process is influenced by rhizosphere microbiota.

It is well established that both endo as well as ectomycorrhizal associations are influenced by the microbial population of the rhizosphere and such interactions are of prime interest. However, the complexity of such interactions hinders the better utilization of these symbiotic associations for crop sustainability (Pravin et al. 2016). Beneficial effects of the PGPR on mycorrhizae had been reflected in many studies which include enhancements in root colonization, soil improvement in terms of nitrogen content and organic matter, potential incrementation of plant resistance against pathogen attack, stress adaptation, etc. These all parameters are influenced by extracellular factors from rhizobacteria as well as the plant which influences the mycorrhizal association and thus there is a need of elucidation of the physical and chemical factors (Beneduzi et al. 2012; Sood 2003).

4.3.2 Other Microorganisms

The effect of plant growth-promoting rhizobacteria on the rhizosphere microbiota has been extensively worked out under different conditions including gnotobiotic, greenhouse, and native natural conditions. Studies related to the microbiota of rhizosphere reflected that it is very variable and changes from crop to crop, soil to the soil as well as during different stages of the plant (Shahbaz et al. 2017). plant growth-promoting rhizobacteria are also seen to be the modifier of rhizosphere microbiota due to their influence on different ecological interactions (Probanza et al. 1996). Zhang et al. 2019 worked on the sweet pepper system and reported the effect of plant growth-promoting rhizobacterial consortium on rhizosphere biota which results in disease suppression and protection against stress conditions. It has been observed that plant growth-promoting rhizobacteria acts on soil health improvement in terms of total nitrogen, organic carbon, ammonium nitrogen form, potassium and phosphorus availability, thus transforming rhizosphere ecology which favours the plant growth and development escaping adverse effects of the abiotic and biotic stress conditions (Cummings 2009). Plant growth-promoting rhizobacteria-mediated induced systemic resistance is determined by the presence of lipopolysaccharides, siderophores, and salicylic acid which are its major determinants.

4.3.3 Soil Fauna

Rhizosphere ecology accompanies efficient nutrient cycling which is controlled by the interrelationship of plant roots, microorganisms, and animals and it acts as a determinant of mineral nutrient availability from soil to plant root system (Mohamed et al. 2019). The release of root exudates initiates the process of the microbial loop in the soil which is responsible for the increase in microbial biomass. Microbial growth results in the sequestration of nutrients which are re-mobilized for plant uptake due to the microbial consumption by soil fauna. Protozoa and nematodes constitute an important population in this process which is responsible for 70% and 15% respectively of total respiration of soil animals. Protozoa and saprozoic nematodes show plant growth-promoting activity indirectly, mainly due to their key role in N-mineralization.

Interactions between protozoa and rhizobacteria in the rhizosphere which acts through the mechanism which is identified as "the microbial loop in soil" are well-known to have positive effects on plant growth. Protozoa-mediated plant growth promotion occurs due to their effect on the plant root system, rhizosphere microbiota composition, and nutrient cycling which also includes the addition of nutrients from the digestion of bacterial biomass consumed by them for their development and growth. This effect is very similar to what we termed as the "plant-growth-promoting" or "hormonal" effect. Nematodes usually inhibit plant growth and development, and thus, plant growth-promoting rhizobacteria-nematodes interactions have mainly been studied from the biocontrol point of view. Several genera are reported to have biocontrol activity against nematodes due to the production of extracellular hydrolytic enzymes, cyanide and oxidizing agents. Nematicidal activities has been reported from the following genera *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Clostridium*, *Desulfovibrio*, *Pseudomonas*, *Serratia,* and *Streptomyces* (Cetintas et al. 2018).

4.3.4 Host Rhizospheric and Endophytic Relationships

Plant-rhizobacteria relationship enhances the nutrient availability to the root system and this involves intricate physical as well as chemical interactions to modify the rhizosphere ecology which favours plant growth and development. Similarly, endophytes are the beneficial microorganisms residing in the apo-plastic space and favours the plant growth and developments due to their traits like siderophore production, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and inorganic phosphate solubilization (Lally et al. 2017). Therefore, plant growth-promoting rhizobacteria and host relationships are mainly categorized into two levels of complexity i.e., rhizospheric and endophytic (Vinayarani and Prakash 2018). Recently, Cheng et al. (2019) worked on the rhizospheric and endophytic PGPR communities of an invasive plant *Sinecio vulgaris,* and reported different types of PGPR imparting plants to develop efficiently due to phosphate solubilization and nitrogen fixation. In the same study, some of these PGPRs also improve plant capacity to potentially fight against different biotic as well as abiotic stresses. Recently, endophytic and rhizospheric culturable bacterial communities were characterized from Maize roots which were irrigated with municipal and industrial wastewater and their findings are interesting to notice that most of the PGPR isolated are salinity and heavy metal resistant. Plant growth-promoting rhizobacteria have more potential of heavy metal tolerance than the endophytic ones along with their increased root colonization under wastewater irrigation in comparison to control (Abedinzadeh et al. 2019).

4.4 Improving Rhizobacterial Potential by Genetic Modification

The identification of genes related to the ability of rhizobacterial strains to plant growth promotion creates the potential to improve the capability of biocontrol strains or to construct novel biocontrol strains by genetic modification tools. With the advancements in molecular biology techniques, the genetically-modified rhizobacteria have been developed with additional features such as contaminant-degrading genes that have been transferred to conduct the bioremediation in the rhizosphere. However, many technologies have been developed including high throughput sequencing of bacterial genomes and genome editing techniques which facilitate the genome modification apart from improvements in the visualization and imaging techniques for more close observation in biocontrol behaviour (Glandorf 2019). Table 4.2 enlists few genetically modified plant growth-promoting rhizobacteria.

Important issues related to the selection of compatible strain for gene modification and incorporation into the rhizosphere includes: (1) Stability of the strain after cloning and the high expression of the target gene in the host strain; (2) Tolerance of the strain towards contaminants or insensitiveness to the contaminants; and (3) Survival of the strains as some strains can survive only in specific plant rhizosphere even after genetic modification. Before marketing genetically modified biocontrol agents registered as a plant protection product, the effect of genetically modified plants on humans and the environment must be ascertained.

Some studies have shown that gene introduction into plant growth-promoting rhizobacteria enhanced the capability of rhizobacteria (Maxime et al. 2014). Several genes are identified through whole-genome sequencing includes, *pqq* gene for Phosphate solubilization, *acdS* gene for ACC deaminase activity, *phzF* gene for phenazine production, *pvd* gene for siderophore production, *chiA* gene for chitinase production, *cry* gene for insecticidal activity, *nif* gene of *Rhizobium* for nitrogen fixation and *phl* gene for phloroglucinol synthesis. Few genes are transferred into PGPR strain such as *nif* gene in *Paenibacillus* strains via horizontal gene transfer from Frankia.

Phosphate solubilizing *Pseudomonas* sp. strain P34 enhanced the growth of wheat and can be utilized as biofertilizer (Liu et al. 2019). The genes transferred from *P. fluorescens* WCS365 have also been shown to improve the competitive colonization capability of other *Pseudomonas* isolates. *Pseudomonas capeferrum* (WCS$_{35}$8r) has been modified to produce naturally occurring secondary metabolites like, phenazine carboxylic acid or 2,4 diacetyl phloroglucinol (DAPG); these secondary metabolites help plants in defence and are indirectly involved in enhancing

Table 4.2 Genetic Engineering of plant growth-promoting rhizobacteria. Various plant growth-promoting rhizobacteria are modified through genetic modification for desired traits for better utilization

S. no.	Plant growth-promoting rhizobacteria	Plants	Genetic modifications	Traits	References
1	*Pseudomonas fluorescens*	*Oryza sativa L.*	EMS mutation; UV mutation	Improved production of plant growth-promoting substances and phosphate solubilization	Sivasakthi et al. (2015)
2	*Bacillus subtilis*	*Oryza sativa L.*	EMS mutation UV mutation	Improved production of plant growth-promoting substances & phosphate solubilization	Sivasakthi et al. (2015)
3	*Pseudomonas aeruginosa*	Tobacco	Expression of salicylic acid biosynthesis genes	Improvement of salicylic acid production	Maurhofer et al. (1998)
4	*Pseudomonas*, *Bacillus*, *E. coli*, *Proteus*, *Shigella*	Fenugreek	Physical agents (UV) and chemical agent (EMS)	Seed germination promotion	Queen et al. (2016)
5	Bacterial isolates	*Withania somnifera*	Physical agent (UV B exposure)	Seed germination promotion	Rathaur et al. (2012)
6	*Bacillus amyloliquefaciens* subsp. plantarum SA5	–	Protoplast fusion technique	Improving the nematicidal potential	Abdel-Salam et al. (2018)
7	*Lysinibacillussphaericus* Amira strain	–	Protoplast fusion technique	Improving the nematicidal potential	Abdel-Salam et al. (2018)
8	*Sinorhizobium* strains	Lettuce (*L. sativa*)	–	High growth-promoting effect	Galleguillos et al. (2000)
9	*Azospirillum brasilense* Sp245	Wheat	ipdC gene expression which codes for IAA production	Better phyto-stimulatory properties	Baudoin et al. (2010)
10	*Azospirillum brasilense* Sp6	*Sorghum bicolor* L.	Gene mutation and tagging	Overproduction of indole-3-acetic acid	Basaglia et al. (2003)

EMS ethyl methane sulfonate, *UV* ultraviolet

productivity (Glandorf 2019). Genetic modified *Azospirillum* strains known for higher production of IAA, promote the growth and nitrogen uptake. Several studies are already done on genetically modified PGPR strains expressing 1-amino cyclopropane-1-carboxylic acid (ACC) deaminase activity gene for plant growth and protection plant from diseases (Gupta and Pandey 2019).

Advancement in technology includes next-generation sequencing, Transcriptome engineering, genetic editing, microarray-based gene expression analysis, gene expression profiling, qRT-PCR, metabolomics, proteomics, and other omics technologies that provides a new direction in understanding rhizospheric microbes ecology, biology, and chemistry. The present need for agriculture is to enhance soil fertility and plant growth for better yield without chemical additives. These technologies provide convenient insight into microbial ecology to fulfil the requirement of present agriculture.

Rhizosphere engineering related to plant growth-promoting rhizobacteria may also contribute to plant development and growth for more production. Microbial exudates such as metabolites, small peptides, lipids, proteins stimulate the growth of plants by different interactions (Canarini et al. 2019). In rhizosphere engineering, synthetic biology techniques are used to incorporate the beneficial PGPR microbial strains in the rhizosphere to harvest maximum benefits of microbes. Besides introducing plant growth-promoting rhizobacterial strains, genetically engineered plants are also exploited to alter the rhizosphere (Rondon et al. 1999). Genetically modified plants change their root exudate profile and manipulate gene expression levels which facilitates interaction with plant growth-promoting microorganisms around the root zone of plants.

The Root exudates serve as communicating molecules for biological and physiological interactions in the rhizospheric zone. Even simple inoculation of plant growth-promoting rhizobacterial strains in plants may alter expression of some of the genes. In spite of specific, these changes are random and it require lots of exercises in order to reduce time for engineering. In context to plants several transporters such as UMAMIT transporter, CAT transporter, GDU transporter for amino acid, SWEET transporter for sugar, ALMT/Malate, MATE/citrate transporter for organic acid has been modified to enhance the root exudates. Under control of constitutive promoter *GOS2* and root-specific promoter *RCc3,* overexpression of *OsNAC10* gene in rice increases plant tolerance to drought, high salinity, and low temperature at the vegetative stage (Jeong et al. 2010).

Gene transition in transgenic plants has been constructed for higher efficiency of remediation in compare to bacterial transgenic. A popular example is a transgenic plant of ACC deaminase gene expression exhibits much more advantages over its expression in the bacterial system: (1) the ACC deaminase activity in bacteria is much lower than in the -in transgenic plants during initial stages; (2) it leads to a higher metal accumulation due to constant stimulation of the plant growth; (3) increase in the shoot/root ratio in some cases. However, sometimes constitutive expression of some genes may lead to such modified plants which may perform worse than the original plant because of the fact that transfer of even single trait can have profound effect on plant-rhizosphere interaction which may have adverse effect on plant performance.

4.5 Commercialization of Plant Growth Promoting Rhizobacteria

Plant Stress management in agriculture needs different strategies for sustainable agricultural productivity. Plant growth-promoting rhizobacterial inoculation is one of the crucial strategies employed for an increase in crop yield. However, the results are not satisfactory in many cases due to the limitation in the accurate knowledge about the plant-PGPR system and its mechanism of action. Commercial use of plant growth-promoting rhizobacteria belonging to the microbial genera like, *Agrobacterium, Azospirillum, Azotobacter, Bacillus, Burkholderia, Paenibacillus, Pantoea, Pseudomonas, Serratia, Streptomyces, Rhizobium* are used commercially for the efficient crop cultivation (Backer et al. 2018). Bacterial bio-inoculants as biocontrol agents are also recently available in the markets which belong mostly to the genera *Azospirillum, Bacillus, Pseudomonas, Serratia, Streptomyces, etc.* for improving the disease-fighting potential of plants (Bushra et al. 2017).

Although commercial use of plant growth-promoting rhizobacteria as biofertilizers, biocontrol agents is very lucrative and presents an eco-friendly solution to the recalcitrant chemical pesticides, fertilizers, their application is limited by the knowledge gap related to the following parameters viz. mechanism of action involved, growth parameters, large-scale production, cost-effective formulation, marketing, ease of use, and farmer awareness (Rachel et al. 2018). These constraints can be tackled by the multidisciplinary study of plant-PGPR-surrounding interactions and identifying the different physical and chemical principles involved in the establishment of conditions favouring the efficient growth and development of plants.

This requires extensive studies of direct as well as indirect mechanisms utilized by PGPR which include growth regulator production, nitrogen fixation, siderophore production, phosphate solubilisation, antibiotics production, extracellular secretion of different hydrolases, and competitive inhibition of pathogens (Bushra et al. 2017). The need for sustainable substitutes to the chemical agro-additives is necessary for the enhancement of crop yield under the changing climatic conditions but it should be carried out keeping the fact that PGPR can also pave potential eco-hazards due to microbial nature (Carlos et al. 2019). This, therefore, necessitates the evaluation of biosafety aspects of different PGPR released for commercialization and used for agricultural production.

4.6 Perspective

Plant growth-promoting rhizobacteria are emerging as an efficient alternative to chemical agro-additives, which are being used under formulated regulations and sometimes forbidden completely (Anwar et al. 2017; Mohd et al. 2019). Agricultural applications depend upon the better elucidation of PGPR properties and mechanisms underlying at both molecular as well as the genetic levels which strongly promotes the performance of PGPR as agro-additive. Such understanding also narrows the gap occurring in beneficial effects under gnotobiotic conditions and at the field applications (Nailwal et al. 2014; Rocheli et al. 2015). This will also impart the potential to enhance the plant growth promotion activity of plant growth-promoting rhizobacteria utilizing the advanced genetic modification methods.

Plant growth promoting activity can also be supplemented using consortium strategies i.e., combination of different plant growth-promoting rhizobacteria in place of using a single strain and in most of the cases this helps in increasing the crop productivity (Carlos et al. 2019). The beneficial effects are also achieved if we include the soil studies and inoculation of plant growth-promoting rhizobacteria as per the native nature of the soil. The success of plant growth-promoting rhizobacterial inoculation depends upon the soil factors including both the abiotic as well as biotic. We know that plant and beneficial microbes form a holobiont system in which plant provides the microbes with reduced carbon and other important metabolites and in return, microbes provide better nutrient bioavailability, assimilation, soil texture modification, root colonization, pathogen control, etc. This takes place by a large array of chemical secretion from the plant as well as microbes (Lally et al. 2017). Thus, presently researches are carried to study plant root exudate which comprises various chemical compounds which act as root colonization signals for the beneficial microorganisms.

Similarly, analysing the microbial signalling in the rhizosphere helps in developing a better agro-inoculant and alleviates problems associated with the failure of most of plant growth-promoting rhizobacteria under the field study. The genetic-engineering technologies and their advanced variations greatly expand the extension and degree of bioremediation (Patel et al. 2016). However, applications are constrained due to a lack of understanding of genetic mechanisms underlying the plant growth-promoting rhizobacterial action especially the establishment of the plant growth-promoting rhizobacteria in its host plant rhizosphere which is affected by various contaminants including high concentrations of heavy metals (Ojuederie and Babalola 2017; Saima and Mohammad 2017; Shukla et al. 2011). Thus, this requires a holistic study of the biochemical nature of the signalling compounds which helps in the establishment of plant-PGPR holobiont system along with the elucidation of genetic changes that control such signalling. Therefore, it is the utmost requirement to decipher the interrelations of this system by genomics, metabolomics, and proteomics high throughput tools.

4.7 Conclusion

The plant-microbe interrelationships as understood by various research works represents the necessity of analysing in detail what we term as “Phytomicrobiome”. Studies related to the composition of Phytomicrobiome, signalling and variations at different stages of the plant as well as in different plants revealed many interesting factors which influence plant growth and development. It is now well established that the success of plant growth-promoting rhizobacteria-based agro-additives doesn't only requires isolation and study of a plant growth-promoting rhizobacterial strain but needs also to carry out the detailed study of compatibility between the plant growth-promoting rhizobacteria, plant, and phytomicrobiome. This will result in the potential application of plant growth-promoting rhizobacteria for sustainable crop management. Presently, we are at infancy in understanding such interrelationships and require studying the phyto-PGPR system by applying advanced methods of analysis. Thus, presently plant growth-promoting rhizobacterial research has a similar impetus as in previous decades, and it's revealing the many intricate plant-microbe relationships in soil with the help of advanced life science technologies.

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Chapter 5
Diversity and Evolution of Nitrogen Fixing Bacteria

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Abstract Nitrogen is a major element for plant life, yet environmental nitrogen is poorly available to plant, and thus classified as a ‘limiting element’. As a consequence, most plants, except the insectivorous florae, rely upon microbial partners to maintain the nitrogen supply. Nitrogen-fixing prokaryotes are widely distributed and account for the fixation of nearly 50–200 megatonnes of nitrogen per year. Nitrogen-fixing microorganisms are potent agents for applications in agricultural fields. Reduction of gaseous dinitrogen to bioavailable nitrogen is mainly done by the molybdenum-dependent nitrogenase in archaea and eubacteria. In plants, the process of nodulation has evolved from 100 million years ago, confering the nodulation capability to about 70% of leguminous plant species. The genes necessary for the nitrogen fixation evolved only after the divergence of bacteria and archaea. Furthermore, the nitrogen-fixing endosymbionts are supposed to have evolved many times in the higher plants, especially in angiosperms. This chapter reviews the diversity and evolution of nitrogen-fixing bacteria.

Keywords Nitrogen · Nitrogen fixation · Endosymbiosis · Rhizobiaceae · Nitrogenase · Symbiotic nitrogen fixation · Associative nitrogen fixation · *Nostoc* · Evolution · Evolutionary analysis

5.1 Introduction

Nitrogen, an unambiguously regulating element meant for plant growth, is primarily present in the atmosphere in the form of dinitrogen gas (N_2), which is an incongruous source for plants. As an alternative, plants depend on reduced forms of

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nitrogen, such as nitrate (NO_3^-) or ammonium (NH_4^+), which can be easily absorbed from the soil. In agroecosystems, nitrogen supply is maintained by introducing plant-available forms in the form of fertilizers fashioned either through various chemical methods, e.g., the Haber–Bosch process that transforms the atmospheric nitrogen into ammonia by expending a large quantity of energy, or, sporadically, from mining mineral deposits, e.g., rocks encompassing potassium or sodium nitrate. Conversely, in most of the unobstructed non-agricultural systems (along with a few agricultural ones), the soluble form of nitrogen (ammonium) is made accessible to the plants through a process commonly acknowledged as biological nitrogen fixation (James 2017). The process of biological nitrogen fixation, which is defined as the capability of converting atmospheric nitrogen (N_2) to its reduced form, is certainly the earliest innovation utterly accomplished by various members of bacteria and archaea and is also one among the ecological services of supreme significance that are offered by microbes to different eukaryotes.

A diverse array of organisms, comprising plants, animals, protists, and fungi form symbiotic relations with nitrogen-fixing bacteria, but such associations appear to be unfastened and only a little indication of any kind of evolutionary amendment is found to be prevailing in either of the partners (Masson-Boivin and Sachs 2018). However, some plants have evolved the ability to make some specific root structures called nodules where diazotrophic bacteria are hosted intracellularly (van Velzen et al. 2018). A diverse community of leguminous and non-leguminous plants hosts a polyphyletic crowd of diazotrophs belonging to α and β proteobacteria which are together known as rhizobia. The additional group of nodulating species of plants is said to be ‘actinorhizal’ as a result of their passion for hosting diazotrophic filamentous Actinobacteria belonging to the genus *Frankia*.

The nitrogen fixation in leguminous plants is well documented; however, it differs from that of nitrogen fixation in actinorhizal plants. The actinorhizal plants develop an endosymbiosis with the nitrogen-fixing soil actinomycete *Frankia*. The establishment of the symbiotic process results in the formation of root nodules in which *Frankia* provides fixed nitrogen to the host plant in exchange for reduced carbon. As in the case of rhizobium–legume symbioses, the compatible interaction between *Frankia* and actinorhizal plants due to fine-tuned exchange of signals between the two partners leads to the development of nitrogen-fixing nodules. On the plant side, although the involvement of flavonoids in symbiosis is poorly understood, several studies indicate that they may play a significant role in the early stage of the interaction. The flavonols present in the root exudates enhance the level nodulation. Several genes of the isoflavonoid biosynthesis pathway are also known to be up-regulated during the early steps of plant-microbe interactions. The *Frankia* root hair deforming factors are known to play a similar role as played by Nod factors in legume-rhizobium symbiosis.

Although, their chemical properties differ from that of Nod factors but these are also known to be structurally similar to *Rhizobium* nod factors owing to the presence of *N*-acetyl-glucosamine, the backbone of *Rhizobium* Nod factors. After the successful completion of plant-microbe interactions, the actinobacterial auxin possibly plays a role in plant cell expansion, cell-wall remodeling, induction of adventitious roots, and in increasing the level of auxin in nodule primordial. The root infection may proceed either intracellularly or intercellularly depending upon the plant species involved. Unlike legume nodules, actinorhizal nodules are modified lateral roots (Santi et al. 2013). However, legume nodule primordia are formed in the root cortex and develop into stem-like organs with a peripheral vascular system and infected cells in the central tissue (Pawlowski and Sprent 2007).

Inside these specialized structures, these microsymbionts are provided with physiological circumstances favoring the catalysis of the conversion of atmospheric N_2 to NH_4^+ by the bacterial enzyme complex nitrogenase which is a complex and energy-intensive enzyme system, demanding 16 moles of adenosine triphosphate for a single mole of nitrogen fixed (Hoffman et al. 2014). Universally, the process of biological nitrogen fixation is solely accountable for the fixation of around 50–200 megatonnes of nitrogen per year added to earthly ecosystems, which makes up about 80% of the total fixed nitrogen. While it is anticipated that terrestrial contributions of biological nitrogen fixation are principally owing to the legume-rhizobial symbiosis, however, it is not easy to estimate biological nitrogen fixation by legumes in the field conditions (James 2017).

The evolution of the process of nodulation in plants is believed to have happened firstly nearby 100 million years ago leading to the possession of nodulation capability in around 70% of the leguminous plant species, and also in several plants, predominantly called as actinorhizal plants, which find their distributionwide three Angiosperm orders (Werner et al. 2014; Van Velzen et al. 2017). In parallel, the capacityof nitrogen fixation within legumes has found its way to hundreds of species in both α-Proteobacteria as well as β –Proteobacteria (Remigi et al. 2016), referred to as rhizobia, while the ability to nodulate actinorhizal plants has been restricted to the *Frankia* genus in Actinobacteria. Incongruously, most of the essential food crops like wheat, rice, corn, and tuber and root crops have not evolved with the nodule-based symbiotic association thereby lacking their nitrogen-fixing partners. However, another kind of, somewhat fewer formal relations, entitled as associative symbioses, is well known among several kinds of grass and bacteria, for instance, the tropical grasses, *Digitaria* and *Paspalum* often subordinate with the bacteria, *Azospirillum brasilense*, and *Azotobacter paspali*, respectively (Döbereiner et al. 1972).

A vast array of nitrogen-fixing microbes, however, is not associated with either animals or plants. They are freely living inhabitants of the soil which fix nitrogen for their own use. These free-living nitrogen-fixing bacteria also encompass some bacteria of agricultural importance such as cyanobacteria. The most comprehensively studied members of free-living nitrogen-fixing bacteria are represented by the members such as *Azotobacter vinelandii* (an obligate aerobe), *Clostridium pasteurianum* (an obligate anaerobe), *Klebsiella pneumoniae* (a facultative anaerobe), *Rhodobacter capsulatus* (a photosynthetic bacterium), and numerous *Nostoc* and *Anabaena* species. The death of such free-living nitrogen-fixing microbes marks the availability of nitrogen fixed by these organisms to the neighboring ecosystems (Fisher and Newton 2002). The present chapter targets the diversity of different nitrogen-fixing

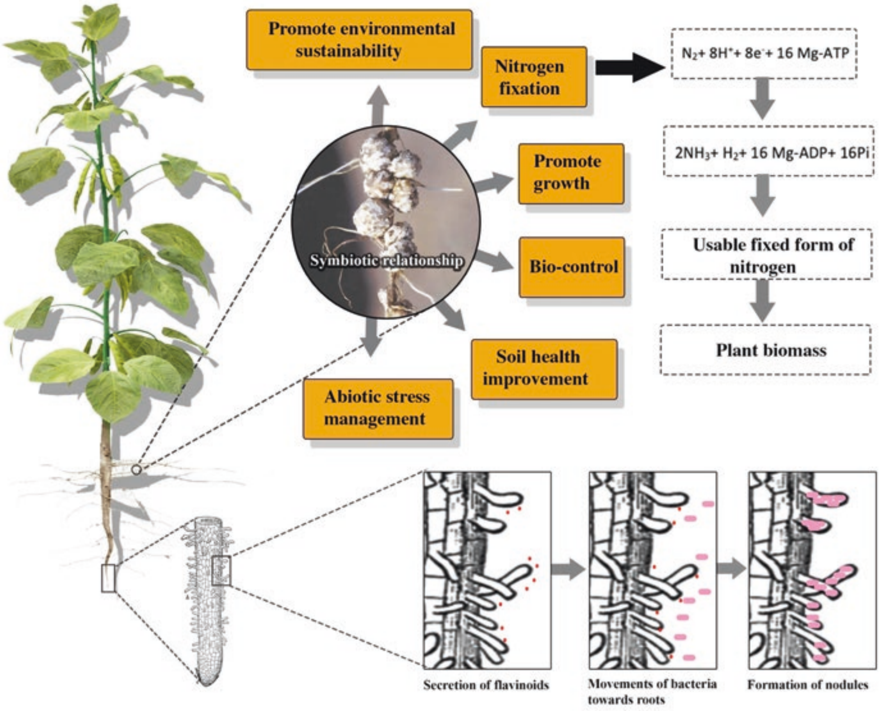

Fig. 5.1 Effects of nitrogen fixing microorganisms on plant growth and soil health

bacteria along with their importance for different ecosystems. The evolution of nitrogen-fixing bacteria is also guided further along with the evolution of the nitrogen-fixingsymbiotic relationship. Figure 5.1 enumerates the overall effects of nitrogen fixation by N_2-fixing microbes.

5.2 Diversity of Nitrogen-Fixing Bacteria

Although nitrogen is present as the most copious compound on the crust of the earth, it is however inaccessible for the majority of microorganisms. Nonetheless, nitrogen is the central part of almost all the proteinaceous compounds as well as nucleic acids; it is a fundamental need of all active living organisms. The conversion of nitrogen into ammonia via a natural process catalyzed by an enzyme complex known as nitrogenase requires an excessive amount of energy at its end (Canfield et al. 2010). This enzyme complex is usually present in some selective members of bacteria as well as archaeal taxa (Young 1992).

5.2.1 Diversity of Free-Living Nitrogen-Fixing Bacteria

The cycling of nitrogen in natural systems, including the agricultural sector, depends upon the fixation of nitrogen by biological means mainly by diazotrophic bacteria which are remarkably diversified and broadly dispersed athwart Bacteria and Archaea phyla. The preeminently acknowledged nitrogen-fixing bacteria make symbiotic relationships with their host plant and contribute to fixing a large amount of nitrogen (about 80%) by biological means (Peoples et al. 1995). However, these nitrogen fixers are restricted to only selective plants factions *e.g.,* leguminous plants, alders as well as cycad plants, whereas a very wide variety of plants is not able to make a symbiotic association with these nitrogen fixers. Free-living nitrogen fixers have got lesser consideration because of their lesser nitrogen-fixing efficiency as well as lower population size as compared to symbiotic fixers in the clement cultivation sector. These free-living nitrogen-fixing bacteria are usually heterotrophs residing in soil and contribute to fixing a noticeable amount of nitrogen devoid of any straight relationship with the plants. The advantageous characteristic of free-living nitrogen fixers over symbiotic nitrogen fixers is that the benefits of free-living nitrogen fixers are accessible to all the plants and not restricted to a particular plant group, thereby playing a good task in nitrogen delivery to the ecological system (Belnap 2002; Yeager et al. 2007).

Nitrogen-fixing bacteria free-living in soil exemplify a diverse group of bacteria counting saprophytes residing on plants litters and bacteria residing in close vicinity of plants roots with intimate relationships. For instance, bacterial species belonging to genera *Azotobacter, Bacillus, Clostridium Pseudomonas*, *Azospirillum, Klebsiella,* and cyanobacteria are examples of different types of nitrogen-fixing bacteria living freely in soil (Table 5.1). These saprophytic, freely living bacteria make their peculiar way to arrange energy supply either by oxidation of natural compounds discharged by other life forms or by the decay of residual plant materials. Some freely residing nitrogen fixers are chemolithotrophic in nature; therefore, having the potential to utilize inorganic molecules to accomplish their energy needs. In a particular set of conditions, freely residing nitrogen fixers might be able to fix a considerable amount of nitrogen (0–60 kg N ha^{-1} $year^{-1}$) (Kahindi et al. 1997; Burgmann et al. 2004). In aquatic organizations, cyanobacteria can fix up to 300 kg N ha^{-1} $year^{-1}$ in relationship with *Azolla* sp. whereas, in earthly organizations, these can fix approximately 30 kg N ha^{-1} $year^{-1}$ in close interactions with grasslands and approximately 3–5 kg N ha^{-1} $year^{-1}$ in different cultivationsystem (Paul and Clark 1989). *Azotobacter* sp. has been found to fix approximately 1 kg N ha^{-1} $year^{-1}$ while residing freely and approximately 1–20 kg N ha^{-1} $year^{-1}$ when present in baggy relationships with grasses (Paul and Clark 1989). These fixations of nitrogen occur under peculiar conditions where the readily accessible nitrogen to these bacteria is usually limited (Rosen and Allan 2007). These conditions can be created by amendments of farming lands with such organic compounds which are having lesser accessible nitrogen contents.

Table 5.1 Grouping of nitrogen-fixing bacteria

S. no.	Family	Bacteria
	Associative N_2-fixers	
1.	Rhodospirillaceae	*Azospirillum lipoferum, A. brasilense, A. amazonense, A. halopraeferens, A. irakense, A. oryzae, A. rugosum, A. formosense, A. palatum, A. canadense, A. doebereinerae, A. thiophilum, A. picis, A. zeae, A. humicireducens, A. fermentarium, A. agricola.*
	Free-living N_2-fixers	
2.	Azotobacteraceae	*Azotobacter vinelandii, A. chroococcum, A. paspali, A. salinestris, A. tropicalis, A. nigricans, A. beijernckii, A. armeniacus*
3.	Clostridiaceae	*Clostridium pasteurianum*
4.	Pseudomonadaceae	*Pseudomonas stutzeri*
	Symbiotic N_2-fixers	
5.	Rhizobiaceae	*Rhizobium hainanense, R. indigoferae, R. leguminosarum, R. lupine, R. lusitanum, R. miluonense, R. tropici, R. cellulosilyticum, R. daejeonense, R. etli, R. gallicum, R. giardinii, Sinorhizobium meliloti, S. americanum, S. fredii, S. medicae, Allorhizobium borbori, A. vitis, A. oryzae, A pseudooryzae*
6.	Bradyrhizobiaceae	*Bradyrhizobium japonicum, B. elkanii, B. yuanmingense, B. liaoningense, B. betae, B. cytisi*

Free-living nitrogen-fixing bacteria perform nitrogen fixation under such conditions where the amount of oxygen is either limited or absent due to the sensitivity of the nitrogenase enzyme towards oxygen which is the main enzyme responsible for nitrogen fixation. The abundance of free-living nitrogen-fixing bacteria in soil is generally low due to the limited amount of suitable carbon and energy resources. Furthermore, the conditions to access the suitable carbon and energy resources also happen to be least favorable (Smercina et al. 2019). However, the prevalence of favorable conditions and availability of suitable energy sources can encourage these bacteria to fix a considerable amount of nitrogen, accessibility of which is not limited to specific plant taxa but it is widespread.

5.2.2 *Diversity of Symbiotic Nitrogen-Fixing Bacteria*

The highly proficient form of nitrogen fixation is established with a symbiotic relationship with higher plants where the energy for fixing nitrogen, usually, the oxygen defense organization is provided by the plant counterpart. The two major groups of the symbiotic association are represented by the prokaryotic companions which are mainly soil bacteria, rhizobia in case of symbiotic associations with legumes, and *Frankia* in case of actinorhizal symbiotic relationships. However, in the case of a symbiotic association of *Gunnera*, the microbial counterpart responsible for fixing nitrogen is the cyanobacterium, *Nostoc* sp. Although, the habitats where the symbionts are located in differs from the companion microbe, for instance, in *Gunnera* the cyanobacterial partner reside in already present stem glands, while in the case of leguminous and actinorhizal plants, specialized organs called as the root nodules, are fashioned by the plant when it comes in contact with symbiont followed by the infection process.

All the above-mentioned systems harbor prokaryotes for fixing nitrogen inside the host cells; however, they are also disjointed from the plant cytoplasm utilizing the membranes which find their origin from the plant plasmalemma (Mylona et al. 1995). The most studied symbiotic relationship involves the leguminous plant and bacteria which are cooperatively regarded as rhizobia. Rhizobial partners collaborate in this symbiotic relationship of major biological significance occurring on all the landforms and account for one-fourth of total biological nitrogen fixed yearly on earth. This relationship involves around 18,000 legume species organized with a mounting assortment of alpha and beta-Proteobacteria. The term “rhizobia” is used here as the collective term that designs bacteria to be able to form a nitrogen-fixing symbiosis with legumes (Masson-Boivin et al. 2009). Along with a greater deal of phylogenetic variety, rhizobia also display a great piece of metabolic possessions (Prell and Poole 2006).

However, only one member, *Azorhizobium caulinodans*, seems to be a candid diazotroph, which can propagate *ex planta* that too at the expenditure of fixed nitrogen (Dreyfus et al. 1988) at a relatively high oxygen concentration. Along with the presence of diazotrophy, the other amazing metabolic features are, methylotrophy in *Methylobacterium nodulans* and the ability to photosynthesize in *Bradyrhizobium* which brings nodulation in the legumes belonging to the genus *Aeschynomene.* Both of these metabolic sorts are supposed to subsidize the symbiotic relationship (Masson-Boivin et al. 2009).

5.2.3 Diversity of Associative Nitrogen Fixing Bacteria

Symbiotic nitrogen fixation is well acknowledged in different actinorhizal and leguminous plants along with its occurrence in non-nodulating species. In grasses, nitrogen fixation takes place via loose associations with different rhizospheric and endophytic bacteria. Such a type of nitrogen fixation is recognized as associative nitrogen fixation. It can solely justify for about 60% of sugarcane’s yearly nitrogen supplies. Associative nitrogen fixation has been principally premeditated in tropical species; however, it also ensues in various moderate grasses where mesocosm reports have interpreted that such kind of nitrogen fixation process contributes up to 50 kg N ha^{-1} yr.$^{-1}$ (Roley et al. 2019).

It is quite indistinct that which microbial taxa are involved when the process of fixation takes place along with uncertainty in the place of fixation in a plant that whether it is rhizosphere or any other part supporting the fixation. In the case of sugarcane, both endophytes, as well as other present rhizosphere soil, account for nitrogen fixation (Dobereiner 1961; Boddey 1987; James 2000). The site for associative nitrogen fixation is largely presumed to happen in rhizomes and roots, coupled with the subsequent transfer of fixed nitrogen to the above ground tissues. However, the presence of diazotrophs throughout the plant's structures has been highly acknowledged which clearly indicates the occurrence of nitrogen fixation in above ground plant tissues (Roley et al. 2019). The most encouraging aspirants for nitrogen fixation in grasses are *Acetobacter diazotrophicus, Azoarcus* spp. in the case of kallar grass and *Herbaspirillum* spp. in case of sugar cane, and several other species of *Pseudomonas*, *Bacillus*, *Enterobacter*, *Alcaligenes*, *Azospirillum*, *Herbaspirillum*, *Klebsiella*, and *Rhizobium*, in case of rice and maize (James 2000). Several associative, non-endophytic, and diazotrophs, nitrogen fixers find their habitat on the root surfaces, predominantly in the elongation zones and root hair, or inside dislocated epidermises.

Conversely, the endophytic diazotrophs, for instance, *Azoarcus spp.*, *Acetobacter diazotrophicus*, *Herbaspirillum spp.*, and a few strains of *Azospirillum brasilense*, incline on colonizing the root cortex, and also pierce the endodermis layer to occupy the stele, so that there can be a subsequent and perpetual translocationtowards the aerial plant parts. Another endophytic bacterium, *H. seropedicae* enters through cracks shaped at root junctions which act as another apparent site for entry in rice plants.

5.3 Importance of Nitrogen Fixing Microbes

Extensive utilization of chemical fertilizers and pesticides has directed to severe upshots counting worsening of soil as well as ecological attributes. Maintenance and reinstatement of soil health attribute to buttress the proper development of plants are necessary to sustain agricultural production. The effect of soil deterioration on gross domestic production can be remunerated using superior management practices (Meena et al. 2016). It is vitally important to increase productivity not merely to meet increased food requirements but to improve soil fruitfulness as well. The nitrogen-fixing microorganisms utilizing their communication with other microbes contribute considerably to improve soil health and environmental sustainability.

5.3.1 Maintenance of Soil Health

The constancy and production efficiency of bio-network mainly depend upon the health of the soil. Thus, the management of agricultural systems should necessarily contemplate the health of the soil and its sustainability. The properties of soil are elementary not merely for the production of good food or to sustain functional bio-network but for prevention of soil attrition and minimizing ecological influences as well. The functions of soil microorganisms in elevated input agricultural systems have received lesser interest for the reason that organically mediated practices such as regulation of soil edifice; nutrient supply and management of ailments have been majorly substituted with manmade inputs including chemical fertilizers and pesticides utilization which eventually depends upon the use of non-renewable energy reservoirs (Barrios 2007). Substantial information are mounting up about maintaining various living microorganisms and their functioning in soil reflecting elemental tasks to soil health (Barea et al. 2005).

Positive communications between plants and microorganisms in soil are supposed to be fundamental determinants of the healthiness of plants as well as soil. Nutrient cycling in the soil is a very crucial ecological task that is vital to life on this planet. Degradation and elemental conversion have recognized greater interest as well due to their necessary position in the natural cycling of nutrients (Coleman et al. 2004). The affirmative effects of soil microorganisms present in symbiotic associations on crop productivity enhance because of the increase in the number of accessible nutrients by plants, particularly nitrogen which is fixed using N_2-fixing microorganisms. The fixation of N_2 by biological means plays a significant part in increasing crop production by various modes including, (1) assimilation of fixed nitrogen straightly in crop products, (2) assimilation of fixed nitrogen in silage, (3) addition to perpetuation and reinstatement of soil health (Giller 2001).

The edifice of soil is a result of the organization of sand, silt, and clay particles in addition to soil organic matter into accumulates of various sizes using living and non-living agents. The proper dimension, amount, and constancy of soil accumulates indicates a proper equilibrium among accumulates materializing things (such as the addition of organic materials, various microbes dwelling in soil, and different vegetations) and the factors responsible for their disruption such as bioturbation and agriculture (Six et al. 2002).

The importance of soil-dwelling microorganisms in soil edifice alteration had been identified earlier by planters but the effects of soil microorganisms on accumulated construction were first hypothesized in the shape of the hierarchical model of soil aggregation just merely 25 years back (Tisdal and Oades 1982). It was additionally projected as the 'aggregate dynamic model' which connects aggregate construction and degradation in a straight line in soils to the turnover of particulate organic matter which are arbitrated by activities of microorganisms as well as macro-fauna (Six et al. 1998). This model suggests that various natural practices *viz.*, various fungal and bacterial activities, roots of plants, and macro-fauna in soil result in the construction of 'biological macro-aggregates, and steadiness of these aggregates accounts to soil edifice.

Being a major part of the plant rhizosphere, nitrogen-fixing microbes play a very crucial role in the construction and maintenance of these aggregates signifying their role in soil health maintenance. A beneficial soil community has a very distinct food network that prevents various phytopathogens and diseases in control by antagonism, predation, and parasitic interaction (Susilo et al. 2004). Therefore, there is a sturdy connection between soil-dwelling microorganisms, soil health, and plant healthiness. For instance, the cultivation of crops in impecunious soil is feebler due to deprived nutrients making the cropincreasingly vulnerable to the attack of phytopathogens and diseases assail (Altieri and Nichols 2003). The restraint of various diseases is mainly due to enriched nutrients in the soil leading to healthier plants which in turn are more capable to combat phytopathogens as well as various diseases. Thus, diverse nitrogen-fixing microorganisms help to enrich the soil with different nutrients via several means, thereby, rendering the plants healthier. It is a harmony that diversity of soil communities not merely assist the prevention of losses because of various pathogens and ailments but stimulate various central natural processes in the soil as well (Wall and Virginia 2000).

5.3.2 Environmental Sustainability

Although non-conventional agricultural practices can produce high crop yield yet it brings about considerable amounts of adverse consequences by significant utilization of inorganic fertilizers and pesticides as well as utilization of non-renewable energy sources (Sharma et al. 2020). Nitrogen-fixing microbes involved in nitrogen dynamics, as well as numerous aspects of sustaining ecology, are thus well-thought-out as a vital means to improve soil healthiness (Fig. 5.2). The present explorations draw attention to the magnitude of scheduling agricultural systems through environmental standards and bio-network facilities for augmentation of ecological as well as agricultural sustainability and productivity, reducing the amount of chemicals utilization and utilization of nonrenewable energy sources. The long-standing sustainability of the agriculture system should majorly depend on utilization and effectual dealing of natural resources (Rani et al. 2019; Sharma et al. 2019; Singh et al. 2019).

Fixing of nitrogen using microorganisms presents an inexpensively appealing and economically sensible means of decreasing exterior inputs as well as enhancing the qualities and quantities of natural sources. Biologically fixed nitrogen is a chief supplier of nitrogen by the use of symbiotically associated microorganisms. A diverse variety of nitrogen-fixing microbes present in soil plays a vital role in handling the performance as well as the sustainability of microbes in the soil, thereby maintaining the ecological constancy. The substantial alteration in types of soil or amateurish agricultural approaches including inappropriate utilization of agrochemicals has overwhelming effects on the quality of soil that in turn is destructive to soil-dwelling microbes resulting in severe damaging effects on the ecosystem (Huang et al. 2013). Numerous bacteria including nitrogen fixers present in the rhizosphere have received noticeable interest due to their handy interactions with plants in the rhizosphere and their capabilities to impart advantages to the plants by enhancing crop production, resistance to stressful conditions, and improving defense systems in plants.

Additionally, these microorganisms alleviate ecological apprehensions by imparting constancy amid plants and surroundings as well (Ahkami et al. 2017). The presence of endophytic microorganisms either bacteria or fungi in associations with plants make a multifaceted, lively as well as enthralling cross-talk environment towards prevalent applications for attaining environmental sustainability (Kusari

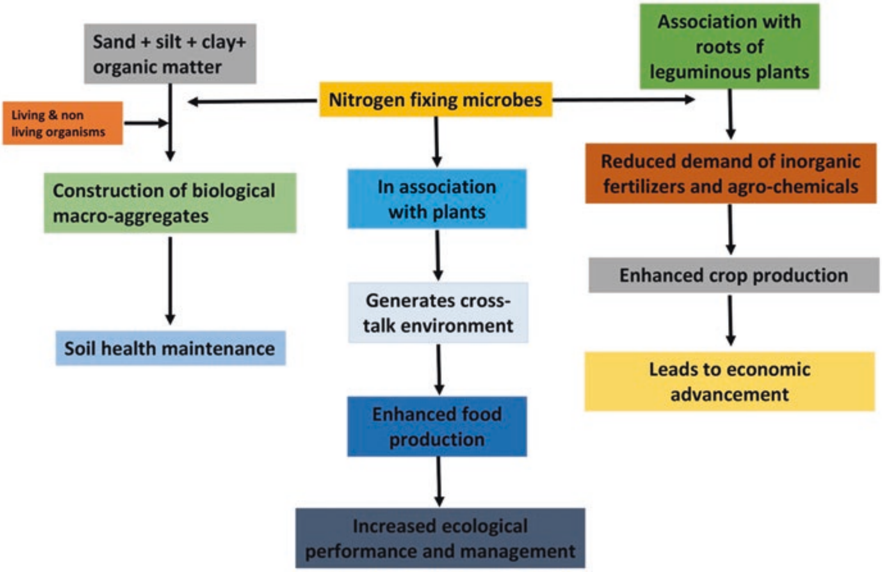

Fig. 5.2 Benefits of dinitrogen (N_2) fixing microbes

et al. 2014). Also, with the biologically fixed nitrogen, worldwide food production scaffolds can be additionally reliable in comparison to the utilization of nitrogen fertilizers. The complicated interchange among numerous microorganisms at various crossing points is imperative to feature the ecosystem and evolution for enhanced food production, ecological performance, and ecological management. The comprehension of these associations among plants and microorganisms will offer novel possibilities for sustaining the agriculture system, maintenance of vital microorganisms as well as plants, and protection of soil qualities and fruitfulness.

5.3.3 Economic Importance

According to the estimates of various studies, more than 925 million people worldwide are living in famine conditions (Hunger Statistic World Food Programme 2020). Furthermore, more than 2 billion people are anticipated to be added by 2050 (Eldakak et al. 2013). To overcome the doubts of food certainty and productivity in these circumstances, the traditional agriculture system is majorly reliant on the utilization of inorganic agrochemicals (Stehfest and Bouwman 2006). In certain mounting countries, farmers utilize inorganic fertilizers in a very intense amount in their agricultural terrains to obtain enhanced crop production because of being short of consciousness and appropriate guidance concerning modernization of agricultural approaches and their effects on ecological systems. The enlarged food products can be allocated to enhance the utilization of inorganic nitrogen fertilizers

(Rahman and Zhang 2018) and by viewing current agriculture approaches, it is anticipated to increase additionally in nearby future (Vance 2001). Because of the massive utilization of inorganic fertilizers across the world, the escalation in expenses of inorganic N_2 fertilizers has come about 104 million tonnes in 2006 from 11.6 million tonnes in 1961 (Mulvaney et al. 2009) that is anticipated to enhance additionally to a very larger extent.

Rhizobacteria present in the rhizosphere make associations with the roots of legume plants through a thread-like structure and make polymorphological structures on the roots denoting the main site of nitrogen fixation. The biologically fixed nitrogen is conferred to the plants present in symbiotic associations and makes the plants propagate in the scarcity of external nitrogen supply (Bardos et al. 2011). Furthermore, if the portion of this fixed nitrogen becomes available to the subsequently grown crop, nitrogen-fixing microorganisms play a key task in diminishing the demand for inorganic fertilizers (Crozat and Fustec 2006). The biologically fixed nitrogen symbolizes a crucial reservoir of nitrogen in an ecological system that is estimated to be about 122 million tonnes per year (Hirel et al. 2011). As a result, nitrogen-fixing microorganisms assist the decrease in the amount of chemical N-fertilizers inputs, therefore advancing the economy of the agricultural system.

5.4 Evolution of Nitrogen-Fixing Bacteria

Different life forms necessitate fixed bases of nitrogen and its accessibility frequently restricts efficiency in different natural systems. A major proportion of nitrogen on Earth is represented by dinitrogen (N_2), and its non-bio-availability is an unhidden and unquestionable phenomenon. On initial Earth, some abiotic developments, for instance, electrical oxidation of dinitrogen, were solely responsible for secure nitrogen sources (Boyd and Peters 2013).

The principal enzyme, responsible for reducing the dinitrogen to the bio-available form (ammonia), is a molybdenum-dependent nitrogenase. However, there are other phylogenetically related forms of nitrogenase divergent in the arrangement of metals at the active site, to contribute to ammonia in environments that face limitations of molybdenum (Joerger and Bishop 1988; Kessler et al. 1997). The nitrogenase enzyme is solely accountable for the fabrication of more than half of the total fixed nitrogen on Earth (Falkowski 1997). This progression of nitrogen fixation utilities to discharge limitation of fixed nitrogen in the natural environments (Zehr et al. 2003) and seems to have an unequal upshot on the working of any bio-network, comparative to involvements from any further inhabitants. Accordingly, the description of organisms capable of nitrogen fixation in the natural communities makes them keystone species (Hamilton et al. 2011).

Nature has strongly constrained the dispersal of enzyme nitrogenase to archaea and bacteria only, with no reported example of genes coding for a similar course taking place within the members of eukaryotes. The enzyme nitrogenase finds only a restricted circulation among Archea with only methanogens as the sole representatives distributed among the orders Methanococcales, Methanosarcinales, and Methanobacteriales. Any further evidence, which supports the occurrence of nitrogenase enzyme in the members of the remaining groups of Archea, is, however, has not been reported so far. Similarly, the nitrogenase enzyme also displays a narrow distribution among the bacterial communities.

For instance, the occurrence of *nif* has been advocated in numerous aerobic soil bacteria and has been identified in various cyanobacterial genomes, accounting for the inhabitants of different terrestrial as well as marine atmospheres. Furthermore, the persistence of *nif* gene clusters is frequently perceived in the genomes of Chlorobi, Chloroflexi, Firmicutes, and Bacteroidetes and numerous lines of Proteobacteria and Actinobacteria (Boyd and Peters 2013). The microorganisms exhibiting the ability of nitrogen fixation exhibit an extensive variability of physiologies ranging from strict anaerobes to obligate aerobes (Raymond et al. 2004; Boyd et al. 2011a; Dos Santos et al. 2012).

The extreme sensitivity of nitrogenase to oxygen exposure has allowed a diverse community of nitrogen-fixing microbes to evolve several strategies to perform the functioning of nitrogenase enzyme effectively under toxic environment (Gallon 1981; Berman-Frank et al. 2003). The evolution of symbiotic nitrogen fixation provides the microbial partner with an environment that can maintain low oxygen concentration. Leghemoglobin, a protein synthesized by the host plant, plays a unique role in lowering oxygen tension due to its advanced empathy for binding oxygen (Ott et al. 2005).

The evolution of another stratagem is confiscating oxygen which permits the symbiont to uphold respiration aerobically while catalyzing nitrogen fixation. Additionally, the process of nitrogen fixation takes place in the absence of oxygen in obligate anaerobes, and in the case of facultative anaerobes, it takes place merely in the course of anaerobic progression. Another member of nitrogen-fixing microbes, cyanobacteria, is the solitary diazotrophic descent which releases molecular oxygen as its metabolic product, have established numerous tools for nitrogen fixation (Fay 1992; Berman-Frank et al. 2003). For instance, the cyanobacteria which are non-filamentous in nature lean towards a diurnal cycle where the fixation of nitrogen is promoted during the night time when the concentration of oxygen has plunged significantly as a result of simultaneous reductions in the oxygen production coupled with the enlarged consumption of oxygen by co-dwelling heterotrophic inhabitants.

On the other hand, there is joint existence of oxygen production and nitrogen fixation in filamentous cyanobacteria which are made probable by the segregation of enzymes in some specialized structures called as ‘heterocysts’ where the photoreduction of oxygen to water upsurges the shield of the nitrogenase complex (Milligan et al. 2007), and this process is also said to be the Mehler reaction (Mehler 1957). Conversely, in the case of strict aerobes, the machinery of nitrogen fixation is sheltered by the cytochrome-reliant respiratory defense machinery whereby the oxygen consumption is ensured by a higher respiration rate thus contributing towards the maintenance of low intracellular oxygen (Poole and Hill 1997).

However, such mechanisms developed far ahead in the evolutionary history attributable to the augmented complication of *nif* gene groups accompanying microorganisms evolved with the ability of nitrogen fixation in an oxygen-rich environment. Table 5.2 enumerates the reported *nif* genes which play a role in nitrogen fixation. The modest congregations of genes specifically accompanying nitrogen fixation ensue in strict anaerobes. Nonetheless, sketching the evolutionary path of the entire process and ascertaining the earliest nitrogen fixers in existing biology have always been a challenge (Boyd and Peters 2013). Figure 5.3 predicts the 16S rRNA-based phylogeny tree depicting the evolution of nitrogen-fixing bacteria inferred by using the Maximum Likelihood method and Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions with less than 95% site coverage were eliminated, *i.e.*, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 1098 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

The process of biological nitrogen fixation is supposed to be the earliest and possibly even primordial process (Falkowski 1997; Fani et al. 2000). This prevalent

Table 5.2 List of *nif* genes reported for nitrogen fixation

S. no.	Gene name	Enzyme/enzyme unit encoded
1	*Nif H*	Nitrogenasereductase
2	*Nif D*	Nitrogenase molybdenum-iron protein subunit alpha
3	*Nif K*	Nitrogenase molybdenum-iron protein subunit beta
4	*Nif E*	Nitrogenase molybdenum-cofactor biosynthesis protein
5	*Nif N*	Nitrogenase molybdenum-cofactor biosynthesis protein
6	*Nif X*	Iron-molybdenum cofactor processing protein
7	*Nif A*	Nif-specific regulatory protein
8	*Nif B*	Fe-Mo cofactor biosynthesis protein
9	*Nif Q*	Molybdenum ion binding protein
10	*Nif S*	Cysteine desulfurase
11	*Nif T*	Nitrogen fixation protein
12	*Nif U*	Fe-S cluster assembly protein
13	*Nif V*	Homocitrate synthase
14	*Nif W*	Nitrogenase stabilizing/protective protein
15	*Nif Z*	Iron-sulfur cofactor synthesis protein
16	*Nif J*	Pyruvate-flavodoxinoxidoreductase
17	*Nif L*	Nitrogen fixation negative regulator
18	*Nif P*	Serine acetyltransferase
19	*Nif Y*	Facilitate insertion of Fe-Mo cofactor into apodinitrogenase
20	*Nif M*	Nif H maturation
21	*Nif F*	Electron donor to Nif H
22	*Nif N*	Electron donor toNitrogenase
23	*Nif R*	No protein encoded (repressor binding site only)

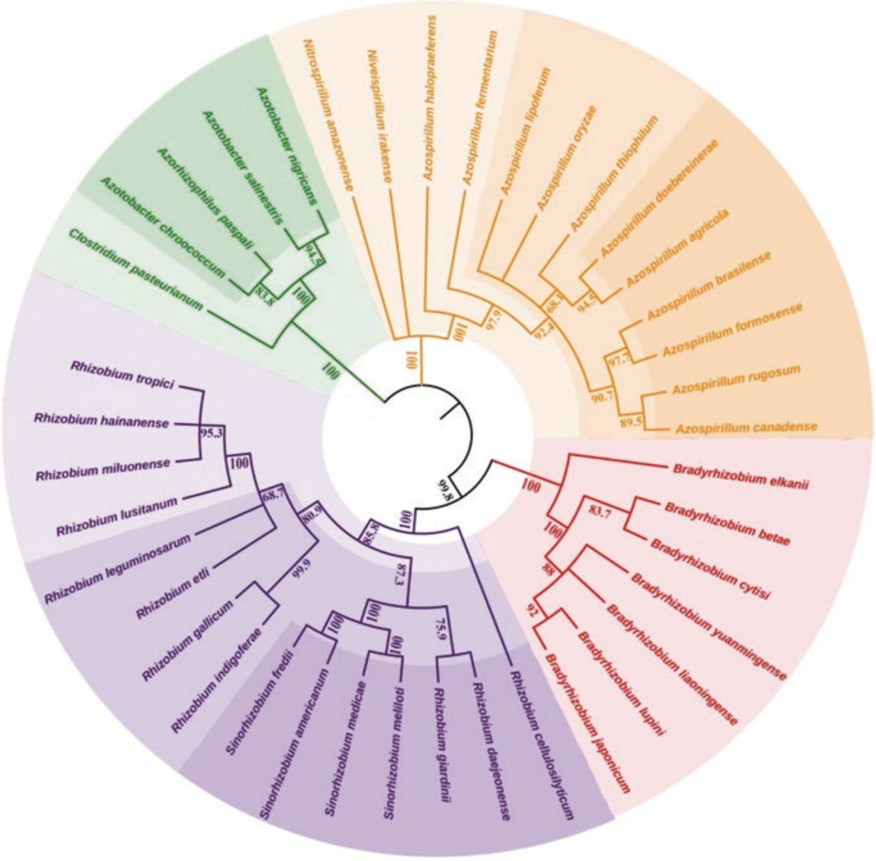

Fig. 5.3 16S rRNA-based phylogenetic tree depicting the evolution of nitrogen-fixing bacteria. The phylogeny is based on the Maximum Likelihood method and Tamura-Nei model

opinion is grounded on simulations of distinctive chemistry of Archaean community that oppose that decreasing concentration of carbon dioxide and a parallel drop in abiotic oxidation of dinitrogen to nitric oxide which headed towards nitrogen crunches at ~3.5 Giga-annum (Kasting and Siefert 2001). The non-biological bases of nitrogen fashioned through several tools such as lightning emancipation or mineral-based catalysis are believed to have become restrictive to an intensifying comprehensive biome. Meanwhile, existent nitrogenase plays an important role to relieve nitrogen restraint in environments (Zehr et al. 2003; Rubio and Ludden 2008), the disparity governing the stock as well as a claim for fixed forms of nitrogen is supposed to have epitomized a robust selective pressure that would have triggered the development of nitrogen fixation (Boyd and Peters 2013). However, if the accessibility to ammonia or other reduced forms of nitrogen is taken into consideration over the progression of geological time, there is the existence of ample pieces of evidence which have suggested enough supply of ammonia that is sufficient enough to upkeep the existence of nitrifying populations in the late Archean >2.5 Giga-annum (Garvin et al. 2009; Godfrey and Falkowski 2009).

The geological records are not that potent till now that they can conclusively resolve when the fixed nitrogen sources started becoming limited, it can be a question of general consideration that whether the distribution and evolutionary history of nitrogenase enzyme along with its accompanying functionalities in existing biology are unswerving with a prehistoric process or an important possession of the last universal common ancestor. Besides, unlike other progressions and functionalities that we attribute towards the possessions of last universal common ancestor, the enzyme nitrogenase is not usually allied with intensely entrenched lines identified by 16S ribosomal RNA evolutionary routes (Boyd and Peters 2013).

The presence of nitrogenase gene clusters has also been acknowledged in the Aquificales genomes (*i.e.*, *Thermocrinis albus* and *Hydrogenobacter thermophilus*) (Wirth et al. 2010; Zeytun et al. 2011). The documentation of the existence of *nif* gene groups in the genetic materials of Aquificales has been considered by sundry as the utmost deeply rooted bacterial line (Reysenbach et al. 2005). The very clear suggestion of this analysis is that profoundly rooted bacteria possess the restricted dispersal of *nif* which is subject to widespread gene loss or was not in the possession of the last universal common ancestor. However, the phylogenetic analyses of a concatenation of the building proteins obligatory for fixing nitrogen (homologs of H, D, and K) clearly specify the paraphyletic attitude of archaea as compared to that of bacteria (Boyd et al. 2011a, b), proposing the emergence of Nif next to the separation of bacteria and archaea.

5.5 Evolution of Nitrogen-Fixing Endosymbiosis

Plants have established several strategies to overcome the deficiency of different nutrients. The paucity of fixed nitrogen is partially fulfilled by living in close connections with the diazotrophic bacteria. These kinds of potent interdependent connections happen as a result of different heights of intimate relationships which range from temporary and adaptable loose links to the enduring and everlasting intracellular lodging of bacteria (Reinhold-Hurek et al. 2015; Santi et al. 2013).

The rhizospheric and phyllosphere portions of plants are inhabited by a diverse array of nitrogen-fixing microbes, thereby fueling opportunities for inaugurating various kinds of symbiotic relations. The endosymbiosis specifically targeting nitrogen fixation is considered to be most lucrative and is supposed to have evolved several times in the higher plants especially angiosperms. Thus, the evolutionary pathway towards endosymbiosis is not a multifaceted one (Geurts et al. 2016). The nitrogen-fixing symbioses are generally represented by two major sections: plant cyanobacterial symbioses and root nodule symbioses.

The symbiotic association between plant and cyanobacteria find a wide distribution through land plants whereas the root nodule symbiosis is constrained, taking

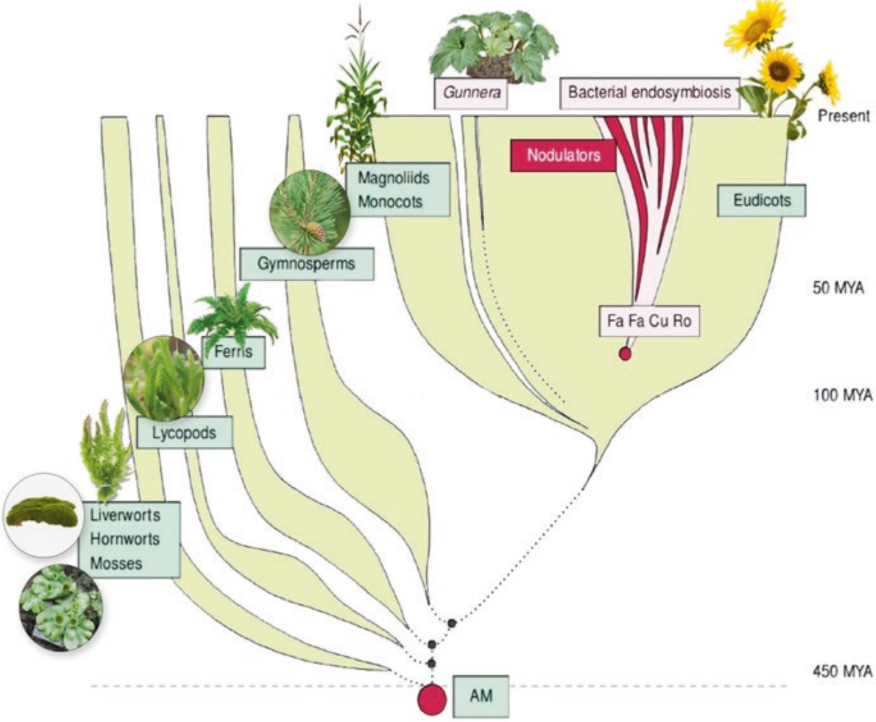

Fig. 5.4 Evolution of the plant root endosymbiosis. FaFa Cu Ro stands for Fabales, Fagales, Cucurbitales, and Rosales members of Eurosid I (Rosids)

place in numerous plant species fitting to only some related orders of flowering plants (Adams 2000; Delaux et al. 2015). The Angiosperms hosts several kinds of symbiotic partners which can be easily differentiated based on the involvement of microbial companions, such as, the cyanobacteria belonging to the genus *Nostoc* is a heterocystous bacteria, which can infect *Gunnera* species (Osborne and Bergman 2009) (Fig. 5.4). The filamentous actinomycetes belonging to the genus *Frankia* also form root nodules in plants called 'actinorhizal' plants (a polyphyletic crowd encompassing 25 genera demonstrating eight taxonomic families) (Li et al. 2015).

There is an appearance of several advances in the plants which range from macroscopic attributes to biochemical alterations. The appearance of such innovations is totally sovereign in different nitrogen-fixing lines. There is a continuous release of signals from the host plant for attracting the symbiont, followed by its enclosure in a freshly advanced assembly, which is later nourished with carbon and an appropriate atmosphere for nitrogen fixation. The development of such multifaceted plant attributes has started with initial adjustments and the later signs of progress are followed through manytransitionalphases (Christin et al. 2015; Delaux et al. 2015).

Despite alterations in the target tissues, the nitrogen-fixing endosymbioses share a collective fact that accommodation of the microbial companion is headed by the stimulation of cell division (Uheda and Silvester 2001; Pawlowski and Demchenko 2012). Cells accommodating the housing of nitrogen-fixing bacteria usually instigate from the mitotic cell divisions which are induced by a signal from the microbial partner.

The cells appearing permissive to infection are usually small in size and have got comparatively thin cell walls. There is a modification in the structure of the cell wall at the point of interactions which makes it less rigid, and also permits invaginations in the cell membrane thereby facilitating the uptake of the microbial partner. Subsequently, the symbiont microbes are not in direct contact with the cytoplasm as they persist walled in some specialized membrane sections (Geurts et al. 2016). The signaling encouraged by the symbiotic bacteria is found to be interfering with the auxin homeostasis in the plants (Kefford et al. 1960). Therefore, the accretion of auxin in the objective cells is prompted indirectly as an outcome of the microbe-induced signaling.

It has been established that the symbiotic signaling grid springs from the cell membrane-localized LysM-type and LRR-type receptor kinases down to a nuclear-localized transcriptional network. Another hallmark in the symbiotic signaling pathways is the generation of consistent oscillatory waves in the calcium concentration of the nuclear region. This calcium−/calmodulin-dependent kinase is often employed by the plant systems for decoding the signal, which sequentially triggers a network of transcription factors crucial for the development of root nodules. Figure 5.5 represents the graphical representation of the signaling cascade involved in root nodule formation. The expression of the *nodule inception* (NIN) gene is much important and adequate for the root nodule establishment (Marsh et al. 2007; Soyano et al. 2013; Vernié et al. 2015). Additionally, it has also been shown that the interdependent signaling elicits Ca^{2+} undulations in *Parasponia andersonii* as well as the actinorhizal plants *Alnus glutinosa* and *C. glauca* (Vernié et al. 2015; Granqvist et al. 2015).

Most of the symbiotic relationships, including the association of rhizobia, instigate the common signaling pathways by secretion of some special compounds called as lipochitooligosaccharides (D'Haeze and Holsters 2002; Persson et al. 2015). Therefore, such kind of symbiotic relationship signifies convergent evolutionary proceedings. The evolution of endosymbiosis of plants with different soil microbes, resulting in the facilitation of nutrient procurement, happened some 450 million years ago and now ensues in more than 80% of the higher plants. The other symbiotic relationships appeared during the preceding 60 million years, comprising the mutually valuable relationship of plants with nitrogen-fixing soil bacteria, harbored in nodules (Granqvist et al. 2015). Additionally, such kind of endosymbiotic relations has evolved several times in the plant kingdom: while rhizobia facing two to five times evolutionary activities whereas it was found to be almost nine times in the case of *Frankia* (Soltis et al. 1995; Doyle 2011; Werner et al. 2014; Li et al. 2015).

Since all the nodulating species of plants mainly find their position in the monophyletic group encompassing the orders Rosales, Fagales, Fabales, and Cucurbitales, and this group is often denoted as the nitrogen-fixing clade (Soltis et al. 1995). However, some of the lineages of this clade have not evolved with the ability to

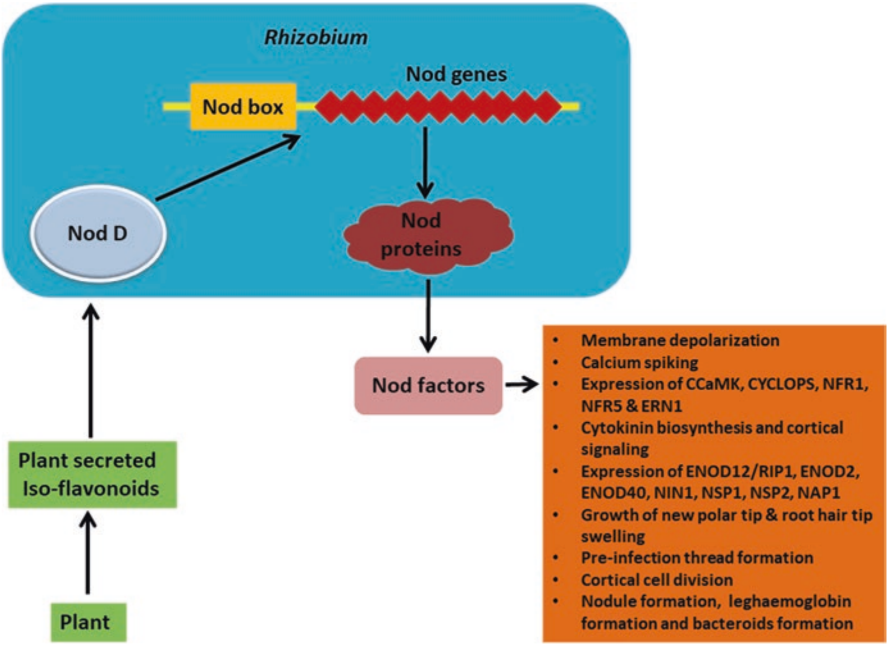

Fig. 5.5 *Rhizobium* bacteria recognize the plant-secreted iso-flavonoids with the help of the transcription factor nod D. nod D upon activation leads to the translation of nod proteins from various nod genes (both common and host-specific. Nod proteins are further involved in the production of Nod factors. These nod factors, in turn, induce several responses in the epidermis and cortex

make symbiotic relationships, two different theories have been proposed to account for such type of phylogenetic relationship: (i) the solitary achievement of nodulation in some antecedent of this clade which was further followed by an enormous and corresponding loss of the same attribute in most of the progenies, or (ii) a parallel progression of nodulation in some progenies along with fewer damages. The former assumption has not been able to get much support thus has received a universal dismissal, although the latter has got extensive acceptance (van Velzen et al. 2018).

Though the latter hypothesis has also got several constraints and one among those is confinement of nodulating species to the nitrogen-fixing clade only. To guide this kind of ostensible clash, one common assumption is that a genetic predisposition happening directed towards an originator state for the process of nodulation developed in some mutual antecedent of this clade about 110 million years ago. This kind of precursor state simplified the equivalent origin of the nodulation process in diverse inheritor roots (Soltis et al. 1995; Werner et al. 2014). However, even after the long-term devotional research in this field, the existence of this proposed precursor state has endured vague and also lacks any pragmatic sustenance. The consideration of the phylogenetic perspective of this nitrogen-fixing clade, for the hypothetical single gain of nodulation to be true, there is a requirement of minimum

7, 5, 17, and 36 losses in Fagales, Cucurbitales, Rosales, and Fabales, respectively. The precise refurbishment of all the evolutionary happenings that ensued 110 million years ago is a fallacious job (vanVelzen et al. 2019).

5.6 Conclusion

The evolution of the process of nitrogen fixation is one of several perfect examples of nature's success stories. Nitrogen is a vital element along with being part of the nucleic acid composition, therefore, often limits the growth and acts as a limiting agent. A vast array of microbehas evolved with the ability to fix atmospheric nitrogen, which otherwise is unavailable, to usable forms. Such microbes are harbored by plants and they form various kinds of associations thus increasing the availability of nitrogen to the plants and thereby decreasing the input demand of chemical fertilizers which is a major economic concern.

The process of evolution of nitrogen fixation seems to be a multifaceted one and appears to have evolved many times. Although, linking the events in a correct sequence that happened millions of years ago seems to be impossible but the understanding of 16S rRNA, which is a conserved sequence often used for tracing phylogeny, gives an idea of the evolutionary history of different nitrogen-fixing microbes. Since the evolution of the ability to harbor nitrogen-fixing microbes is restricted to some genera of the higher plants, a major proportion of crops rely heavily on the use of chemical fertilizers for meeting nitrogen demands. Therefore, a clear understanding of the evolutionary history of nitrogen fixation and evolution of different endosymbiotic relationships could pave a way for various genetic engineering experiments which could enable us to engineer the trait of nodulation in various crops like cereals and thereby would help in saving a vast amount of energy spent for the production of chemical fertilizers along with the promotion and safeguarding environmental sustainability.

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Chapter 6
Encapsulation of Biofertilizers, Biopesticides and Biocontrol Agents

Geeta Singh and Ishani Paithankar

Abstract Increasing the yield of crop plants is possible by alleviating biotic and abiotic stresses and by improving fertilization. Classical agrochemicals are gradually being replaced by biological inputs such as biofertilizers, biopesticides and biological plant growth enhancers. Biofertilizers and biopesticides are, for instance, soil microorganisms that contribute to plant growth and protect plants from diseases. Here, the targeted delivery of these microbes at their site of action is important. In this chapter we review the encapsulation process for targeted delivery of biofertilizers, biopesticides and biocontrol agents. Strategies include microbial encapsulation, and encapsulation in natural and artificial polymers. Spray drying, freeze drying, extrusion, and emulsion are used to prepare capsules or beads or formulations. We present materials for microbial encapsulation, preparation of encapsulated microbial formulations, and applications.

Keywords Biofertilizers · Biopesticides · Plant growth promoting microorganisms · Encapsulation · Microcapsules · Beads

Abbreviations

ACC	Aminocyclopropane-1-Carboxylate
CFU	Colony forming units
IAA	Indole 3-acetic acid
OSAN	Octenyl succinic acid anhydride

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6.1 Introduction

Biofertilizers, biopesticides and biocontrol agents together encompass groups of microorganisms that contribute to the growth and development of plants in an environment friendly manner. Biofertilizers are microbial formulations which help in availability of nutrients using their metabolic activities and thus, improve soil health and fertility (Noumavo et al. 2016). The availability of macronutrients nitrogen, phosphorous, potash as well as secondary and micronutrients to the crop plants are significantly regulated by the diverse group of soil microorganisms. Some bacteria and fungi are able to reduce molecular nitrogen (N_2) to ammonia (NH_3) and make it available for plants through the action of nitrogenase enzyme (Newton 2000; Franche et al. 2008; Dixon and Kahn 2004). These microbes exist as symbiotic or asymbiotic associations with plants. Some well-known examples include *Rhizobium*, *Bradyrhizobium*, *Klebsiella, Azospirillum*, and *Burkholderia*. *Rhizobium* is known to fix N_2 in association with leguminous plants of Fabaceae family (Willems 2007).

High reactivity of phosphate renders it into insoluble forms including inorganic phosphate or mineral phosphate (e.g., apatite) and organic phosphate (Ionositol phosphate, phosphomonoesters, phosphodiesters) (Khan et al. 2009). The soluble forms of P ($H_2PO_4^-$ and HPO_4^{2-}) are available for assimilation by plants. The conversion of these insoluble inorganic and organic phosphate compounds into soluble forms is primarily mediated by soil microorganism. This is accomplished by production of organic acids carboxylic and gluconic acids resulting in lowering of pH leading to dissolution of phosphates (Rodriguez and Fraga 1999). Organic phosphates are solubilised by production of phosphatases enzymes hydrolysing phosphate mono- and diesters (Rodriguez and Fraga 1999; Tao et al. 2008).

Besides, enhancing the plant nutrient availability microbial biofertilizers also stimulates the plant growth and development by production of some phytohormones including auxins, gibberellins, and cytokinins. Plants often are unable to produce optimal levels of auxin required for root growth (Pilet and Saugy 1987). However, there are some soil bacteria that are able to synthesize indole 3-acetic acid (IAA), precursor of auxin hormone, from L-tryptophan released from root exudates. Most common IAA producing bacteria include *Rhizobia* (rice), *Azospirillum* (wheat), *and Pseudomonas* (radish) (Badenoch-Jones et al. 1984). Another method by which IAA producing bacteria affect plant growth is by reducing ethylene levels in plants. The IAA secreted by bacteria works with endogenous IAA to activate synthesis of ethylene synthesis pathway enzyme 1-Aminocyclopropane-1-Carboxylate (ACC) synthase (Penrose and Glick 2001). ACC synthase synthesizes ACC from S-adenosyl-methionine. This ACC synthesized by plants is assimilated by bacteria and degraded to ammonia and α-ketoglutarate using enzyme ACC deaminase. Thus, these microbes act to regulate the levels of ethylene in plants and prevent it from inhibiting plant growth.

Biocontrol agents are the microbial organisms that protect plants against biotic stresses. Their mechanism of action against plant pathogens includes production of antibiotics (Compant et al. 2005; Haas and Keel 2003; Mazurier et al. 2009), synthesis of lytic enzymes (Frankowski et al. 2001) or production of siderophores (Dowling et al. 1996; Kloepper et al. 1980), competition for plant nutrients. Soil borne microorganisms often synthesise lytic enzymes including glucanases, cellulases, chitinases proteases, lipases that hydrolyse cell wall components of pathogens and thus inhibiting them from colonising or infecting plant parts. The siderophores take up/deplete iron from rhizosphere thereby limiting the colonization of pathogenic fungi (Dowling et al. 1996; Kloepper et al. 1980). The well-known examples of the biocontrol organisms include fungi of *Trichoderma sp, Pseudomonas*. Currently bio-formulations having biofertilizers as well as biocontrol agents are primarily available as powder form (solid) or as liquid formulations. The major constraint encountered in these is loss of viability of the active organisms over the period of storage, transportation as well as at the time of application. In addition, the problem with contamination with undesirable organisms is also a major limitation. After their application at the target site, the sustained and gradual release is also not possible in these formulations. Therefore, by resorting to bioencapsulation process these limitations can be successfully overcome.

6.2 Encapsulation

Encapsulation is defined as confinement of any solid, liquid or gaseous material within a semi-permeable wall of polymeric material resulting in formation of small microcapsules (da Silva et al. 2014; Martinis et al. 2013; Nedovic et al. 2011). The capsular wall serves as a protective shield against external conditions including pH, temperature, humidity etc. that may adversely affect the activity of the core / enclosed material. In this manner, the capsule facilitates regulated release of encapsulated material only in the presence of conditions favouring its activity at the desired place (Suave et al. 2006). Encapsulation is classified as one of the immobilization techniques along with entrapment and covalent bonding/cross linking. Entrapment is the irreversible immobilization technique, in which the immobilized material is entrapped in a matrix or fibres for support (Górecka and Jastrzębska 2011). Encapsulation differs from immobilization. In immobilization, the material in entrapped entirely within the matrix, while in encapsulation a coating material is used to enclose the matrix, which is contained within capsule forming core of entrapped material.

Immobilization allows exposure of small portion of material surface, while encapsulated material is totally enclosed within capsule (King 1995). Encapsulation harbours a number of advantages over immobilization. Encapsulation involves enclosure of material within a semi-permeable membrane, facilitating diffusion of nutrients and also high strength of the wall material enables retention of the material. Encapsulation is categorised on the basis of bead size as microspheres (10–100 μm) and macrospheres (>100 μm) (John et al. 2011; Rathore et al. 2013). It can also be classified on the basis of bead structure or morphology (John et al. 2011). Solid spheres are known as beads while hollow spheres made of a liquid core are referred as capsules. Capsules are further classified as microcapsules (1-1000 μm) and macrocapsules (mm to cm) (Rathore et al. 2013). Thus, encapsulation process is divided into two different types- Microencapsulation (bead size 1–1000 μm) and Macroencapsulation (bead size mm to cm).

This technique is employed for immobilization of diverse substances including enzymes, pharmaceuticals, flavours, cell organelles, plant and animal cells (Rathore et al. 2013). Recently, this technology has captured the imagination of biologists for entrapment of microorganisms. The encapsulated microorganisms have found applications in food industry, pharmaceutical, environment, agriculture etc. It has also been widely used for treatment of industrial waste water (Martinis et al. 2013), formulation of probiotics for yoghurt preparation (Krasaekoopt et al. 2003). In agricultural sector, it is being exploited for producing formulations of biofertilizers, biopesticides or biocontrol agents.

6.2.1 Advantages of Encapsulation

The most commonly used inoculants include liquid inoculants, that are cultures of broth in water, organic or mineral oils, or peat carrier formulations. The liquid formulations are applied as dips or sprays for seeds. Peat formulations are directly inoculated into the seeds. However, both of these formulations decrease microbial survival as they are unable to provide protection to the material from external conditions and also the products are rendered to higher chances of contamination during storage, transport or application in soil, which reduces the shelf life of product (Bashan et al. 2002). The encapsulated formulations harbour a number of advantages over conventional inoculants in terms of preserving microbial viability, shelf life, protection against unfavourable external conditions and regulation of release in target environment.

6.2.2 Microcapsule Structure

Microcapsules are made of natural or synthetic polymers. These are formulated as gel beads or as dried powder form. Due to presence of pores in their smooth or irregular walls, they lack encapsulation efficiency and stability (Mortazavian et al. 2007; Favaro-Trindade et al. 2008). Thus, these capsules are coated with suitable wall materials (Mortazavian et al. 2007). Structurally, a microcapsule consists of an inner, centrally located core enclosed by a polymer layer forming wall or membrane of the capsule.

6.2.2.1 Coating Material

The essential features deciding the suitability of a given material for it usage for making capsule membrane are non-reactive response towards core material or active agent, provision of protection to the core against external, adverse conditions, ensure proper sealing of the material inside the capsule and economic viability. It should also facilitate the efficient release of the material under suitable, favourable conditions at the target place (Gharsallaoui et al. 2007; Nazzaro et al. 2012).

A number of materials can be employed for coating microcapsules. Most commonly used materials include both natural and synthetic polymers. These include carbohydrates such as starch, modified starch, dextrins, sucrose, chitosan; gums, Arabic gums, alginate, carrageenan; lipids, wax, paraffin, hydrogenated oils and fats; proteins, gelatine, casein, albumin; and inorganic compounds: Calcium sulphate, silicates (Favaro-Trindade et al. 2008). Synthetic polymers used for encapsulation include polyethylene glycol, polyvinyl alcohol, polyurethane, polypropylene, sodium polystyrene sulphate and polyacrylate (acrylonitrile-sodium methallylsulfonate). Khorramvatan et al. (2013) used three different natural polymers starch, gelatine and sodium alginate as coating material of encapsulated formulation of *Bacillus thuringiensis*. It was found that sodium alginate was most effective coating material against UVB (385 nm) and UVC (254 nm).

6.2.2.2 Common Natural and Synthetic Polymers

Various natural and artificial polymers used for preparation of microcapsules and their properties are listed in Table 6.1 (Gasperini et al. 2014; Wandrey et al. 2010; Olabisi 2015).

6.3 Techniques for Formulation of Microbial Inoculants

The entire process of production of encapsulated particles is completed in two phases: encapsulation and drying. This section describes the microbial encapsulation process for selected organisms. Mainly two types of microcapsulation methods have been described and used by various researchers.

6.3.1 Microencapsulation Phase

6.3.1.1 Extrusion Method or Droplet Method

It involves dripping of encapsulation matrix containing cell suspension through an orifice into a hardening bath. The mixture dripped in the form of droplets is converted into gelled spherical capsules upon contact with hardening solution

Table 6.1 Properties of commonly used polymers for microbial encapsulation

Name	Chemical/structure	Origin	Commercial modifications	Nature	Properties
Agarose	β-D-galactopyranose, 3,6-anhydro-α-L-galactopyranose	Red algae-*Gelidium* and *Gracilaria*	–	Carbohydrate	Cost effective Readily available in number of pore sizes Mechanical resistant Modulation of particle size
Carrageenan	βD-galactose, αD-galactose (anionic polymer)	Red algae-Rhodophyceae	*Kappa, iota, lambda* forms	Carbohydrate	Water soluble at 60°–80 °C Forms gels at room temperature Form viscous solutions Thermally reversible gels upon cooling in presence of cations Stability at neutral-alkaline pH
Alginate	β-1,4 linked β-D-mannuroic acid, α-L-guluronic acid (anionic polymer)	Brown sea weed, *Pseudomonas* and *Azotobacter*	Sodium alginate	Carbohydrate	Large pore size Form gels in presence of cations Water soluble at 60°–80 °C Insoluble in acid media
Chitosan	N-acetyl β-D-glucosamine (cationic polysaccharide)	Crabs and shrimp shells	–	Carbohydrate	Soluble at pH less than 5.4
Cellulose	β-1,4-D-glucose	Cell wall of plants, algae, oomycetes	Sodium carboxy-methyl cellulose, Ethylmethyl cellulose, Ethylcellulose, Hydroxypropyl cellulose, Hydroxypropyl methylcellulose	Carbohydrate	Thermogelation Soluble in cold water High strength Flexible films and transparent Low cost Requires treatments before use for entrapment

Starch	α-1,4 D- glucose units linear (amylose) and α-1,6 branched (amylopectin)	Plant parts including leaves, roots, tubers, stems	Corn starch, maize starch, rice starch OSAN (octenyl succinic acid anhydride) modified starch	Polysaccharide	Low cost Readily available Lower gel strength
Dextrin (starch gums)		Degradation of starch		Polysaccharide	Formulations dried easily Lower tensile strength
Collagen	Triple helix with every third amino acid glycine, proline and hydroxyproline	Extracellular matrix, fibroblast and osteoblasts		Protein	High strength Biocompatible and non-toxic
Fibrin	Glycoprotein, 3 polypeptides linked by disulphide bonds	Blood clots		Protein	
Polydextrose	Branched polymer of D-glucose	Heating dextrose with acid catalyst		Synthetic polysaccharide	Soluble Low calorific value Non-digestable High degree of polymerisation
Poly (ethylene glycol)	Ethylene glycol units	Reaction of ethylene oxide and water, ethylene glycol or ethylene glycol oligomers	Polyethylene glycol-vinyl sulfone, dimethacrylated polyethylene glycol	Synthetic	High strength
Polyvinyl alcohol	Vinyl alcohol units	Polyvinyl acetate		Thermoplastic	Non-toxic
Polyurethane		Reaction of polyol and di-isocyanate		Elastomer	
Polypropylene	Propylene units	Polymerisation of propylene with Ziegler-Natta catalyst		Thermoplastic	

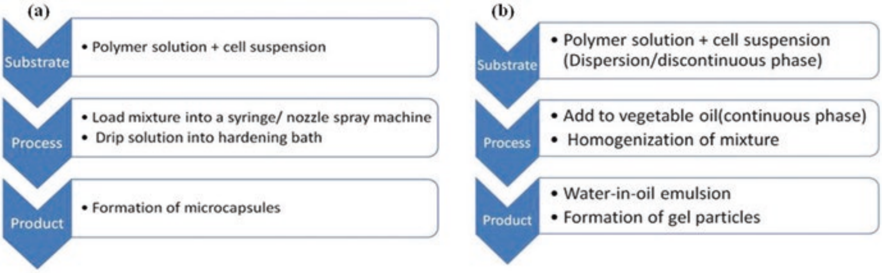

Fig. 6.1 Steps involved in bio-encapsulation (**a**) extrusion or droplet method and (**b**) emulsion technique

(Fig. 6.1a). The size of microcapsules formed is determined by diameter of orifice, viscosity of matrix, distance from hardening solution, and the concentration and temperature of hardening material. Based on the gelling method, this technique is further divided as Thermal gellation, ionic gellation and complex coacervation (Vemmer and Patel 2013).

Ionic gelation is used mainly for hydrocolloid biopolymers alginates, carrageenan and pectin. In case of alginate for encapsulation, the method involves following steps: Hydrocolloid solution preparation in water, adding cells to the hydrocolloid to form suspension, dripping droplets of cell suspension via a syringe into a hardening solution ($CaCl_2$) (Chen and Chen 2007). This hardening solution is made of divalent cations including Ca^{2+}, Mg^{2+} etc. In the $CaCl_2$ solution, the Ca^{2+} ions enable alginate polymers to form 3-D lattice around the cells by forming cross-linkages. The mechanism behind gel formation involves a bond formation between carboxylic free radicles of polymers and the positively charged cations in the solution (Champagne and Fustier 2007). This result in gel formation and the droplets formed are called beads (Gbassi and Vandamme 2012). The main advantage of this method lies in its easy procedure, gentle operations with minimal injury to cells high viability and low cost. Due to slow formation of microcapsules, the method cannot be employed for large-scale productions. It produces relatively larger beads of size 2–5 mm. Also, it often lacks compatability with high viscosity matrices.

6.3.1.2 Emulsion Technique

It involves two different phases the dispersion phase and continuous phase. Here, slurry of cells and polymer serve as dispersion phase and vegetable oils including sunflower, corn or paraffin oils act as continuous phase. The dispersive phase is added to continuous phase resulting in formation of water in oil emulsion. The resulting capsules are collected using centrifugation or filtration (Sheu and Marshall 1993; Gbassi and Vandamme 2012). For alginate beads, the process includes mixing of encapsulation solution with fat soluble acetic acid to lower the pH. This is followed by addition to water to separate oil phase. Figure 6.1b briefly gives steps involved in the process. Overall, this technique is better than extrusion in that it can be used for large scale productions and it produces relatively small-sized beads (25-2 μm). However, requirement of additional purification steps for removal of oil phase and lack of control over size of microcapsules produced, create roadblocks in the use of this technique (Ding and Shah 2009; Rathore et al. 2013).

The above techniques have been used for encapsulation of microorganisms employed for number of purposes. In case of probiotics, extrusion method is employed for formation of alginate beads. Alginate is often used with a number of different polymers acting as coating materials. Jankowski et al. (1997) encapsulated probiotic bacteria *Lactobacillus acidophilus* using a formulation of alginate and starch. Krasaekoopt et al. (2006) used alginate alongwith chitosan coating material for formulation of alginate beads of *Lactobacillus acidophilus*, *Bifidobacterium bifidium*, *L. casei*. Another well known coating polymer for alginate beads is poly L-lysine. Champagne et al. (1992) used a alginate beads coated with poly L-lysine for encapsulating *Lactococcus lactis* for probiotics production. Other most widely used materials for formulation of probiotics include gellan gum and xanthan gum, Κ-carrageenan and Cellulose acetate phthalate. Gellan and Xanthan gums were used in combination for encapsulation of *Bifidobacterium lactis* (McMaster et al. 2005). Κ-carrageenan was used for encapsulation of *Bifidobacterium bifidium* by Dinakar and Mistry (1994). Rao et al. (1989); Favaro-Trindade and Grosso (2002) encapsulated *Bifidobacterium pseudolongum* using cellulose acetate phthalate.

Alginate is also used in agricultures for producing formulations of biofertilizers, biocontrol agents. Farhat et al. (2014) used alginate for encapsulation of two plant growth bacteria *Serratia marcescens, Enterobacter sp*. Santos et al. (2018) used alginate and clay for encapsulation of plant growth promoting microbes including *Azospirillum brasilense, Burkholderia cepacia, B. thuringiensis, B. megaterium, B. cereus, B. subtilis, Trichoderma spp*. Ivanova et al. (2005) encapsulated *Azospirillum brasilense* using Na-Alginate, standard and modified cornstarch. Bashan (1986) encapsulated *Azospirillum brasilense* using Na-Alginate with skim-milk. Young et al. (2006) used alginate and humic acid for encapsulation of bacteria *Bacillus subtilis*. Van Elsas et al. (1992) tested three combinations of Na-alginate for encapsulation of *Pseudomonas fluorescens*. These combinations included: Na-alginate, Na-alginate and skim-milk and Na-alginate, skim-milk and bentonite. Other plant growth microorganisms encapsulated were *Bradyrhizobium japonicum* with carboxymethyl cellulose with starch coating (Júnior et al. 2009) and *Rhizobium japonicum* with synthetic polymer polyacrylamide (Dommergues et al. 1979). Alginate has also been employed for formulation of biocontrol agents in agriculture. Fravel et al. (1985) used alginate, pyrax (clay) for encapsulation of *Talaromyces flavus, Gliocladium virens, Penicillium oxalicum*. Shah et al. (1998) used only Na-alginate for formulation of biocontrol agent *Erynia aphidis*.

Synthetic polymers including polyvinyl alcohol, polyurethane and polysulfone have been used for bioremediation purposes. Cunningham et al. (2004) encapsulated hydrocarbon degrading bacteria with the help of polyvinyl alcohol. Briglia et al. (1990) used Polyurethane foam for encapsulation of Pentachlorophenol degrading microorganisms *Rhodococcus chlorophenolicus, Flavobacterium sp.* Ben-Dov et al. (2009) encapsulated waste-water bacteria using agar and polysulfone.

6.3.2 *Drying of Encapsulated Cultures*

The microcapsules produced are dried in order to convert them into minute particles (granules) or powder form. This is required to improve the shelf life and stability of the cultures during storage. Here, a few commonly used methods are presented.

6.3.2.1 Spray Drying

Spray drying method involves conversion of a fluid product into a solid in the form of powder (Fig. 6.2). This is accomplished by dispersion of the droplets of the fluid by using hot air within a hot chamber (Rodríguez-Hernández et al. 2005). The energy from hot air acts to disintegrate the liquid, dividing it into small particles, which results in mist or spray of droplets (Finney et al. 2002). It is one of the most widely used methods for microencapsulation of biological materials and food products. The reason behind its wide applicability is lower exposure time of the product

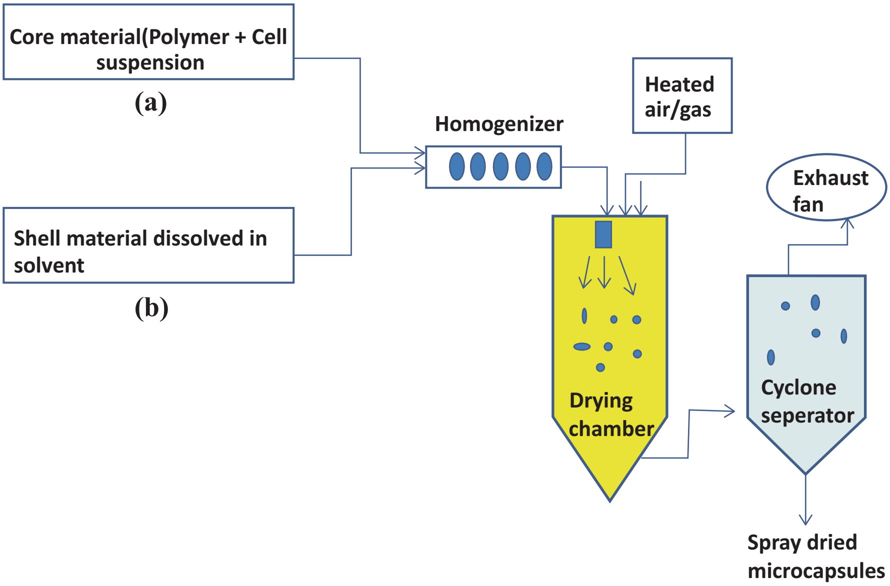

Fig. 6.2 Spray drying (**a**) The core material and coating solutions are homogenized. (**b**) The shell material is dissolved in solvent. The solution is passed through drying chamber where hot air acts to disintegrate it into small particles to form mist or spray of droplets. The spray dried particles are recovered in cyclone separator

to high temperatures, minimal thermal damage and higher yields. However, this method results in increased losses in viability. The origin of this technology dates back to nineteenth century, when it was used for drying eggs. However, the industrial application of this method began only in 1920s.

Milk and washing powder were the first industrial products to be produced by this method. O'Riordan et al. (2002) used spray drying method to encapsulate *Bifidobacterium* using gelatinised modified starch. Amiet-Charpentier et al. (2008) encapsulated rhizobacteria *Pseudomonas fluorescens-putida* using methacrylic copolymer from Eudragitrange. Jin and Custis (2011) used spray drying for producing conidia of *Trichoderma harzianum* using three different sugars, sucrose, molasses or glycerol for encapsulation. Paul et al. (1993) used dry air for encapsulation of *Azospirillum lipoferum* using alginate. It was observed that very high rate of drying of beads, adversely affected the cell viability than lower drying rate. Sinkiewicz-Enggren et al. (2015) encapsulated *Lactobacillus reuteri* using spray drying with following parameters of spray dryer: inlet temperature (120 °C), outlet temperature (73–74 °C), aspirator: 100%, pump: 20%, nozzle cleaner: 6–8. Spray drying device used was BUCHI, mini-spray dryer B290, Essen, Germany. Behboudi-Jobbehdar et al. (2013) found that an inlet temperature of 133.34 °C and feed rate of 7.14 ml/min were optimum for production of highly viable encapsulated *L. acidophilus*.

6.3.2.2 Spray Chilling

In this method, a dry core material is sprayed with a lipid-based material to serve as coating. The lipid-based material is sprayed in form of mist on the core material, which is kept in motion. This is followed by solidification of coating by using cold air with temperatures between 10–50 °C. This technique has been used for encapsulation of various food materials including vitamins, minerals, and other heat sensitive materials (Gibbs et al. 1999).

6.3.2.3 Coacervation

This method involves separation of a hydrocolloid/polymer from the solution followed by deposition over the emulsified core material. The principle behind the method is that after the phase separation, the polymer coating material forms a coacervate, which coalease to decrease the surface area and total interfacial free energy of the system and this favours its adsorption over the core material surface and form a uniform coating on core particle. This coating material is solidified by crosslinking reaction using thermal, chemical or enzymatic methods (Desai and Park 2005). The main advantages of the process include proper control of release of encapsulated material, a high pay load of 99% and its operation at room temperature, making it suitable for heat-sensitive bacteria. However, the materials used in techniques, result in a higher costs and complexity of the process make it relatively disadvantageous over commonly used techniques like spray drying.

6.3.2.4 Freeze Drying

It involves freezing of solution of carrier matrix and biological agent at low temperature, which is followed by removal of solvent by sublimation by applying low pressure or vaccum. This method is also termed as lyophilisation. Since, the process does not involve melting, it is considered as mild and hence enables preservation of characteristics of microcapsule. However, high cost of the method makes its use disadvantageous (Santivarangkna et al. 2007).

6.3.2.5 Vacuum Drying

It involves sublimation of frozen sample by applying low pressure similar to freeze drying. However, in this method sample solution of matrix and biological agent is not frozen but is converted from liquid to solid by phase transition. The application of this method for microbial encapsulation is however limited (Broeckx et al. 2016).

6.3.2.6 Fluid-Bed Agglomeration and Coating or Fluidized Bed Drying

This technique was first developed by pharmaceutical industry with purpose of making dry, enteric coatings for targeted and controlled release of drug in gastrointestinal tract (Dewettinck and Huyghebaert 1999). Today, it is being widely utilised by other industries like food, feed, agrochemicals, cosmetics for formulation and preservation of various products (Boerefijn and Hounslow 2005; Guignon et al. 2003; Saleh et al. 2003). A dehumified and filtered air is used to fluidise particle bed of the product. The technique is divided into three processes, the dehydration, heating and cooling. It finds applications, primarily in food industry, where it is used for commercial production of baker's yeast *Saccharomyces cerevisiae.* In agriculture, it is used for drying process of microencapsulated biocontrol formulations of fungi, bacteria, yeast or protein toxins of *Bacillus thuringiensis* (Brar et al. 2006).

6.3.2.7 Co-crystallization

This method involves dispersal of core material in a supersaturated sucrose solution, which is maintained at a high temperature. This is followed by a gradual dissipation of the heat resulting in crystallization of solution and core material. The crystals formed are dried and sieved (Bhandari et al. 1998). Table 6.2 briefly describe the microbial encapsulation process for selected organisms with their advantages and limitations. It shows bioencapsulation of beneficial microorganisms used as biofertilizers, biocontrol agents or biopesticides using spray drying and freeze drying and their advantages or disadvantages.

Table 6.2 Bioencapsulation of biofertilizers, biocontrol agents or biopesticides using spray drying and freeze drying

Microorganisms	Encapsulation material/ parameters	Technique	Advantage/disadvantage	References
Trichoderma harzianum	MaltodextrinDE10, MaltodextrinDE20, gum arabic, 1:1 MaltodextrinDE10 – gum arabic	Spray drying	Survival (storage and drying): MaltodextrinDE10 – gum arabic formulation 86%	Muñoz-Celaya et al. (2012)
Trichoderma harzianum	Sucrose (0.5, 1, 2, 4, 6 and 8%), molasses, glycerol BUCHI mini spray dryer B-290 Inlet temperature: 40–140 °C Outlet temperature: 20–81 °C	Spray drying	Survival (storage and drying): Increase survival CFU 7.5×10^{10}/g of dried conidia Optimum sucrose concentration: 2% Inlet temperature: 60 °C Outlet temperature: 31 °C	Jin and Custis (2011)
Bacillus cereus C1L	Maltodextrin (0–25%) and gum arabic (0–25%) Outlet temperature: 60–100 °C Inlet temperature: 150–250 °C	Spray drying	Viability: 42% at outlet temperature of 73.5 °C Best yield at maltodextrin (18.3) and gum arabic (12.5%)	Chen et al. (2013)
Beauveria brongniartii	Skim milk and polyvinylpyrrolidone (PVPK90) Outlet temperatures: 53+/−2 °C	Spray drying	Yield: 25% Viability: 92%	Horaczek and Viernstein (2004)
Beauveria brongniartii and *Metarhizium anisopliae*	Skim milk and polyvinylpyrrolidone (PVPK90) Inlet temperature: 60 °C Outlet temperature: 40 ± 2 °C	Spray drying	Loss of fungal material Lower germination rates	Horaczek and Viernstein (2004)
Beauveria bassiana	Dextrin (10%), skimmed milk (10%), polyvinylpyrrolidoneK90 (5%)	Spray drying	Viability (after 6 months storage at 4 °C): 80%	Liu and Liu (2009)

(continued)

Table 6.2 (continued)

Microorganisms	Encapsulation material/ parameters	Technique	Advantage/disadvantage	References
Beauveria bassiana	Sodium humate Inlet temperature:175 °C Outlet temperature: 86.5+/−1.3 °C	Spray drying	Good viability after 6 months storage at room temperature	Qureshi et al. (2014)
Tsukamurella paurometabola C-924	Starch cream (10% w/w) Mobile Minor™ (Niro atomizer, Denmark) atomizer Inlet temperature: 130 °C Outlet temperature: 55° or 65 °C Feed flow rates: 1.9 or 1.7 L/h	Spray drying	Stability (at room temperature and 4 °C): More for encapsulated Shelf life: longer than 1 year	Hernández et al. (2007)
Nitrogen-fixing bacteria associated with lupin nodules	Sodium-alginate: Maltodextrin (0:15,1:1, 2:13) LabPlant SD-05 dryer Nozzle diameter:1.5 mm Inlet temperature: 100 °C Outlet temperature: 65 °C Air-flow rate: $73 m^3 h^{-1}$ Feed rate: $5.3 g min^{-1}$	Spray drying	Highest survival at Na-alginate: maltodextrin ratio 1:14: 79% Microcapsule yield: 27%	Campos et al. (2014)
Bradyrhizobium japonicum	Gum-acacia (1,3,6%) and maltodextrin (13,15,30%) Buchi mini spray dryer(B-290) Inlet temperature: 65°–70 °C Outlet temperature: 30–32 °C Air flow rate: 40 units Feed pump rate: 20–23%	Spray drying	Viable cell count: 1×10^8 CFU/g with gum-acacia (1%) and maltodextrin (15%) Survival: 90% Increased root of soyabean biomass by 2%	Dey et al. (2018)
Pantoea agglomerans, Trichoderma harzianum	Alginate-glycerol-kaolin	Freeze drying	Shelf life: Increased Protection from UV-C radiation	Nussinovitch (2016)

(continued)

Streptomyces sp. Di944	Alginate beads Durum flour (starch) granules, Talcum powder	Freeze drying	Stability (4 °C): Talcum powder (14 week)-stable and 100% viable Granular forms (10 week)- stable and 100% viable Alginate beads (12 and 24 week)- less stable Disease suppression (tomato damping-off): Talcum formulation-90% Alginate beads-30% Starch granules-22%	Sabaratnam and Traquair (2002)
Beauveria brongniartii, Metarhizium anisopliae	Skim milk and polyvinylpyrrolidone (PVPK90)	Freeze drying	Viability: *B. brongniartii*: 68% *M. anisopliae*: 4%	Horaczek and Viernstein (2004)
Azospirillum brasilense Cd	Na-alginate (wet and dry beads) Na-alginate and skim milk (wet and dry beads)	Freeze drying	Microbead diameter 10–20 m Some bacteria killed during microbead formation Enhanced growth of wheat and tomato seedlings in unfertile soil	Bashan et al. (2002)

6.4 Encapsulation of Plant Growth Promoting Microorganisms

There have been several studies for encapsulation of plant growth promoting microorganisms and many of them have resulted favourable outcomes (Table 6.3). A method for encapsulation of potential biocontrol agents like – ascospores or conidia of *Talaromyces flavus* (Tf1/Tf-I), *Gliocladium virens* (GL3), *Penicillium oxalicum* or *Trichoderma viridae* (T-1-R9) or cells of *Pseudomonas cepacia* (POP-SI) by mixing with a solution containing sodium alginate (1%) and Pyrax (1%) followed by dripping into a solution of $CaCl_2$(0.25 M) or Ca-gluconate (0.1 M) was attempted by Fravel et al. (1985). It was observed that all strains of fungi but not *Pseudomonas cepacia* (POP-SI) remained viable after forming pellet in $CaCl_2$ and after drying. However, all fungal and bacterial strains were able to retain their viability in Ca-gluconate for a longer time period after pellet formation.

In another study, sodium alginate along with wheat bran, a food carrier base was used for encapsulation of 11 isolates of *Trichoderma spp.* and *Gliocladium virens* to check their biocontrol efficacy against *Rhizoctonia solani* infected seeds of beet in soil (Lewis and Papavizas 1987). The biocontrol activity of isolates was tested in 6 different soils. All the isolates were effective against the pathogen in natural soil. It was found that eight isolates of *Trichoderma spp* were effective in reducing the survival of *R. solani* by 34–78%. Most effective strains were *T. harzianum* (Th-58) and *T. hamatum* (TRI-4). *Trichoderma* isolate TRI-4 was highly effective against the pathogen in all 6 soils (>70%) and against 6 *R. solani* isolates in loamy sand. A minute amount of biomass of isolates showed efficacy comparable to very large biomass. However, the effectivity of all the formulations was reduced after 6 weeks of storage at 5° or 25 °C.

Sodium alginate was used for formulation of *Erynia neoaphidis*, a pathogenic fungus of aphid pests. It was observed that the optimal concentration of sodium alginate for effective encapsulation of fungal mycelium was 1.5%. 0.1 M and 0.25 M $CaCl_2$ were found to be equally efficient as gelling agents. Freshly produced alginate beads with fungal conidia showed an infectivity of 27–32% in aphids of pea. However, the performance did not differ significantly from fresh mats of mycelia or plugs from petri dish cultures. A reduction in survival (63–97%) of conidia was observed after drying and storage of beads in comparison to freshly prepared beads (Shah et al. 1998). In further studied the factors involved in production of alginate granules of *Erynia neoaphidis*. Granules were formed by entrapment of fungal mycelia in alginate polymer. It was found that addition of sucrose, potato starch or chitin to alginate significantly improved conidia production from granules (Shah et al. 2010). The performance of alginate pellets of entomopathogenic fungus *Beauveria bassiana* was evaluated for biocontrol of *Solenopsis invicta* (Red Imported Fire Ant) under field conditions (Bextine and Thorvilson 2002).

Many commercial formulations of biocontrol, biopesticide and biofertilizer agents have been prescribed by several researchers are in different plants (Table 6.4). A comparison of the performance of sodium alginate beads of mycoherbicide

Table 6.3 Encapsulation of microbes used in agriculture

Formulation	Microbe used	Results	Reference
Alginate-glycerol-kaolin	*Pantoea agglomerans, Trichoderma harzianum*	Increased shelf life. Protection from UV-C radiation.	Nussinovitch (2016)
Alginate-humic acid	*Bacillus subtilis* CC-pg104	Increased cell viability. Storage till 5 months without much cell loss. Successful growth promotion of lettuce.	Young et al. (2006)
CM-cellulose/xanthan	*B. subtilis*	Bacterial release efficiency: Xanthan: 90.2% CM-cellulose: 76.6% Xanthan formulation showed better biocontrol activity against *Meloidogyne* incognita, Xanthan beads inoculated tomato plants showed decreased galls	Pacheco-Anguirre et al. (2016)
Na-alginate-bentonite	*Pseudomonas putida* Rs-198	Better survival than non-encapsulated cells.	Li et al. (2017)
Na-alginate (2–3% w/w)	*Bacillus thuringiensis* sub sp. *kurstaki* (Bt-KD2)	70% spore viability.	Khorramvatan et al. (2017)
Na-bentonite and alginate	*Raoultella planticola* Rs-2	100% encapsulation efficiency. Survival rate of 81% at 4 °C and 88.9% at room temperature. Increased survival during drying. Increased stability during storage.	He et al. (2015)
Na-alginate	*Klebsiella oxytoca* Rs-5	High degree of root colonization. Increased survival rate. Increased retention time. Relieves salt stress of cotton seeds.	Wu et al. (2013)
Na-alginate and starch	*Azospirillum brasilense*	76% viability after one year storage.	Schoebitz et al. (2012)
CM-cellulose, corn, starch, potato starch, autoclaved baker's yeast	*Metarhizium brunneum*	Max. Survival 82%.	Przyklenk et al. (2017)
Chitosan	*Rhizobium, Azotobacter, Azospirillum*	Increased plant growth.	Namasivayam et al. (2014)

(continued)

Table 6.3 (continued)

Formulation	Microbe used	Results	Reference
Alginate, bentonite, skim milk	*Pseudomonas fluorescens*	Increased colonization in soils. Better survival. Less sensitivity to dry/wet fluctuations in soils. Drying beads resulted in reduced survival than moist beads. Moist beads colonized wheat roots after 63 days.	Trevors et al. (1993)
Na-alginate (wet and dry beads) Na-alginate and skim milk (wet and dry beads)	*Azospirillum brasilense* Cd	Microbead diameter 10–20 m. Some bacteria killed during micro-bead formation. Enhanced growth of wheat and tomato seedlings in unfertile soil.	Bashan et al. (2002)
Na-alginate (2–4%) Agarose Polyurethane	*Flavobacterium sp.* (ATCC 39723)	All three formulations showed capacity of Pentachlorophenol degradation. All encapsulated cells showed stability upon storage at 4 °C and retained biodegradable activity.	Stormo and Crawford (1992)
Na-alginate	*Glomus versiforme*	Encapsulated spores able to germinate and retained ability to infect plant roots.	Declerck et al. (1996)
Na-Alginate prills (0.2%)	*Trichoderma koningii* (biocontrol to phytopathogens)	*T. koningii* alginate prills+wheat bran (2 g) remained activity on 2-year storage at 5 °C.	Mónaco and Rollán (1999)

Alternaria cassia with kaolin or corn cob as filler material and fermentation medium with or without Potato dextrose broth was attempted. It was observed that in case of un-supplemented fermentation medium alginate beads with Corn cob grits filler materials performed better in terms number of spores than kaolin alginate beads. Using fermentation media added with Potato dextrose broth enhanced spore production in both the cases. Potato dextrose broth and corn cob grits act as nutrient source for encapsulated mycelia, accelerating spore production. Therefore, a higher spore yield was observed when corn cob grits were used as fillers for alginate beads and the yields improved when corn cob grits were supplemented with Potato dextrose broth (Daigle and Cotty 1992).

Studies were undertaken to evaluate appropriate concentration of chitin with Na alginate to be used for effective encapsulation of *Beauveria bassiana*. Among the different concentrations of chitin used with or without wheat bran, three times increase in conidia production was observed with 2% chitin and 2% wheat bran upon 21 days storage. It was observed that increasing chitin content of alginate

Table 6.4 Commercially-used biocontrol, biopesticide and biofertilizer agents

Name	Microbe	Form	Application	Use	Plant	Reference
Mycostop®	*Streptomyces griseoviridis*	Wettable powder	Seed dressing	Biocontrol of *Fusarium* and *Alternaria*	Crucifers, carnations	Sabaratnam and Traquair (2002) and White et al. (1990)
Galtrol®	*Agrobacterium radiobacter*	Agar plate culture of bacteria	Plant dip treatment as water suspension: spray, root dip, root drench	Control of crown gall disease caused by *Agrobacterium tumefaciens*	Woody plants	Kerr (1989)
Quantum 4000®	*Bacillus subtilis*	Wettable powder	Seed dressing			Connick Jr. et al. (1990)
Dagger-G®	*Pseudomonas fluorescens*	Wettable powder	Seed dressing			Currier et al. (1988)
Blue Circle®	*Pseudomonas cepacia*	Wettable powder	Seed dressing			McLoughlin et al. (1992)
BINAB-TW®	*Trichoderma harzianum*	Granular/solid (alginate beads)		Control of soil-borne pathogenic fungi	Ornamentals and vegetables	Lumsden et al. (1995)
GlioGard®	*G. virens*	Granular/solid (alginate beads)		Control of soil-borne pathogenic fungi	Ornamentals and vegetables	Lumsden et al. (1995)
Lipel ™	*Bacillus thuringiensis* var. kurstaki	Wettable powder (nutrient medium, residues, sodium chloride, dextrose, spores, endotoxin)	Slurry in water: Foliar spray Dry powder: Foliar dusting	Biopesticide		Agri Life, India
Bionemagon ™	*Bacillus firmus* strain NCIM 2673	Wettable powder (Kaolin spores)	Dry: Soil amendment Mixture of water+powder (filtered and decanted): Drip irrigation	Biopesticide		Agri Life, India

(continued)

Table 6.4 (continued)

Name	Microbe	Form	Application	Use	Plant	Reference
Sheathguard™	*Pseudomonas fluorescens* strain IIHR-PF2	Wettable powder (Carboxymethyl cellulose, talc, cells)	Slurry in water+sugar: seed coating Dry: nursery bed treatment, soil application and compost enrichment	Biopesticide		Agri Life, India
Serenade® Opti	*Bacillus subtilis* QST713	Wettable powder	Dilution with water: Foliar spray, soil drench	Biopesticide		Bayer Crop Science LP, USA
FZB24® TB	*Bacillus amyloliquefaciens ssp. plantarum*	Dry powder (talcum, corn starch, skim-milk powder, glycerol, spores (lyophilized)	Dry treatment of seeds and tubers, soil amendment	Biofertilizer		ABiTEP GmbH, Germany
FZB24® WG	*Bacillus amyloliquefaciens ssp. plantarum*	Wettable powder (corn starch, skim-milk powder, glycerol, spores (lyophilized)	Slurry with water: tuber treatment, seed treatment, soil drench	Biofertilizer		ABiTEP GmbH, Germany
NITROFIX™ – AC	*Azotobacter chroococcum* strain MTCC 3853	Wettable powder (kaolin, dextrose, lignite, spores)	Slurry with water+ sugar: seed coating Slurry with water+manure: Seedling root dip Mix with compost: soil amendment Add to irrigation stream: soil drench	Biofertilizer		Agri Life, India

pellet decreased conidial numbers. However, using wheat bran with any concentration of chitin resulted in increased number of conidia. Also, chitin incorporation in alginate pellet reduced saprophytic fungal contamination (González et al. 2007).

Testing of Sunflower oil, Groundnut oil and talc for encapsulation of entomopathogenic fungus *Lecanicillium lecanii*. The three basic carriers (Sunflower oil, Groundnut oil), talc was evaluated independently as well as in composition with chitin and chitosan. The most suitable proportion of carrier material: technical ingredient and viability were evaluated by using CFU (Colony forming units) count of the formulations during 3 months storage period. It was observed that enrichments of both Groundnut Oil and Sunflower Oil with chitin exhibited best viability and thus, were found to be best carriers for fungal encapsulation (Nithya and Rani 2017).

Encapsulation of Na-alginate granules *Trichoderma hamatum* for biocontrol of *R. solani* colonization along with wheat bran, rice straw, oat bran, eucalyptus leaves and corn meal were attempted. Their addition was found to reduce the *R. solani* saprophytic activity and maintained 100% viability after 3 months storage. Wheat bran was found to be the most effective (Mafia et al. 2003). Alginate encapsulated chlamydospores of *Trichoderma spp.* and *Gliocladium virens* with bran as bulking agent showed a higher survival and viability than alginate encapsulations of conidia and kaolin bulking agent in soil (Lewis and Papavizas 1985).

Application of the encapsulated *Trichoderma hamatum* (TRI-4) and *Gliocladium virens* (GI-3, GI-21, GI-32) for biocontrol of *R. solani* damping-off of eggplant led to a decrease in saprophytic growth of pathogen. It was effective in a reduction in post-emergence damping-off in other plants including cucumber and pepper seedlings (Lewis et al. 1998). However, A biocontrol formulation comprising of *Cladorrhinum foecundissimumto* consisting of bran, alginate prills and flour/clay was found effective for damping off pathogen control in eggplant and pepper (Lewis and Larkin 1998).

Material like wheat bran, rice straw, oat bran, eucalyptus leaves and corn meal were employed to encapsulate Na-alginate granules. These formulations showed 100% viability and it was observed that addition of food sources to Na-alginate reduced the saprophytic activity of *R. solani* (Mafia et al. 2003). Higher survival was observed in the alginate encapsulated chlamydospores of *Trichoderma* spp. and *Gliocladium* (Lewis and Papavizas 1985). Application of formulations having encapsulated *Trichoderma hamatum* (TR 4) and *Gliocladium virens* (GI 3, GI 21, GI 32) using Na alginate and Biodac (cellulose) on soilless mix showed reduction in the disease of eggplant and decreased saprophytic growth of *R. solani* (Lewis et al. 1998).

Further, formulations of *Cladorrhinum foecundissimumto* developed using bran could successfully reduce the disease and plant stands produced were comparable to those in non-infected control plants after 4 weeks (Lewis and Larkin 1998). Entrapment of the wet biomass of *Trichoderma viridae* in gluten matrix yielded 10^6–10^7 CFU/g soil and was more effective at lower dose as compared to non-encapsulated counterparts (Chen-Fu and Wen-Chien 1999). In the second week after inoculation, the formulations produced a biomass of 10^6–10^7 CFU/g soil.

Different food bases additives wheat bran, corn cobs, peanut hulls, soy fiber, castor pomace, cocoa hulls and chitin for encapsulation of *Gliocladium virens* and *Trichoderma spp*. were evaluated and found to be effective against damping-off. All combinations of alginate prills could perform well in soil against damping-off of cotton plants (Lewis et al. 1996). They also believed that the biocontrol effect of the formulation depended on the intrinsic activity of the isolate used.

In order to use alginate prills, organic carriers were used for encapsulation of *Talaromyces flavus* for biocontrol of *Verticillium dahlia* causing wilt in eggplant (Fravel et al. 1995). The results of green house experimentation using three different soils (two loamy sands and silt clay) revealed their suitability as an efficient carrier for an effective delivery of the bioformulation.

Encapsulated biocontrol agents *Bacillus subtilis* and *Pseudomonas putida* against the root rot pathogen *Pythium aphanidermatum* and *Fusarium oxysporum* f.sp. *cucurbitacearum* were able to survive over a range of temperature up to 45 days. Immobilization using materials like kaolin clay, vermiculite, bacterial broth carriers showed more population than other carriers. Vermiculite, peat moss, wheat bran, bacterial broth carriers were found to be best for survival and population growth of *P. putida* (Amer and Utkhede 2000). Use of alginate with or without wheat bran for encapsulation of *Beauveria bassiana* for biocontrol of green bug (*Schizaphis graminum*) infecting wheat crop was undertaken and superiority of alginate-wheat bran combination was observed in terms of improved shelf life (Knudsen et al. 1990).

Jain et al. (2010) studied the efficacy of phosphate solubilising activity of fungus *Aspergillus awamori* using two different polymers for encapsulation- Ca-alginate and agar. Two types of insoluble phosphates were used namely, Udaipur Rock Phosphate and Tri-Calcium Phosphate. When the three formulations were compared, Agar encapsulation showed maximum solubilisation of Udaipur Rock Phosphate followed by Ca-alginate and un-encapsulated (free) cells, while Ca-alginate encapsulation showed maximum solubilisation of Tri-Calcium Phosphate as compared to agar and free cell formulations.

In addition to biocontrol agents the technique of encapsulation is also widely applied to biofertilizers like phosphate solubilising and nitrogen fixing bacteria and fungi. Bioencapsulation of nitrogen fixing *Azospirillum* by formulation of alginate (3%) standard starch (44.6%) and modified starch (2.4%) to yield biodegradable formulations which were characterized with high viability (Ivanova et al. 2005). Similar advantage of encapsulation of P-solubilizing bacterium *Enterobacter* using alginate combined with skim milk (Vassileva et al. 1999) was observed. It was recorded that encapsulated bacteria could induce better growth in plants than non-encapsulated bacteria. The alginate-skim milk beads also enhanced plant growth. Similar observations pertaining to the use of alginate along with skim milk as the matrix for the encapsulation of phosphate solubilising rhizobacterial strains *Pseudomonas fluorescens* BAM-4 and *Burkholderia cepacia* has been found successful (Minaxi and Saxena 2011). Hence, it is concluded that alginate is best polymer with easy application and low cost for development of N-fixing biofertilizers.

6.5 Conclusion

In this article, various techniques available for encapsulation of microorganisms for various purposes have been discussed. The variety of naturally obtained biodegradable materials as well as artificially synthesised polymers is available. These can be used individually or in combinations optimized in proportions for making of biological formulations providing best possible yields and their viability and activity. The methods available for production of microbeads or capsules containing biological agents are used keeping in mind the suitability of the organism to be immobilized. Much research has been done in encapsulation of biofertilizers and biocontrols for agricultural delivery. Alginate is the most widely used biomaterial in combination with various other natural or artificial materials for coating. Another most widely used polymer matrix for microbial encapsulation is Carboxy-methyl cellulose.

These two polymers are used with a number of coating materials including starch, wheat bran, talc, bentonite, skim milk etc. It has been observed that supplementing of main carrier matrix with wheat bran, corn starch, talc or peat significantly enhance the performance of the formulations. Spray drying and freeze drying are the primary methods for drying of the capsules. More biodegradable and cheaper materials need to be explored for encapsulation of biofertilizers and biocontrols. A major challenge is the loss of viability of most formulations during drying phase and storage. However, research conducted over the years has shown that encapsulated microbes are superior to their non-encapsulated counterparts in terms of all the parameters like viability, shelf life, survival, activity and efficiency.

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Chapter 7
Induced Systematic Resistance and Plant Immunity

Deepshikha Satish and Sahil Mehta

Abstract The demand for more food production and the pollution of ecosystems by pesticides is calling for sustainable methods to improve crop yields, such as the management of rhizobacteria that grow in the root zone. For instance, rhizobacteria induce systemic resistance against a large number of pathogens in plants. Here we review induced systemic resistance in plants with focus on plant immunity, systemic versus local resistance, molecular mechanisms, signaling, the role of salicylic acid, hormones and genes, and the control of crop diseases.

Keywords Plants · Resistance · Induced systemic resistance · Signalling · Hormones · Plant-immunity · Systemic acquired resistance · Commercial products

Abbreviations

ISR	Induced systemic resistance
NPR1	Natriuretic peptide receptor1
PR	Pathogenesis related
SAR	Systemic acquired resistance

7.1 History of Resistance

Defining induced resistance in the plants is a daunting task due to the absence of defense-dedicated cells (Ruano and Scheuring 2020). Some pioneer scientists define induced resistance as an enhanced expression of the natural defensive attitude of plants in the presence of pathogenic invaders. This defensive behaviour is

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aggravated by alien factors of diverse type and subsequent infection lead to the expression of deterrence (Edreva 2004). The concept of systemic resistance in the plant is more than a century old. Beauverie published a paper titled, “Testing the immunization of plants against fungal diseases”. He discovered that begonia plants become more resistant to vigorously virulent strains of *Botrytis cinerea* fungus when a feebly virulent strain of this fungus (*Botrytis cinerea*) is inoculated to the begonia plants (Beauverie 1901). In the same year, in a different study, titled “Cryptogamic diseases of plants” Ray also indicated that exposure to various external or alien factors/pathogens; impart immunity to plants against different invaders. This provocation enhances the explicit expression of the natural defense mechanism of plants (Ray 1901). Thus, both of them, in independent studies proposed, plants previously exposed to a pathogen could better withstand second exposure.

Further, Chester in his famous study summarised different reports regarding the existence of various induced disease resistance phenomena in plants and gave the first-ever generic concept of plant defense mechanism (Chester 1933). J. Kuć is widely known as “the father of induced resistance research”. He gave biochemical evidence of induced resistance in the 1960s. He explained the phenomenon of induced systemic resistance with the help of phenylalanine (amino acid derivative). He studied the effects of phenylalanine regarding its resistance imparting properties against apple scab disease (*Venturia inaequalis*) (Williams and Kuc 1969). Kuc and co-workers became the torch-bearer in the area of induced resistance and its use as a method in plant defense mechanisms (Dalisay and Kuć 1955). After this initiation, induced systemic resistance phenomenon had been widely applauded and promoted by numerous authors from all around the globe (Benhamou and Picard 1999; Bokshi et al. 2003; Cohen 2001; Schönbeck et al. 1993; Gozzo 2003; Kessmann et al. 1994; Schneider et al. 1996; Soylu et al. 2003).

The terminology “induced systemic resistance” was envisaged specifically for soil-borne non-pathogenic bacteria; when these non-harmful rhizobacteria infect roots, the resistance was found to be induced in the leaves of the infected plants. This newly introduced type of defense system was unveiled in *Arabidopsis thaliana* plants. Roots of a few *A. thaliana* plants were inoculated with the strain of *Pseudomonas fluorescens* (non-pathogenic). Leaves of the plant in reference became somewhat resistant to *Pseudomonas syringae* (bacterial leaf pathogen) after a one-time exposure to infection (Pieterse 1998). Further, the induced systemic resistance involving non-pathogenic bacteria also demonstrates resistance against bacteria, viruses, and fungi in cucumber, tomato, tobacco, *Arabidopsis*, bean, and radish (van Loon et al. 1998). Later on, systemic resistance was characterized as plants’ ability to recall past experiences and as an example of “plant memory” (Conrath 2006). A variety of biotic and abiotic agents was found to be the reason behind the induction of such resistance. The resulting broad-spectrum and long-lasting resistance was called by different terms for example “plant immunity”, “resistance displacement”, “acquired physiological immunity” and “induced system resistance” (Conrath 2006).

7.2 Plant Systemic Immunity

Understanding of plant immunity mechanism is still in its infancy. Scientists are not certain of the underneath mechanisms involved in Induced systemic resistance for many plant species, plant diseases, or pests of these plants, despite the elucidation of the presence and identification of several pathways and chemical signals related to induced systemic resistance. For example, only a few studies are available regarding the resistance mechanisms involved in plant viruses (Satish et al. 2019). But it is well established that like animal immunity mechanisms, plant immunity also has layered characteristics. In the plant fraternity, the first line of defense i.e., pathogen-associated molecular patterns-triggered immunity is composed of pattern recognition (Boller and Felix 2009; Zipfel 2009). This shield prevents most potential enemies' invasion. Further, the component of the second line of defense is a bit different than the components of the first line. For instance, Nucleotide-binding leucine-rich repeat receptor proteins [similar to Resistance (R) gene] is part of the second line of defense in plants. These resistance proteins identify specific effector molecules of an attacker, causing Effector-triggered immunity. This second line of defense is genetically more specific and generally followed by apoptosis in order to prevent further infection. The programmed killing of infected cells and extensive host cell reprogramming is part of the local immune response against pathogens. The first line and second line of defense, generally pave the way for enhanced defensive capacity in plant parts that are still not damaged by the invader. When such induced resistance is developed in a distant location from point of infection, resistance is known as systemic resistance.

7.3 Systemic Acquired Resistance Versus Local Acquired Resistance

Depending upon the area and method of its expression, invader-derived immunity in plants might be classified as 'local acquired resistance' and 'systemic acquired resistance'. Carefully conducted laboratory experiments with the *Tobacco Mosaic Virus* system helped Ross and co-workers in coining the terminology of local acquired resistance and systemic acquired resistance (Ross 1961a, b). In this historic experiment, leaves of the *Nicotiana tabacum* (cv. Xanthi-nc) were inoculated with the tobacco mosaic virus. After this tobacco mosaic virus infection, small necrotic abrasions protruded on the leaves. After a few days, the same leaves were again infected with Tobacco Mosaic Virus but this time smaller-sized and less numerous lesions appeared. Hence severity of infection was reduced to a great extent.

Local acquired resistance was defined by an experiment involving the secondary infection to the nearby leaves where the first inoculation was done. It was found that due to previous exposure to the inoculation, leaves in close vicinity also got immune. In the same system, Ross and colleagues infected the leaves, which were not exposed

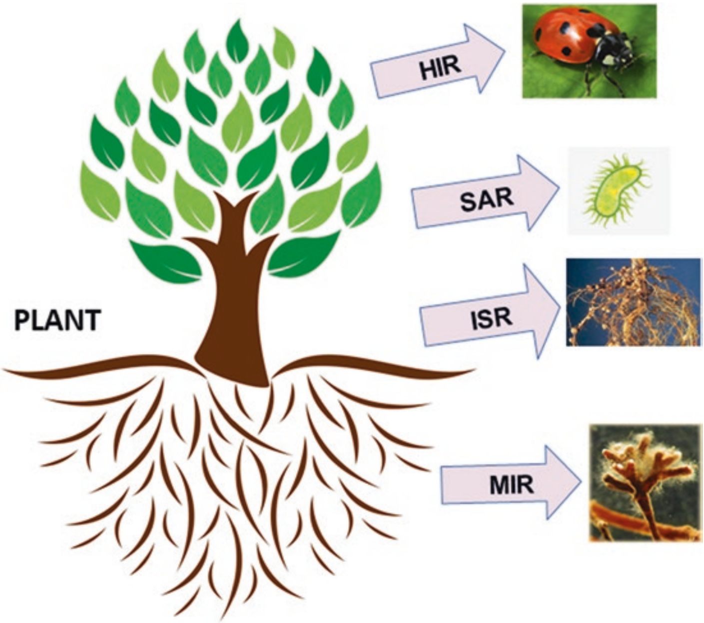

Fig. 7.1 Various types of phyto-invaders induce a variety of resistance in plants. *ISR* induced systemic resistance, *SAR* systemic acquired resistance, *HIR* herbivore-induced resistance, and *MIR* mycorrhizal induced resistance

to the infection previously. They observed that these leaves also showed signs of resistance. They term this phenomenon as 'local acquired resistance' (Ross 1961a). Ross successfully induced resistance to tobacco mosaic virus in the tobacco leaves (which were distantly located on the same plant), by inoculation (primary) of an aerial part of the plant with the virus. They refer to this phenomenon as 'systemic acquired resistance' (Ross 1961b).

This systemic immunity response is known by different names depending upon the invader nature (Fig. 7.1) for example, if elicitor is a pathogen then immunity imparted will be known as 'pathogen-induced systemic acquired resistance' if the infection is caused by mycorrhiza 'mycorrhiza-induced resistance', while in case of herbivores, name of resistance will be 'herbivore-induced resistance', and if immunity is triggered by a non-pathogenic invader or beneficial soil-borne microbes then it will be acknowledged as 'induced systemic resistance'.

7.4 Induced Systemic Resistance

Induced plant resistance is a broad terminology for the defense mechanism of plants, evoked by infection or chemical or biological infections. Despite knowing systemically acquired resistance in plants, the concept of induced systemic resistance became apparent very late. During the early years of the 1990s, three research groups independently demonstrated that root colonization by certain non-pathogenic bacteria leads to enhancement in the defense capabilities of plants against pathogens/invaders (Alström 1991; van Peer et al. 1991; Wei et al. 1991). It has been demonstrated that after root infection by *Pseudomonas fluorescens* WCS417r, aerial plant parts attained an amplified immunity against *Fusarium oxysporum* infection and produced significantly more antimicrobial phytoalexins (van Peer et al. 1991). Thus, van Peerandco-workers gave testimony that *P. fluorescens* infection to the root system can provide systemic resistance in plants. Similarly, Wei and co-worker and Alström explicated enhanced plant immunity after infection of *Pseudomonas* and *Serratia* PGPR strains on cucumber and PGPR strain *P. fluorescens* S97 infection on common bean roots, respectively (Wei et al. 1991; Alström 1991). Among these pioneer research teams in the induced systemic resistance area, gave evidence about the spatial difference between the site of plant growth-promoting rhizobacterial infection and challenging pathogen (Wei et al. 1991; van Peer et al. 1991). Thus, after rigorous contemplation concept of induced systemic resistance came to the scientific sphere and the search for causing agents begun.

Different research groups suggested that induced resistance can be triggered by various elicitors for example; avirulent forms of pathogens or by virulent pathogens under certain environmental conditions, non-pathogens, chemicals, incompatible races of pathogens, etc. After the establishment of the correlation between bacteria and induced systemic resistance, rhizo-fungi (*Trichoderma* spp. or *Piriformospora indica*) were also shown to have a similar impact on plant immunity (Boller and Felix 2009; van Peer et al. 1991; Wei et al. 1991). Induced immunity provides enhanced resistance against a broad spectrum of invaders, for example, foliar, root and fruit pathogen, parasitic plants, and pests (even in some cases, invertebrates such as nematodes). Inoculation of *A. thaliana* roots by *Pseudomonas fluorescens* (WCS417r) bacteria, insulate the plants from various other invaders (bacteria, fungus, and oomycete). Various research groups demonstrated this induced resistance against bacteria (for example, bacterial leaf pathogens *P. Syringae* pv. tomato and *Xanthomonas campestris* pv. Armoraciae), fungi (for example; root-infecting fungi – *Fusarium oxysporum* f.sp. *raphani*, leaf infecting fungi – *Alternaria brassicicola*), and the oomycete leaf pathogen (*Phytophthora parasitica*) (Pieterse 1996; Ton et al. 2002; van Wees et al. 1997). A comprehensive list of induced systemic resistance-inducing beneficial microbes is given in Table 7.1.

7.5 Molecular Mechanism of Induced Systemic Resistance

Induced resistance in plants comprises of an intensified expression of resistance against several invaders simultaneously. This is a plants' way to avoid infection from plant pathogens. Both induced systemic resistance and systemic acquired resistance lead to resistance against a wide range of invaders hence *prima facie* the mechanism of both was assumed to be similar. But many factors indicated

Table 7.1 Various induced systemic resistances induced by beneficial microbes

S. No.	Induced systemic resistance-inducing beneficial microbes	References
1.	Protective strains of *Fusarium oxysporum*	Alabouvette et al. (2009)
2.	Arbuscular mycorrhizal fungi (*Glomus intraradices*, *Glomus mosseae*)	Cameron et al. (2013), Jung et al. (2012), and Pozo and Azcón-Aguilar (2007)
3.	Endophytes (*Piriformospora indica*)	Franken (2012)
4.	*Bacillus* spp. (*Bacillus sphaericus*, *B. mycoides*, *B. cereus*, *B. pumilus*, *B. pasteurii*, *B. subtilis*, and *B. amyloliquefaciens*)	Kloepper et al. (2004)
5	*Pseudomonas* spp. (*Pseudomonas aeruginosa*)	Fatima and Anjum (2017) and Bakker et al. (2007)
6.	Biocontrol fungi (*Trichoderma asperellum SKT-1*, *T. harzianum T39*, *T. harzianum/ T. atroviride*, *T. atroviride* and Sebacinales)	Shoresh et al. (2010)
7	*Rhizobium* spp.	van der Ent et al. (2009b)
8.	Plant growth-promoting rhizobacteria (*Ochrobactrum lupine*/ *Novosphingobium pentaromativorans*, *Azospirillum brasilense* REC3)	Beneduzi et al. (2012), De Vleesschauwer and Höfte (2009), and van Loon (2007)
9.	Plant growth-promoting fungi (*Fusarium equiseti*)	Saldajeno and Hyakumachi (2011)
10.	*B. amyloliquefaciens* strain Blu-v2	Li et al. (2015)
11.	*P. simiae* WCS417r	Pangesti et al. (2016)
12.	*Bacillus amyloliquefaciens*	Wang et al. (2017)
13.	*Flavobacterium* sp. and *Pseudomonas simiae* WCS417	Sommer et al. (2021)

otherwise; for example, salicylic acid is both necessary and sufficient for systemic acquired resistance whereas induced systemic resistance can work without accumulation of salicylic acid and is reliant on jasmonic acid and ethylene reaction pathways in plants (Yuan et al. 2019).

In the case of induced systemic resistance, no damage/localized necrosis is involved, thus evoking factors generated by induced systemic resistance-triggering bacteria are generally not similar to elicitor molecules produced by pathogenic invaders (Ebel and Mithöfer 1998). In an interesting study, it was revealed that induced systemic resistance-engineered *P. Fluorescens* treated *Raphanus raphanistrum* plant, did not amass pathogenesis related proteins, even though these plants exhibit amplified immunity against wilt disease caused by *Fusarium.* But if we take cognizance of the mechanism of systemic acquired resistance, pathogen related protein accumulation is indispensable (Hoffland et al. 1995). A brief description of the differences between systemic acquired resistance and induced systemic resistance is given in Table 7.2.

Despite having many differences induced systemic resistance and systemic acquired resistance pathway converge at the NPR1 protein (Cao 1994). NPR1

Table 7.2 Differences between the two major induced resistance pathways in plants

S. No.	Features	Systemic acquired resistance	Induced systemic resistance
1.	Definition	The phenomenon in which uninfected systemic plant parts become more resistant in response to a localized pathogenic infection elsewhere in the plant is known as systemic acquired resistance (Ross 1961a).	An increased expression of natural defense mechanisms of plants against different pathogens provoked by external factors of various types and manifested upon subsequent inoculation is known as induced systemic resistance (van Peer et al. 1991; Edreva 2004).
2.	Characteristic signaling compound	Salicylic acid	Jasmonic acid and ethylene
3.	Mode of action	Increase in salicylic acid production and accumulation upon inoculation	No increase in production, just sensitivity increases regarding jasmonic acid and ethylene.
4.	Accumulation of pathogen-related proteins	Yes (indispensable in case of systemic acquired resistance)	No accumulation is required
5.	Impact of resistance	The resistance imparted during systemic acquired resistance is long-lasting (sometimes lifelong for the plant), and efficacious against several plant invaders, including oomycetes, bacteria, viruses, and fungi.	Comparatively more specific. Not explored completely.
6.	Damage/ localized necrosis	Present	Not required
7.	Salicylic acid	Both necessary and sufficient	Can work without accumulation of salicylic acid
8.	Defense gene involved	PR-1 and PR-2	PDF1.2
9.	Chemical induction (non-living)	Possible	Not possible

Note: *PR* Pathogenesis related, *PDF* Plant defensin gene

protein is downstream of the salicylic acid in the systemic acquired resistance pathway, whereas in induced systemic resistance it is situated downstream of ethylene response. This protein is found to be necessary for both systemic acquired resistance and induced systemic resistance as mutant *npr1* plants are unable to express induced systemic resistance after exposure to WCS417. Hence, it has been proved that NPR1 protein might be involved in a critical reaction in achieving the evoked state in both the cases of systemic acquired resistance and induced systemic resistance. But perhaps in the mechanism after NPR1, the signaling reactions and pathways might become deviated as pathogen related proteins are not accumulated in the case of induced systemic resistance (Fig. 7.2). A deep insight into the induced

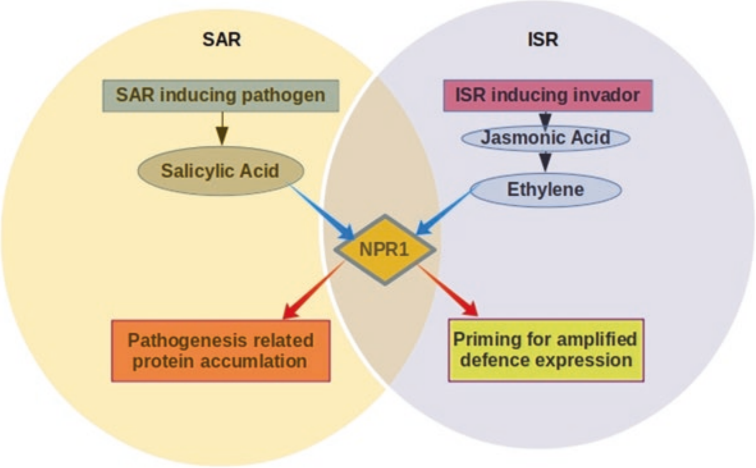

Fig. 7.2 Inter-sectioning of the systemic acquired resistance (SAR) and the induced systemic resistance (ISR) resistance pathways

systemic resistance mechanism can be achieved if we explore three main and distinctive steps of induced systemic resistance i.e., induction, signaling, and expression.

7.5.1 Induction

During initial research findings, salicylic acid produced by bacteria was considered to be the inducing factor in the case of bean (De Meyer and Höfte 1997), tobacco, and tomato (De Meyer et al. 1999), but later on, many other elicitors were shown to be effective in triggering induced systemic resistance. In the light of limited knowledge present in the scientific literature about bacterial determinants that trigger induced systemic resistance, few important conclusions can be made. For example, crude microbial cell wall preparations of the WCS358 (rhizobacterial strain) can invoke induced systemic resistance if absolute components of this strain are inoculated to roots of *A. thaliana* plants (Bakker et al. 2003; Meziane et al. 2005). A brief list of induced systemic resistance triggering agents has been enlisted in Table 7.3.

It has been identified that few 'plant growth-promoting fungi' also have cell components that explicitly behave as an elicitor of defense mechanisms (Conrath 2006). For example, Sm1 from *Trichoderma virens* is a protein that has a particular defense-eliciting function (Djonovic et al. 2007). Not only this, few cellulases and xylanases also act as defense-elicitor. Further, the concept of "differential induction of systemic resistance" emerged in various experimental reports related to different plant species or ecotypes (Ton et al. 1999; van Wees et al. 1997). According to the notion of differential induction pattern, few beneficial microbe strains have an

Table 7.3 Redundant induced systemic resistance elicitors

S. No.	Induced systemic resistance elicitor	References
1.	Lipopolysaccharides	Meziane et al. (2005)
2.	Iron-regulated metabolites pyoverdin	De Vleesschauwer and Höfte (2009) and van Loon et al. (1998)
3.	Salicylic acid	De Meyer et al. (1999)
4.	2,4-diacetylphloroglucinol	Iavicoli et al. (2003)
5.	Pyocyanin	Audenaert et al. (2002)
6.	Flagella	De Vleesschauwer and Höfte (2009)
7.	N-acyl homoserine lactones	
8.	Iron-regulated siderophores	
9.	Biosurfactants	
10.	2R,3R-butanediol produced by *B. subtilis* GB03	Ryu (2004)
11.	C13 volatile emitted by *Paenibacillus polymyxa*	Lee et al. (2012)
12.	2,3-butanediol	Ryu (2004)
13.	Siderophore	Maurhofer et al. (1994)
14.	Acibenzolar-S-methyl	Ren et al. (2012)
15.	β-Aminobutyricacid	Quaglia et al. (2011)
16.	Probenazole	Yang et al. (2011)
17.	Saccharin	Srivastava et al. (2011)
18.	Potassium phosphite	Pinto et al. (2012)
19.	Thiamine	Pushpalatha et al. (2011)
20.	Silicon	Shetty et al. (2012)
21.	Biochar	Elad et al. (2010)
22.	*Ulva armoricana*	Jaulneau et al. (2011)
23.	*Ulva lactuca*	El Modafar et al. (2012)
24.	Coumarins	Siwinska et al. (2018)
25.	*TH12* and *CF*	Alkooranee et al. (2017)

impact on a variety of plant species i.e., they can elicit systemic resistance in various plant species, while other bacteria exhibit more specificity i.e., they can impact the defense mechanism of only a few plant species. This differential induction pattern indicates bacteria act according to a species-specific recognition pattern. It is proposed that bacteria recognize receptors, on the root surface before eliciting induced systemic resistance (van Loon et al. 1998). For example, WCS374 strain elicits induced systemic resistance in *Raphanus raphanistrum* but not in *Arabidopsis thaliana*, whereas WCS358 elicits induced systemic resistance in *A. thaliana*, *Phaseolus vulgaris*, and *Solanum lycopersicum*, but not in *Daucus carota* or *Raphanus raphanistrum* (Duijff et al. 1993; Leeman et al. 1995; van Wees et al. 1997; Meziane et al. 2005).

7.5.2 Priming of Infected Plants

Preparation of the whole plant to better resist the invader's attack is called priming. In response to the primary attack of the induced systemic resistance-inducing invader, some of the induced systemic resistance-related compounds are produced in uninfected plant tissues. But the explicit induced systemic resistance-associated compounds are expressed only after secondary infection. This second exposure is characterized by comparatively faster and stronger defense responses. It is often not possible to assess if a plant is primed, in the absence of the invader. Only after the secondary exposure to the invaders' attack, amplified/altered transcriptional changes in a plant can be observed.

After extensive study of priming, it has been ascertained that priming enhances the fitness quotient of a plant, and the advantage of priming seems to be greater than its costs during invader combat. This indicates that priming serves as a survival mechanism of the plant against damage caused in an adverse environment. During conditioning/sensitization, augmentation of structural barriers and elevation in several miRNAs of various transcription factors genes is observed.

In a study, it has been shown that *strain* WCS417r (*P. fluorescens*) inoculated *A. thaliana* plant display crucial changes. On the entry of *Hyaloperonospora arabidopsidis* in *A. thaliana*, an enhanced frequency of callose accumulation was witnessed, which efficiently arrests the invasion of the pathogen (van der Ent et al. 2009a). Callose is a polysaccharide comprised of β-1,3-glucan with β-1,6-branches. Callose is generally present in the cell walls of higher plants. Further, the same study threw some light on the fact that this phenomenon of ingression hindrance is controlled by abscisic acid (plant hormone). In a different but related study of structural barriers by priming, stomata closure to a significant extent has been reported after a second exposure to infection. In this study, *A. thaliana* leaves were inoculated by *P. syringae.* This primary exposure of *P. syringae* results in the infection of the FB17 strain of *Bacillus subtilis* to the roots of *A. thaliana* FB17 triggered induced systemic resistance, which provides immunity to the unexposed plant parts from *P. syringae* infection (Rudrappa et al. 2008). This plant growth promoting rhizobacteria-induced priming indicated a potent structural barrier that can delay/inhibit the progression of disease in induced systemic resistance-primed plants.

Along with structural barriers, transcriptional factors play an important role during priming. This induced systemic resistance-involved transcription factors often remain inactive in plants which not exposed to the invaders, but upon the cognizance of pathogen/pest presence, these transcription factors provide the plant accelerated defense response. Several members of the APETALA2/ethylene-responsive factor family are predicted to have a crucial role in the regulation of jasmonic acid- and ethylene-dependent defences (Memelink 2009). Priming effects are an integral part of the induced systemic resistance mechanism and can be elicited by biological, chemical-induced systemic resistance inducers. After the primary infection/inoculation, responses such as cell wall lignification or phytoalexin synthesis occur more

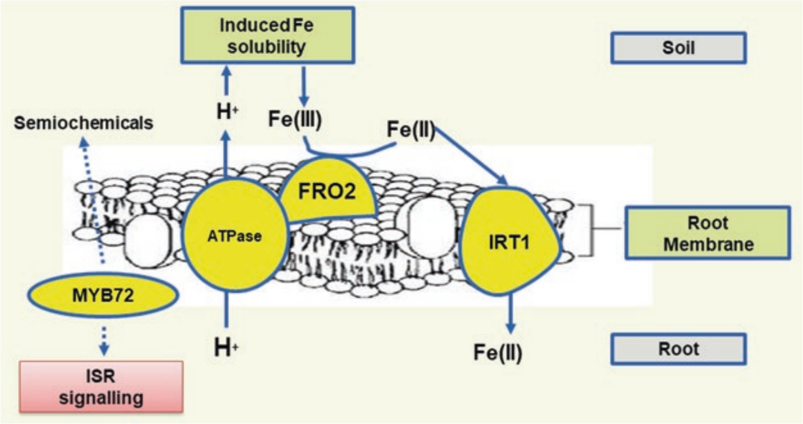

Fig. 7.3 Iron deficiency response in *Arabidopsis* roots

expeditiously. The in-depth molecular mechanisms behind priming and its significance in the overall plant immunity are still not explored to the fullest.

7.5.3 Signalling in Beneficial Microbe-Induced Systemic Resistance

Signaling in induced systemic resistance is a considerably complex phenomenon. For example, several induced systemic resistance-evoking rhizobacterial strains produce salicylic acid, whereas others do-not. Some rhizobacteria are capable of producing salicylic acid on minimal media *in-vitro* in the absence of iron (Fig. 7.3). If the soil confronts a similar environment in the rhizosphere, these bacteria are expected to show analogous expression. But in nature i.e., under *in-vivo* conditions, salicylic acid is not released into the rhizosphere and is destined to the salicylic acid-containing siderophore. Thus, induced systemic resistance induction can happen only when the determinant agent is siderophore and no resistance happens if siderophore is not able to act as elicitor (Aznar and Dellagi 2015).

7.5.4 Expression of Induced Systemic Resistance

Induced systemic resistance articulation is pretty much analogous to systemic acquired resistance, this is the reason why the discovery of induced systemic resistance took too long while the mechanism of systemic acquired resistance had been already explored to a great extent. Due to overlapping responses and few proteins like NPA1, finding induced systemic resistance-specific mechanisms has become a challenge to the scientific community. Both, the induced systemic resistance and systemic acquired resistance, reduce the disease severity and in some cases, the extent of invasion in plants is minimized along with depreciated growth of the pathogen itself. These signs are a testament to the enhanced immunity of plants.

Though the plant seems to be well equipped against the invader, expression of neither induced systemic resistance nor systemically acquired resistance saves plants thoroughly from all types of infections. But the reduction in disease development generally saves a crop to some extent from the natural attack of invaders. As stated earlier, the complete mechanism of induced systemic resistance has not been discovered yet thus the entire conclusions drawn are based on the limited information available. Systemic acquired resistance has characteristic pathogenesis-related genes but the search for characteristic protein for induced systemic resistance was not able to produce substantial results. Further, there is no major shift found in the defense-related gene expression during induced systemic resistance activation (Heil 2002).

Using, transcriptome analyses (cDNA microarrays technique) Verhagen and coworkers confirmed the hypothesis that beneficial microbes interfere in plant transcriptome to only a negligible extent (Verhagen et al. 2004). For example, WCS417-infected *Arabidopsis* plants were taken for the analysis of over 8000 genes. Out of these whooping 8000 genes, only 102 genes in the roots exhibit changes in expression and no gene showed the change in expression in leaves at all, despite full-blown expression of induced systemic resistance by leaves. As the studies to date are in primitive stage, we are not able to draw any concrete conclusion regarding players involved in induced systemic resistance. We can assume only that, plants must possess some undiscovered defense-related products, which impart resistance to plants against broad spectrum invaders. Transgenic plants with altered enzyme activities, exposed to induced systemic resistance-evoking invader can help in exploring the molecular mechanism behind induced systemic resistance. But so far, no such successful studies are known to the scientific world.

7.6 Is Salicylic Acid Necessary for Induced Systemic Resistance?

Many studies have been conducted to ascertain if induced systemic resistance-triggering strains cause activation of the salicylic acid-independent pathway. Association of pathogen-related proteins with induced systemic resistance and *nahG* gene mutant studies clear the air to a great extent. Induced activation study on tobacco plant demonstrated that induction of induced systemic resistance by 7NSK2 strain was salicylic acid independent and there was no sign of enhanced production of the pathogenesis related PR-1 protein along with the enhanced immunity (De Meyer et al. 1999). Interpretation of results revealed that salicylic acid produced in this case is insufficient for generating pathogen-related proteins but certainly enough for eliciting induced systemic resistance mechanism. Further, Press and co-workers (1997) demonstrated that mutants of *Serratia marcescens*, which could not produce salicylic acid, were able to induce defense mechanisms in tobacco against *P. syringae* and in cucumber against *C. Orbiculare*. In a different but related study, van Wees et al. (1997) provide experimental proof that WCS358 does not produce salicylic acid but it can elicit systemic resistance in *A. thaliana.*

On the other hand, they also proved that WCS374 when invade, *A. thaliana* can produce salicylic acid in-vitro but this incidence does not provoke any induced systemic resistance (van Wees et al. 1997) or elicit induced systemic resistance in a "Salicylic acid-independent" pathway (Press et al. 1997). In the light of the above-mentioned evidence and observation, it can be concluded that salicylic acid production by rhizobacteria is not imperatively required for the expression of systemic resistance.

7.7 Role of Hormones in the Signaling of Induced Systemic Resistance

Jasmonic acid and ethylene are known to be the central players in induced systemic resistance signaling (Fig. 7.4). In the series of events where beneficial microbes (present in the soil) impart salicylic acid-independent systemic resistance in plants, jasmonic acid and ethylene play crucial role (Audenaert et al. 2002; Hossain et al. 2008; Pieterse 1998, Korolev et al. 2008; Ryu et al. 2004; Stein et al. 2008, Ahn et al. 2007; De Vleesschauwer et al. 2008; Weller et al. 2012, Hase et al. 2008; van der Ent et al. 2009b; Yan et al. 2002). The essentiality of jasmonic acid in the induced systemic resistance pathway is ascertained using *jar1* mutant plants. *jar1* gene is supposed to encode a jasmonic acid amino acid synthetase, required for jasmonic acid signaling activation. *Arabidopsis* plant mutants; *jar1*, *jin1*, and *coi1* are found to be defective in induced systemic resistance signaling (Kloepper et al. 2004; Pieterse 1998) whereas ethylene signaling mutants *etr2*, *ein1*, *ein3*, and *eir1*,

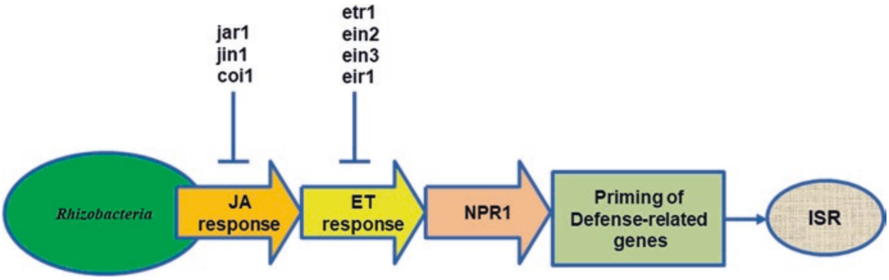

Fig. 7.4 Mechanism of signaling in induced systemic resistance. *JA* jasmonic acid, *ET* ethylene, *NPR1* natriuretic peptide receptor1, *ISR* induced systemic resistance

were found non-functional in WCS417r-induced systemic resistance strain of *P. fluorescens* (Pieterse 1998; Kloepper et al. 2004; Pozo et al. 2008). A *jar1–1* plant (Jasmonic acid response mutant) and the *etr1–1* plant (ethylene insensitive mutant) were checked for the capability to showcase induced systemic resistance in *A. thaliana*. Even after inoculation of roots by *WCS417r* strain of *Pseudomonas fluorescens* both mutants were not show-casing any sign of increased resistance against *P. Syringae* pv. Tomato (Pieterse 1998). Moreover, exogenous application of jasmonic acid and ethylene also often results in an elevated level of resistance against *Pseudomonas syringae*. This leads to the conclusion that both of the hormones are equally essential in induced systemic resistance mechanism, a defect in the synthesis mechanism of any one of them hamper the induced systemic resistance expression.

Further, Pieterse et al. (1998) postulated that jasmonic acid and ethylene components are successively engaged in the induced systemic resistance pathway, which causes deeper insight into the essentiality of jasmonic acid and ethylene simultaneously. Regarding jasmonic acid and ethylene, van Wees et al. (1999) reached an interesting conclusion during a study. They suggested jasmonic acid and ethylene involvement in induced systemic resistance have relation to enhanced sensitivity to these hormones and the production level of these concerned hormones is not enhanced during induced systemic resistance response. To prove this point, van Wees et al. (1999) examined the expression of a combination of jasmonic acid and ethylene-responsive genes (i.e., *PDF1.2*, *VSP*, *LOX1*, *LOX2*, *PAL1*, *CHI-B*, and *HEL*) in *A. thaliana* plants expressing *Pseudomonas fluorescens* WCS417r-ISR. But there were no signs of change in the regulation pattern of jasmonic acid and ethylene genes in inoculated plants, neither locally or systemically. Hence it was suggested that production levels of either jasmonic acid or ethylene have little/no role in resistance achieved. Further, Pieterse et al. (1998) using transgenic line S-12 confirmed that induced systemic resistance involved enhanced sensitivity of ethylene and jasmonic acid rather than elevated production.

7.8 Genes Involved in the Induced Systemic Resistance Mechanism

The repertoire of genes involved in the induced systemic resistance mechanism is neither complete nor well understood (Fig. 7.5). Till now the role of transcription co-regulator of pathogen-related genes (for example, *NPR1*) could not be ascertained in the case of induced systemic resistance, because in the induced systemic resistance mechanism there is no accumulation of pathogen-related genes. But it is quite clear that the induced systemic resistance mechanism cannot take place in the absence of *NPR1* (Pieterse 1998). Apart from systemic acquired resistance and induced systemic resistance, plant growth-promoting rhizobacteria, and plant growth-promoting fungi also witness the crucial role of *NPR1* (Iavicoli et al. 2003;

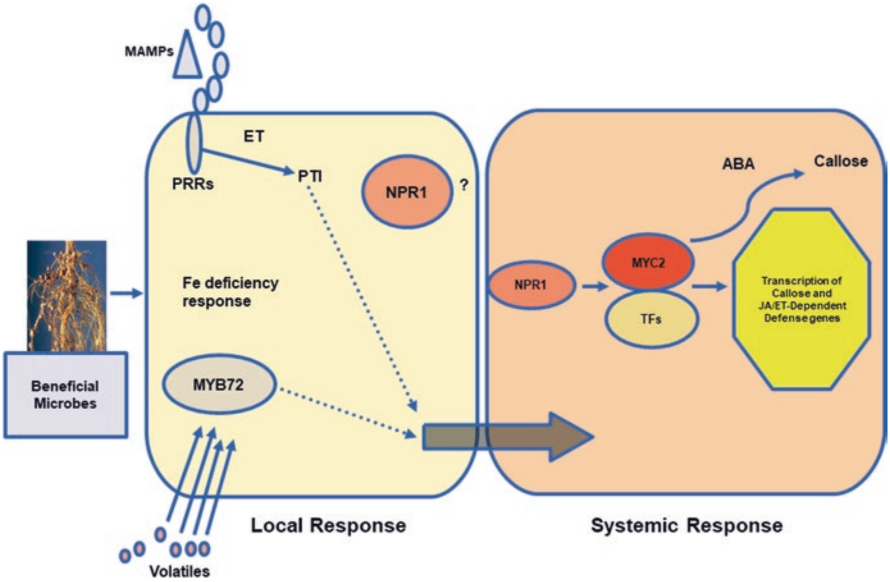

Fig. 7.5 Genes involved in the induced systemic resistance mechanism. Solid purple lines mark established interactions; dotted purple lines are indicative of hypothetical inter-connections. A visibly large arrow indicates long-distance translocation of molecular signals. *MAMP* microbe-associated molecular pattern, *ABA* abscisic acid, *ET* Ethylene, *JA* jasmonic acid, *PRR* pattern-recognition receptor, *PTI* pattern-triggered immunity, *SA* salicylic acid, and *TF* transcription factor

Ahn et al. 2007; Hossain et al. 2008). Further, in contrast to nuclear function in the case of systemic acquired resistance, many pieces of evidence indicate the cytoplasmic function of NPR1 protein in the case of ethylene/jasmonic acid signaling (Pieterse et al. 2012; Ramírez et al. 2010; Spoel 2003). The sequence of induced systemic resistance signaling events established that the role of *NPA1* is downstream that of ethylene and jasmonic acid.

In the quest of searching role of various genes in the mechanism of induced systemic resistance, induced systemic resistance was induced chemically in wild-type *Arabidopsis* plants by external application of 1-aminocyclopropane-1-carboxylic acid. 1-aminocyclopropane-1-carboxylic acid was capable of inducing induced systemic resistance in the *jar1* mutant plants. This hints towards the fact that ethylene is involved in the reaction after the jasmonic acid-involved signaling reaction. Contrarily, 'methyl jasmonate' is not capable of triggering induced systemic resistance in the plants with the mutated *etr1* gene. Thus, receptivity to jasmonic acid and ethylene are structured in precedence, where jasmonic acid is required first. Neither 'methyl jasmonate' nor '1-aminocyclopropane-1-carboxylic acid' could trigger induced systemic resistance in the plants with mutated *npr1* genes. This indicates the requirements for both jasmonic acid and ethylene upstream of *NPR1* in the signaling pathway. Hence it is very much clear that *NPA1* has a crucial role not only in the accumulation of pathogen-related genes in the case of systemic acquired resistance but also in the case of ethylene/jasmonic acid signaling pathways.

As NPR1 is common and inseparable to both systemic acquired resistance and induced systemic resistance, it is a matter of scientific interest to know, how the selection of downstream reaction takes place. A major challenge in this regard is the identification of components necessary for signaling in the induced systemic resistance and systemic acquired resistance so that NPR1-dependent defense gene activation can be ascertained. Along with NPR1, another most important gene involved in induced systemic resistance is *MYB72* (Segarra et al. 2009). This is the regulator of induced systemic resistance, which can be identified during early infection events in roots.

7.9 Controlling Crop Diseases Using Induced Resistance

Induced systemic resistance in plants is a long-lasting mechanism but the main hindrance in using this phenomenon as an alternative to available disease management programs is that it is generally not complete. Along with this, many of the induced systemic resistance-inducing agents minimize disease impact by 20–70% only. In the presence of highly specific, easily available, and immensely effective chemical reagents, the use of non-specific and less effective plant resistance inducers does not seem to be a lucrative and easily acceptable idea by the average farmer. Using non-toxic plant resistance inducers in agriculture to combat plant diseases is a very advantageous concept. It has the potential to reduce the use of conventional pesticides hence their indiscriminate addition to the environment.

Further, by easing the financial burden on consumers and small/marginal farmers, plant resistance inducers could become a potential product for use in modern agriculture. Plant resistance inducers might also be easily used in combination with organisms used as “biocontrol agents”, in comparison to conventional pesticides. In addition to this, induced systemic resistance induction might prolong the effective time of resistance (R) genes. From the economic perspective, some of the plant resistance inducer compounds are relatively cheaper than chemicals available in the market, for example, Probenazole (commercially available as Oryzemate) was the first plant resistance inducers. It was registered in Japan as a chemical resistance activator in 1975 (Iwata et al. 2004). Since then, many plant resistance inducers have been listed for commercial use. Some commercialized plant resistance inducers popular in the market are as follows (Table 7.4).

Table 7.4 Commercial products for induced systemic resistance

S. No.	Commercial name	Active ingredient	Firm/Company
1.	Bion® or Boost® or Actigard®	Acibenzolar-S-methyl (50% w/w)	Syngenta
2.	Axiom	Harpins	Rx Green Solutions
3	Cabrio®, Headline®	Strobilurin fungicide and Pyraclostrobin	BASF
4.	Myco-Grow™ Micronized Endo/ Ecto Seed Mix	An agglomeration of 8 species of ectomycorrhizal and endomycorrhizal fungi	Blue-Sky Organics
5.	Milsana®	Extract of *Reynoutria sachalinensis*	Bioscience
6.	Elexa®	Chitosan	Safe Science
7.	Messenger®	Harpin protein	Plant Health Care
8.	Oryzemate®	Probenazole (PBZ; 3-allyloxy-1,2-benzisothiazole-1,1-dioxide)	Not commercially available now

7.10 Conclusion

Systemically induced resistance expressed itself through elevated defense response upon the attack of the invader. The discovery of systemic acquired resistance dates back to the eighteenth century, while induced systemic resistance is newly discovered and still not completely explored by the scientific community. Systemic acquired resistance and induced systemic resistance, both exhibit resistances against invaders, but the major difference is that systemic acquired resistance negatively affects plant growth while the induced systemic resistance has plant growth-promoting properties. Though, the extent of correlation between plant growth and induced systemic resistance cannot be established with certainty. Both induced systemic resistance and systemic acquired resistance can act independently as well as can have an additive effect. This in-built plant immunity after induction can reduce the plant disease to some extent but it will take scientific time and effort to replace chemical methods of pest control with induced systemic resistance and systemic acquired resistance.

Induced disease resistance i.e., induced systemic resistance and systemic acquired resistance are good and attractive solution against potential environment degrading chemical agents. The molecular mechanism behind systemic acquired resistance is well discovered but molecular mechanism detail behind the induced systemic resistance is still the bottleneck. As systemic acquired resistance and induced systemic resistance pathways to act independently as well as additively, the experimental revelation of induced systemic resistance molecular biology can be proved instrumental in the development of an environment-friendly crop protection method. By exploiting the unique and natural plant potential to contest against pathogens, the induced systemic resistance might help in minimizing the use of toxic and eventually extremely harmful chemicals for plant ailment control.

Due to the least harmful nature, induced systemic resistance could be seen as a substitutive, non-genetically modified organism, non-traditional and eco-friendly approach for the protection of plants against diseases. Thus, induced systemic resistance can be envisaged as one of the foundation stones of the major pillar of sustainable agriculture. This unique and inherent plant power to combat pathogens can be exploited as an alternative, non-conventional, non-biocidal, and eco-friendly approach for plant protection, sustainable agriculture, and the welfare of humanity at large.

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Chapter 8
Microbial Elicitors for Priming Plant Defense Mechanisms

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Abstract Some microrganisms have evolved to be associated with plants, receiving nutrients from plants, and helping plants to fight pathogens by producing microbial elicitors, which are compounds that trigger plant defenses. Elicitors are thus safe compounds that can replace harmful pesticides for a sustainable agriculture. Here we review plant immunity and microbial elicitors with focus on antibiotics, volatile organic compounds, siderophores, antimicrobials, enzymes, salicylic acid, methyl salicylate, benzoic acid, benzothiadiazole and chitosan.

Keywords Plants · Microbes · Pathogen · Immunity · Defence · R genes · Metabolites · Antibiotics · Siderophores

Abbreviations

PRR	Pattern-recognition receptors
MAMP	Microbe-Associated Molecular Pattern
DAMP	Damage-associated molecular patterns
MTI	MAMP triggered immunity
RLP	Receptor-like proteins

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8.1 Introduction

While the demand for food increases exponentially, crop productivity gets relentlessly haunted by an increased number of biotic and abiotic stress combinations generally associated with global warming (Schlenker and Roberts 2009; Challinor et al. 2014; Zhao et al. 2017; Mehta et al. 2019, 2020). Abiotic stress conditions like drought, salinity, low and high-temperature etc. also influence the biotic stress factors (microbes, insects, weeds, and phytopathogens) (Seherm and Coakley 2003; McDonald et al. 2009; Ziska et al. 2010; Peters et al. 2014) (Fig. 8.1). These stress conditions likewise influence the interactions between plants and microbes present in their rhizosphere which built up quite a long time ago. The more fascinating fact is that these plants are established on land with the help of symbiotic fungal associations. It suggests that plants are invariably exposed to microbes via associations since their first existence on land, and these disagreements between microbes and plants resulted in mutative coexistence cycles which further shaped their habitats, lifecycles, distribution, and genomes of both organisms.

Based on their nature, these microbes are either beneficial or harmful to the plants. The harmful microbes act as pathogens and delimit productivity by causing a large number of diseases in multiple crops (Lamichhane and Venturi 2015; Rahman et al. 2019; Singh et al. 2019). It is supported by the fact that these biotic

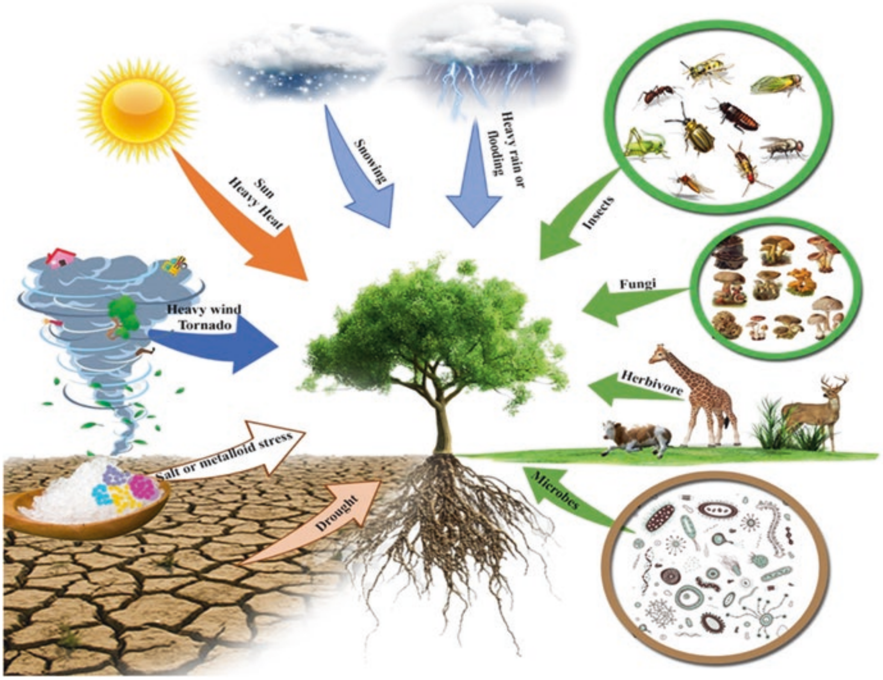

Fig. 8.1 Abiotic and biotic stresses which reduce plant productivity

Table 8.1 Characteristic features of necrotrophs and biotrophs

Features	Necrotrophs	Biotrophs
Uptake of nutrients	From dead plant cells	From live cells
Type of pathogen	Opportunistic (non-obligate)	Specialized (obligate)
Death of host cell	Rapid	Not rapid and induce hypersensitive cell death in incompatible interactions
Mode of entry	Enter through wounds or natural openings thus considered as unspecialized	Specialized entry via direct (mechanical) entry or through natural openings
Secretion of lytic enzymes	Cell-wall degrading enzymes and toxins	Few lytic enzymes or toxins
Systemic	Seldom	Often
Host range	Wide	Narrow
Survival on host	As saprotrophs	On host or as dormant propagules
Host preference	Weak, young, or damaged plants	Plants of all ages
Control	By quantitative resistance genes	By specific (gene-for-gene) Resistance genes
Growth within-host	Intercellular and intracellular through dead cells	Intercellular
Defense pathways of plants against pathogens	Jasmonate and ethylene-dependent	Salicylate-dependent
Examples	*Botrytis cinerea* – Grey mold, *Pythium ultimum* – Damping-off in seedling	*Uromyces fabea* – Rust, *Ustilago maydis* – Maize smut

factors constrain the yield up to 26% globally. They invade the plants either through the leaf (stomata), stem (lenticels), and root surface directly or through injury. After the invasion, they employ a variety of strategies to impair plant growth. These pathogens are comprehensively divided into two types- necrotrophs (bacteria, fungi, insects, and also herbivorous animals), hemibiotrophs, and biotrophs (basically viruses). The former type kills their host and feeds on the dead material, unlike biotrophs that complete their life cycle in a living host. Being sessile by nature, the plants have evolved their immune system to prevent themselves from pathogens as they can't escape their enemy, unlike vertebrates. The characteristic features of necrotrophs and biotrophs are tabulated in Table 8.1.

8.2 Plant Immunity Against Harmful Microbes

An enormous set of pathogens have the potential to kill or damage plants and it goes on through the entire ecosystem. Plants utilize preformed defenses intended to avert pathogen and herbivore attacks. The first line of defense in plants is provided by the thick waxy or cuticular skin of the plant body along with the presence of antimicrobial products (Dangl and Jones 2001). Although pathogen finds a broad spectrum of strategy to invade. For the passive form of invasion, intercellular space like apoplast, stomata, hydathodes, lenticels, or local wounds are the frequent target, and in the active, plant-pathogen develop specialized organs like nematode and aphid have stylet, fungi have hyphae as well as haustoria (Jones and Dangl 2006). On successful plant invasion, the plants utilize their immune system consisting of mainly two interconnected tiers to fight against pathogens (Jones and Dangl 2006; Boller and Felix 2009; Thomma et al. 2011; Spoel and Dong 2012). One of these innate immunity strategies utilizes cell surface pattern-recognition receptors (PRRs) to perceive Microbe-Associated Molecular Patterns (MAMPs) and host-derived damage-associated molecular patterns (DAMPs) present in a large variety of microbes (Boller and Felix 2009). Receptor-like kinases and receptor-like proteins (RLPs) are the cell surface pattern-recognition receptors in plants. The canonical structure of receptor-like kinases has an extracellular domain to recognize ligands, an intracellular kinase domain with only one pass transmembrane domain (Couto and Zipfel 2016; Zipfel and Oldroyd 2017). Receptor-like proteins lack the kinase domain (Zipfel 2014; Couto and Zipfel 2016; Zipfel and Oldroyd 2017) (Fig. 8.2).

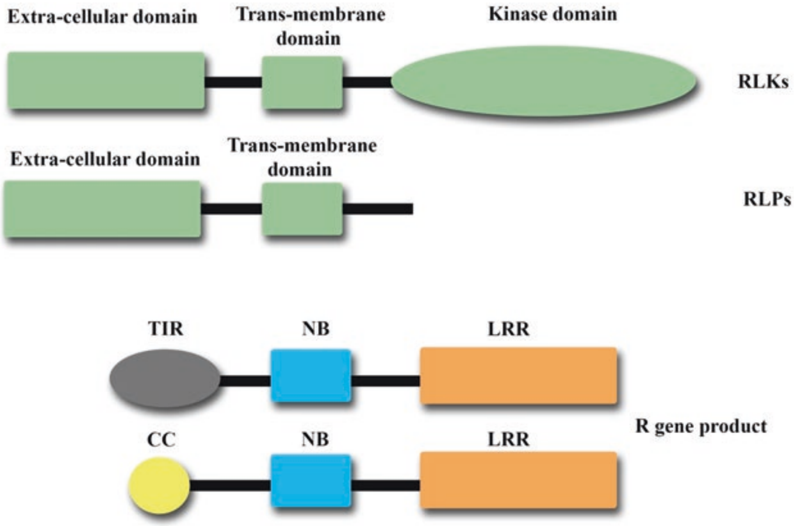

Fig. 8.2 Mechanism of plant immunity against harmful microbes. Receptor-like kinases (RLKs) and receptor-like proteins (RLPs) are potent membrane molecules to identify Microbe-Associated Molecular Patterns (MAMPs) and Pathogen-Associated Molecular Patterns (PAMPs). R gene products (NB-LRRs) recognize the released Avr factors from pathogens. *TIR* Toll-interleukin-1 receptor, *NB* Nucleotide-binding, *LRR* Leucine-rich repeat, *CC* Coiled coil, and *R gene* Resistance gene

Pattern-Recognition Receptors have a highly variable ligand recognition domain and thus recognizes a wide range of microbes. They along with their co-receptors (known to have the same extracellular domain as PRRs) triggers a signaling cascade to establish pattern-triggered immunity (Jones and Dangl 2006; Zipfel 2014). Microbe-Associated Molecular Patterns (MAMPs) are shared similar molecular patterns such as lipopolysaccharides, peptidoglycan, flagellin, etc. (Jones and Dangl 2006) which exist in pathogen cell wall or extremities to represent own group identity as well as potent virulence (Table 8.2). On the other hand, damage-associated molecular patterns (DAMPs) encourage the inflammatory responses by activating the PRRs (Table 8.3). They are endogenous molecules that are released from the stressed or dead cell eliciting the immune system activation (Gust et al. 2017). As, e.g., tomato systemin generated by the wound, influences the processing of prosystemin and it induces adjacent cells as well as vascular bundle elements to produce Jasmonic acid which finally activates the expression of proteinase inhibitor genes (Pearce et al. 1991).

Table 8.2 Common potent microbe-associated molecular patterns with their respective pattern recognition receptors

S. No.	Microbe associated molecular patterns	Origin	Family of pattern recognition receptors	Associated pattern recognition receptors	Plant species
1.	RaxX	*Xanthomonas oryzae pv. Oryzae*	LRR XII	XA21	*Oryza longistaminata*
2.	Flagellin	*Pseudomonas syringae pv. Tabaci*	LRR XII	FLS2	*Arabidopsis thaliana*
3.	csp22	*Staphylococcus aureus*	LRR XII	CORE	*S. lycopersicum*
4.	EF-Tu	*Escherichia coli*	LRR XII	EFR	*A. thaliana*
5.	Chitin	*Agaricus bisporus*	LysM	AtCERK1, AtLYK5	*A. thaliana*
6.	SnTox1	*Stagonospora nodorum*	WAK	Snn1/ TaWAK	*Triticum aestivum*
7.	Lipopolysaccharides	*P. fluorescens*	G-Lec	SD1–29/ LORE	*A. thaliana*
8.	Avr3/ Six1	*Fusarium oxysporum*	G-Lec	I-3	*S. lycopersicum*
9.	NLP	*Pythium aphanidermatum*	LRR	RLP23	*A. thaliana*
10.	Elicitin	*Phytophthora cryptogea*	LRR	RLP85/ ELRb	*S. microdontum*

Source: Google scholar-based literature survey 1995–2020. *LRR* Leucine-rich repeats, *WAK* Wall-associated kinase, *LysM* Lysine motif, *RLP* Receptor-like protein

Table 8.3 Potent damage-associated molecular patterns and respective host plants

Category	Potent Damage associated molecular patterns	Host plants
Protein	PAMP-induced secreted peptides	*Arabidopsis thaliana*
	Rapid alkalinization factors	*A. thaliana*
	AtPep1	*A. thaliana*
	High mobility group box 3	*A. thaliana*
Carbohydrate	Glucose (monosaccharide)	*Nicotiana tabacum*
	Sucrose (diholoside)	*A. thaliana*
	Trehalose (diholoside)	*A. thaliana*
	D-allose (monosaccharide)	*Oryza sativa*
Lipid	Hydroxystearic acid (cutin monomer)	*Hordeum vulgare*
Nucleotide	Extracellular ATP	*A. thaliana*

Source: Google scholar-based literature survey 1995–2020

After recognizing microbe-associated molecular patterns or damage-associated molecular patterns, pattern recognition receptor-dependent response triggers the downstream cell signaling to initiate the immune response (Schwessinger and Ronald 2012). The Pattern-recognition receptors have many kinds of an extracellular domain, *viz*., leucine-rich repeats, lectin, lysine motif, epidermal growth factor-like domains which are intended to provide a more significant range of ligand recognition. The co-receptors that form the complex to activate the different downstream signaling molecules namely Receptor-like proteins, receptor-like kinases, etc. also have a role in plant growth, abiotic stress, and mutualism with beneficial microbes. Finally, Calcium-dependent protein kinases, Mitogen-activated protein kinase cascades, reactive oxygen species production, and cellulose deposition get activated, which leads to modification in transcriptional products (Boutrot and Zipfel 2017).

Through evolution, microbes have developed a vast repertoire of effector molecules or elicitors for successful infection establishment in their hosts, while responsive plants persistently produce disease resistant R proteins to combat these effector molecules. As the elicitors enter into a plant cell through the type III secretion system (Finlay and Falkow 1997), their recognition in plants triggers the effector-triggered immunity (Jones and Dangl 2006; Spoel and Dong 2012). Most of the knowledge about the effectors and type III secretion system is based on the work conducted on *Pseudomonas syringae*, a highly diverse plant biotrophic pathogen (Baltrus et al. 2011). The pan-genome of *P. syringae* species complex from 494 strains was used to analyze type III secretory effector molecules, and a total 14, 613 putative type III secretory effectors were identified out of which 4636 were unique at the amino acid level (Dillon et al. 2019). To date, this vast repertoire of effector molecules constitutes 66 families. A particular strain from this complex typically expresses 15–30 effector molecules. These effector molecules are encoded by hrp/hrc (hypersensitive response and pathogenicity) genes and named Hop because of their ability to pass through the type III secretion system (Fig. 8.2).

Many effectors from its pangenome are also known as ‘Avr’ because of their discovery in the post-genomic era as avirulence phenotype (Lindeberg et al. 2005). These effector molecules were analyzed in the context of their role in the two-grade innate immunity of plants. According to this model, primarily the immunity elicited by bacterial flagellin, lipopolysaccharide, peptidoglycan, and elongation factor Tu which is commonly known as Pattern-triggered immunity was suppressed by these effector molecules secreted by bacteria. Later, these molecules are perceived by resistance (R) proteins, the second grade of innate immunity familiarized as Effector-triggered immunity (Jones and Dangl 2006). The resistance (R) proteins are characterized by nucleotide-binding site leucine-rich repeats through which they recognize and bind to the effector molecules released by microbes resulting in Effector-triggered immunity response. Sometimes Effector-triggered immunity induced response is called hypersensitive response where programmed cell death occurs eventually. This kind of immune is very effective on biotrophs as their association is within the cell. Pathogenic type III secretory effectors are like ‘double-edged swords’, as on one hand, they trigger Effector-triggered immunity response and on the other, they suppress Effector-triggered immunity response (Hou et al. 2011).

Local cellular responses are delivered throughout the system to generate a large scale of resistance toward similar infections as well as secondary infections. The Effector-triggered immunity response also instigates the synthesis of small, low-molecular-weight, mobile, immune signaling molecules like salicylic acid, glycerol-3-phosphate which are then transported from the site of infection where they were synthesized to the site of non-infection, to prevent the healthy plant tissues from infection (Spoel and Dong 2012; Fu and Dong 2013). After perceiving these immune signaling molecules, uninfected tissue accumulates Salicylic acid resulting in massive transcriptional programming. This instigated immune signaling is known as systemic induced signaling (Spoel and Dong 2012; Fu and Dong 2013) (Fig. 8.3).

Recent studies suggested that plant symbionts and pathogens take advantage of comparable molecular strategies to conquer the defense reactions of plants. The Microbe Associated Molecular Patterns/Pattern Recognition Receptor system also takes part in harmonious reciprocity with symbiotic microbes. This proposes the role of beneficial microbes for disease tolerance against pathogens employing the innate immune system of plants (Hacquard et al. 2017).

8.3 Beneficial Microbes and Their Metabolites

The ecosystem of the soil is one of the most complex and multifarious ecosystems of the earth which is inhabited by a wide range of organisms from fungi, arthropods, nematodes to bacteria (Venturi and Keel 2016). Bacterial diversity is lower in the

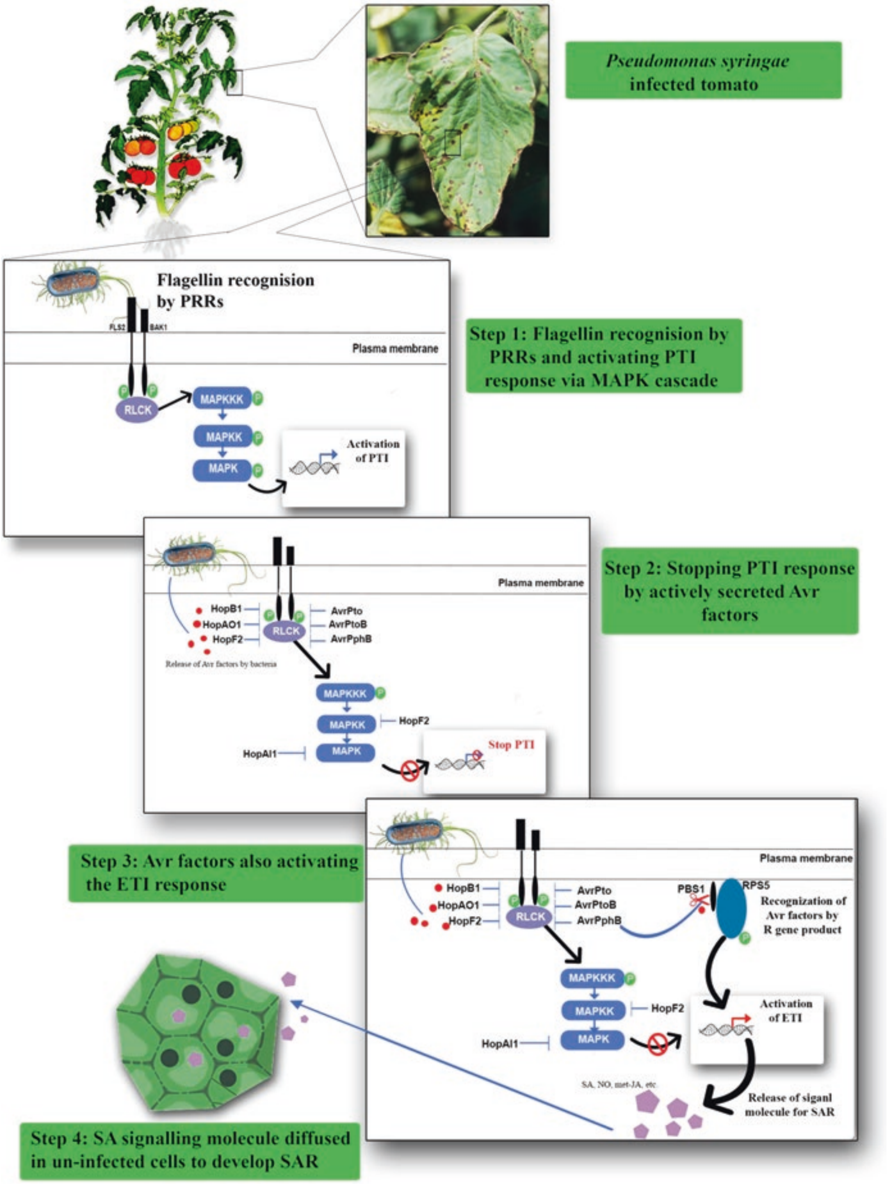

Fig. 8.3 Down-stream signaling in tomato upon recognition of *Pseudomonas syringae* flagellin

rhizosphere but has increased abundance and activity. These bacteria in the rhizosphere are under the selective pressure of plants suggesting a correlation between plant-derived metabolites and microbial metabolites. Through such association, mutual relationships are established between plants and microbes which are essential for root-root interactions, nutrient availability, amassing of microorganisms, and biofilm formation of soil microbes (Mommer et al. 2016; Rosier et al. 2016; Sasse et al. 2018), as well as inhibition of phytopathogens (Bertin et al. 2003; Li et al. 2013).

Based on their effects on plants, plant-associated microbial communities are classified into three categories such as beneficial, deleterious, and neuter. Microbes that play a role in plant growth, nutrient uptake, defense, resistance, and development during stress and normal circumstances are known as plant growth-promoting microbes. The typical plant growth-promoting microbes in the rhizosphere are *Paenibacillus, Burkholderia, Pseudomonas, Bacillus, Acinetobacter, Arthrobacter,* and *Arthrobacter* (Finkel et al. 2017; Sasse et al. 2018; Zhang et al. 2017). These bacteria secrete molecules to establish an association with plants which triggers specific changes in the transcriptome of plants. These plant growth-promoting microbes can produce phytohormones like auxins, abscisic acid, cytokinins, salicylic acid, gibberellins, and jasmonic acid (Fahad et al. 2015).

Additionally, antibiotics, siderophores, antimicrobials, enzymes, volatile organic compounds, and many more helps in priming defense mechanisms in plants. All these metabolites secreted by microbes are known as "elicitors". "Elicitors can be defined as small molecules secreted under stress which induces biosynthesis of specific molecules having an essential role in the adaptations of plants to a stress condition" (Radman et al. 2003). The role of these elicitors for plant growth promotion and ISR priming has been extensively studied for decades, and these are promising substitutes for herbicides, fertilizers, and pesticides (Kloepper et al. 2004; Gupta et al. 2015). Below, we look at the elicitors secreted by plant growth-promoting microbes which are of paramount importance in priming induced systemic resistance in plants against phytopathogens.

8.3.1 Antibiotics

The utmost important mechanism employed by plant growth-promoting microbes to hamper the negative impact of plant pathogens is the biosynthesis of a wide range of antibiotics (Couillerot et al. 2009; Raaijmakers and Mazzola 2012). However, the host range of these antibiotics varies and is also dependent on different field conditions. A large range of bacterial antibiotics have been derived from genera *Bacillus* includes zwittermycin-A (Silo-Suh et al. 1994), kanosamine (Milner et al. 1996), Bacillomycin (Volpon et al. 1999) and Plipastatins A and B (Volpon et al. 2000). On the other hand, *Pseudomonas* include cepafungins (Shoji et al. 1989), pseudomonic acid (Fuller et al. 1971), 2,4 Diacetyl phloroglucinol (Shanahan et al. 1992), pyoluteorin (Howell and Stipanovic 1980), oomycinA (Kim et al. 2000), phenazine-1-carboxylic acid (Pierson III and Pierson 1996), butyrolactones (Thrane et al. 2000), rhamnolipids, viscosinamide (Nielsen et al. 1999), cepaciamide A (Howie and

Suslow 1991), ecomycins (Jiao et al. 1996), azomycin (Shoji et al. 1989), and karalicin which is an anti-viral antibiotic (Lampis et al. 1996).

These metabolites serve as antioxidant, antimicrobial, phytotoxic, antiviral anti-helminthic, insect and mammalian antifeedant, cytotoxic, and plant growth-promoting activity agents and are best studied in disease management. For example, a novel antibiotic secreted by *B. cereus* UW85 is Zwittermicin A, which is highly active against Oomycetes, algal protists and moderately active against a vast range of gram-negative bacteria and fungi and few gram-positive bacteria. When it is combined with another antibiotic, kanosamine secreted by the same organism they act synergistically against *E. coli* (Laura et al. 1998). *P. flouorescens* produce 2,4 Diacetyl phloroglucinol which inhibits *Sclerotium rolfsii* – a soil-borne pathogen (Asadhi et al. 2013). It also secretes another antimicrobial compound, phenazine-1-carboxylic acid (Lohitha et al. 2016) which is responsible for oxidation-reduction reactions as well as amassing of superoxides in target cells and is efficacious in wheat disease caused by *G. graminis* var. *tritici* and *S. rolfsii*, resulting in stem rot in groundnut.

8.3.2 Siderophores

Iron is of paramount importance in the photosynthetic system of plants due to being an essential molecule of chlorophyll. However, its soluble concentration in soil is deficient and its insoluble form (ferric, Fe^{3+} hydroxides) is not readily available for plants and microbes (Saha et al. 2013). To find the key to this issue, some plants, fungi, and bacteria secrete iron-binding molecules of low molecular weight (~400–1000 Da) known as "siderophores" the chelating agents for iron (DalCorso et al. 2013; Saha et al. 2013). These molecules have a surprisingly high affinity for iron and thus scavenge it from the soil.

When iron gets bound to the siderophore, it becomes solubilized and is recognized by receptors on the surface of plants or microbes from where it gets internalized followed by reduction to ferrous state (Fe^{2+}). For the most part, siderophores of plant growth-promoting microbes have a higher affinity for iron than plants and fungi (Saha et al. 2012, 2013). They behave as transport vehicles of iron and common iron-binding molecules and include catechols, hydroxamic acid, and hydroxylic acid. In addition to priming growth, siderophores also help to dampen phytopathogens (Tank et al. 2012). For instance, *B. subtilis* secreted siderophores had similar disease suppression activity in chickpea against dry root rot causing fungi (Patil et al. 2014).

8.3.3 Microbial Volatile Organic Compounds

Volatile organic compounds, as the name suggests are organic molecules having high vapor pressure at room temperature. They are products of metabolic pathways and occurs as a composite aggregation of low-molecular-weight compounds that are having an affinity for lipids and are now termed as "volatile" because of their complex nature (Maffei et al. 2011). These are accountable for communication between various organisms like plants and their pathogens, plant growth-promoting microorganisms, and plants (Maffei 2010; Maffei et al. 2011; Garbeva et al. 2014; Lemfack et al. 2014; Kanchiswamy et al. 2015). Due to their volatile nature, they can easily move from the point of their synthesis to the point of their action, thus acting as communication molecules among organisms (Maffei et al. 2011). Volatile organic compounds released by microbes are commonly termed as microbial volatile organic compounds.

These volatile organic compounds serve chemical windows through which information is allowed to leave (Liang et al. 2008). To name a few; furfurals, camphor, acetaldehyde, methanol, geosmin, butanoic acid, 5-hydroxy methylfurfural, camphene are the most commonly secreted molecules (Li et al. 2004; Müller et al. 2004; Leff and Fierer 2008; Gray et al. 2010; Ramirez et al. 2010; Wenke et al. 2010; Perl et al. 2011; Jünger et al. 2012; Sundberg et al. 2013). Among all metabolites secreted by beneficial microbes, volatile organic compounds form the successful primary defense system in plants against phytopathogens along with promoting plant growth (Ryu et al. 2004; Beneduzi et al. 2012; Song and Ryu 2013). For instance, the mycelial growth of *Rhizoctonia solani* has been reported to be inhibited by microbial volatile organic compounds (Kai et al. 2007). In vitro, volatile organic compounds – 2,4decadienal, n-hexadecanoic acid, oleic acid, and diethyl phthalate secreted from *Paenibacillus* spp. and *Bacillus* suppresses the disease activities of *Ascochyta cutrillina, Alternaria brassicae* and *Alternaria solani* (Han et al. 2016).

In addition to all these, many beneficial microbes secrete enzymes like chitinase, glucanases, amylases, and lipases which also aids in the growth, development, and elicitation of defense mechanisms in plants against phytopathogens (Bull et al. 2002; Saraf et al. 2014). Plant receptors recognize lipopolysaccharides, flagellin, and elicitors from both phytopathogens and plant growth-promoting microorganisms in the same manner, and in response, microbe-associated molecular pattern-triggered immunity is activated in both cases but somehow this response does not ward off beneficial microbes or plant growth-promoting microorganisms, the reason is still unknown (Van Wees et al. 2008). Table 8.4 elucidates the various microbes and their respective elicitors in various plant species and Fig. 8.4 depicts the interaction between phytometabolites and microbial metabolites which includes beneficial as well as infectious interactions. Table 8.5 provides insight into the role of the elicitors and their mode of action in plant defense mechanisms.

Table 8.4 Microbial elicitors that instigate systemic resistance in plants

Plants	Microbes	Organic substance	Phytopathogen	References
Arabidopsis	2,4-diacetylphoroglucinol	*Peronospora parasitica*	*P. fluorescens*	Iavicoli et al. (2003)
	B. subtilis GB03, *B. amyloliquefaciens* IN937a	Volatile compounds	*Erwinia carotovora*	Ryu et al. (2004)
	B. subtilis	Surfactin	*P. Syringae*	Bais et al. (2004)
	Transgenic *Arabidopsis*	PevD1 protein	*B. cinerea, P. syringae* pv. Tomato	Liu et al. (2016)
	B. Amyloliquefaciens UCMB5113	Fengycins	*Alternaria brassicicola*	Asari et al. (2017)
	Saccharothrix yanglingensis Hhs.015	BAR11 protein	*P. Syringae* pv. Tomato DC3000	Zhang et al. (2018)
Bt cotton	*Penicillium chrysogenum*	Dry mycelium	*Fusarium oxysporum, Verticillium dahlia*	Chen et al. (2006)
Cotton	*E. coli* (recombinant)	PevD1 protein	*Verticillium dahliae*	Bu et al. (2014)
Tobacco	*E. coli, Alternaria tenuissima*	PeaT1	*Tobacco mosaic virus*	Zhang et al. (2011)
	Alternaria tenuissima	Hrip1	*Tobacco mosaic virus*	Kulye et al. (2012)
	B. subtilis 985, *B. amyloliquefaciens* 5499	Surfactin lipopeptide	*Botrytis cinerea*	Cawoy et al. (2014)
	B. subtilis	Culture supernatant	*Tobacco mosaic virus, Ralstonia solanacearum, Phytophthora parasitica*	Chang et al. (2015)
	Bacillus sp. SJ	Volatile compounds	*Rhizoctonia solani, Phytophthora nicotianae*	Kim et al. (2015)
	B. subtilis SYST2	Albuterol, 1,3-propanediol	*Ralstonia solanacearum* TBBS1	Tahir et al. (2017)
Rice	*E. coli* (recombinant)	MoHrip1	*Magnaporthe oryzae*	Chen et al. (2012)
	Pseudomonas protegens CHAO	Orfamide A	*Cochliobolus miyabeanus*	Ma et al. (2017)
Soybean	*B. amyloliquefaciens* MEP(2)18 and ARP(2)3	Surfactin, Fengycins	*Sclerotinina scleriotorum*	Alvarez et al. (2012)
Bean	*Pseudomonas sp.* CMR12a	Phenazines, sessilins	*Rhizoctonia* web blight	Ma et al. (2016)
Cucurbits	*B. subtilis UMAF6639*	Iturin and fengycin	*Podosphaera fusca* (cucurbit powdery mildew)	García-Gutiérrez et al. (2013)

(continued)

Table 8.4 (continued)

Plants	Microbes	Organic substance	Phytopathogen	References
Grapevine	*B. subtilis*	Surfactin, mycosubtilin	*B. cinerea*	Farace et al. (2015)
Tea	*P. fluorescens* RRLJ134, *P. aeruginosa* RRLJ04	Phenazine analogues	*Fomes lamoensis, Ustulina zonata*	Mishra et al. (2014)
Tomato	*Trichoderma virens, Trichoderma atroviride*	SM1 (small protein1) and EPl1 proteins (eliciting plant response-like protein)	*Alternaria solani, B. cinerea, P. syringae* pv. Tomato (Pst DC3000)	Salas-Marina et al. (2015)
	B. fortis IAGS 162	Phenylacetic acid	*Fusarium oxysporum* f.sp. *lycopersici*	Akram et al. (2016)
	P. aeruginosa PM12	3-Hydroxy-5-methoxy benzene methanol	*Fusarium oxysporum*	Fatima and Anjum (2017)
Maize	*B. Amyloliquefaciens, B. subtilis*	Iturin A, Fengycin, Bacillomycin	*Fusarium moniliforme*	Gond et al. (2015)
	B. subtilis DZSY21	Lipopeptides	*Bipolaris maydis*	Ding et al. (2017)

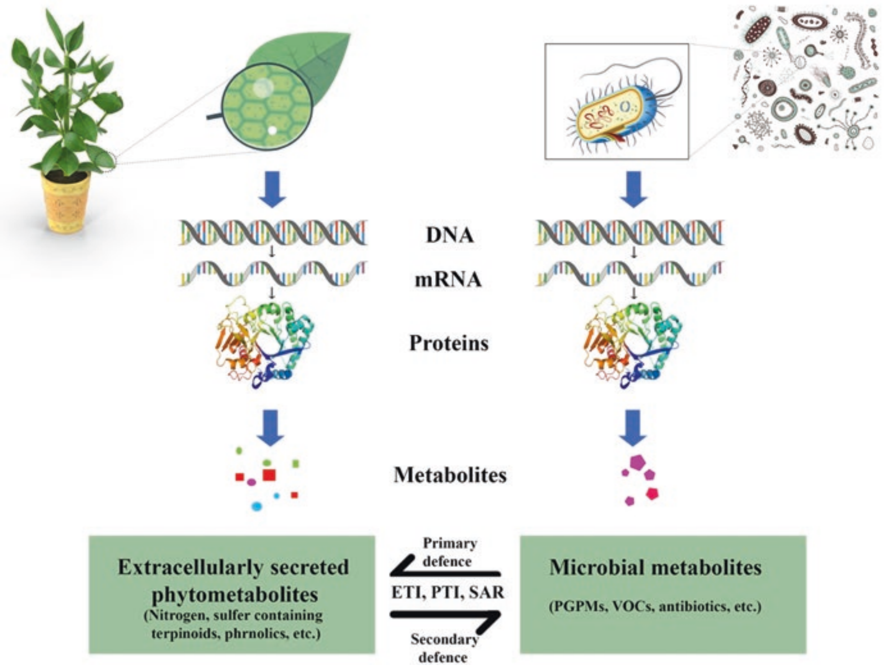

Fig. 8.4 Interaction of phytometabolites and microbial metabolites in the rhizosphere. *ETI* Effector-triggered immunity, *PTI* Pattern-triggered immunity, *SAR* Systemic acquired resistance, *PGPM* plant growth-promoting microorganism, *VOCs* Volatile organic compounds

Table 8.5 Role of elicitors in plant defense mechanisms

Plants	Elicitors/ inducers	Phytopathogens	Mode of action of elicitors/ inducers	References
Tobacco	PeBA1 protein	*Tobacco mosaic virus, B. Cinerea*	Induction of defense responsive genes to produce salicylic acid, phenyl ammonia-lyase, jasmonic acid, H_2O_2, and phenolic compounds	Wang et al. (2016)
	3-Acetonyl-3-hydroxyoxindole (AHO)	Tomato spotted wilt virus	Two differentially expressed genes (PR1 and PR10) were activated for the synthesis of phytometabolites like propanoid, sesquiterpenoid and triterpenoid to protect the wax and cuticle of plants	Chen et al. (2017)
	PevD1	*Verticillium dahlia, tobacco mosaic virus, P. syringae* pv. Tabaci	Interacts with Nbnrp1 to regulate PevD1	Liang et al. (2018)
Tobacco, *Arabidopsis*	Benzoyl salicylic acid	*Tobacco mosaic virus*	WRKY transcription factors, hypersensitive response molecule, mitogen-activated protein kinase as well as NPR1 genes were activated	Kamatham et al. (2016)
Tomato	Benzothiadiazole	Tomato spotted wilt virus and citrus exocortis viroid	Activates salicylic acid signaling pathways of plants	López-Gresa et al. (2016)
	N-decanoyl-homoserine lactone	*Botrytis cinerea*	Induction of jasmonic acid synthesis pathway	Hu et al. (2018)
Sunflower	Benzothiadiazole	*Sclerotinia sclerotiorum*	Hinders growth of fungal hyphae and increase the formation of mycorrhizae in the plant roots	Bán et al. (2017)
Whitebark pine	Methyl jasmonate	*Cronartium ribicola,* mountain pine beetle, *Dendroctonus ponderosae*	Reprogram of defensive genes	Liu et al. (2017)
Cassava	Salicylic acid or methyl jasmonate	*Xanthomonas axonopodis* pv. Manihotis	Elevates the defense action	Yoodee et al. (2018)

Note: *PeBA*1 protein elicitor from *Bacillus amyloliquefaciens* NC6, *NPR*1 Nonexpressor of Pathogenesis-Related Genes 1, *Nbnrp*1 *Nicotiana benthamiana* Neuropilin-1 gene, *PevD*1 Proteinaceous elicitor secreted by *Verticillium dahliae*, *PR*1 Pathogenesis-related protein1, *PR*10 Pathogenesis related protein10

8.4 Conclusion

Thus, in the rhizosphere, plants along with all beneficial and pathogenic microbes are considered as a whole ecological community and referred to as “holobiont”. Plant pathogens are the necrotrophs, hemibiotrophs, and biotrophs had in due course of evolution helped the plant communities to advance their immune responses in one or the other way. The present strategies discussed above include Pattern recognition receptors to perceive Microbe associated molecular patterns and Damage associated molecular patterns to further elicit the downstream signaling cascade involving Calcium dependent protein kinases, Mitogen-activated protein kinase, etc. However, in terms of co-evolution, the microbes developed an enormous repertoire of effector molecules while plants in response co-evolved with disease resistance (R) proteins to counteract these effector molecules.

On the beneficial front or in other terms in a mutualistic way, plant growth-promoting microorganisms promote plant growth via establishing an association triggering the production of phytohormones like auxins, abscisic acid, cytokinins, salicylic acid, gibberellins, and jasmonic acid, antibiotics, siderophores, antimicrobials, enzymes, volatile organic compounds. For example, beneficial microorganisms or plant growth-promoting microorganisms dominated by *Bacillus* and *Pseudomonas* spp. lives in a symbiotic relationship with the plants for food and nutrients and inturn helps plants in their growth, development, and defense against phytopathogens. Plant Growth Promoting Microbes employ direct and indirect mechanisms to hamper the growth of phytopathogens. The direct mechanism involves inhibition of metabolism while the indirect mechanism involves competition against phytopathogens for the nutrients. The metabolism of phytopathogen was inhibited by various mechanisms including secretion of antibiotics (antimicrobial, antiviral, etc.). However, all these mechanisms to surpass, co-evolve, or to involve in symbiotic associations pave the way for further advancements in both the plants and the microbial genome in order to thrive at their utmost capabilities and in future years may evolve or co-evolve in a different mechanism as discussed above under the influence of selection pressure and can lead to different or novel mechanisms.

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Chapter 9
Microbial Mitigation of Abiotic Stress in Crops

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Abstract Abiotic stresses such as drought, salinity, temperature, flooding and heavy metal toxicity reduce crop yield. About 64% of land worldwide is affected by drought. The lack of moisture in crops may lead to 50% yield loss, and increases soil salinity levels. Therefore, the use of plant growth promoting rhizobacteria for increasing plant stress tolerance appears as a sustainable strategy. Here we review abiotic stress tolerance mediated by plant growth promoting rhizobacteria in plants with focus on phytohormones, improved physiological attributes, root system architecture and regulation of the osmotic balance. Improved antioxidant activity result in reduced oxidative damage, which promotes plant growth, and nutrient and water uptake. Other extracellular secretions trap ions and moisture and improve the growth environment. Emissions and elicitors function as signaling molecules that induce genes and transcription factors belonging to the stress responsive pathways. The use of plant growth-promoting rhizobacterial bioinoculants is effective in enhancing tolerance to crop abiotic stress. The formulations and application of these microorganisms is promising for climate smart agriculture.

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Abbreviations

ABA	Abcissic acid
ACC	1-aminocyclopropane – 1-carboxylic acid
PGRR	Plant growth promoting rhizobacteria

9.1 Introduction

Climate change is considered to be one of the most important global environmental challenges facing humankind which can impact natural ecosystems, agriculture and health. Agriculture is very vulnerable to climate-change and crop production is facing increasing stresses due to natural and anthropogenic factors (Selvakumar et al. 2012). Adverse effects of abiotic stresses on plant growth, production and yield of agricultural crops have been reported by many authors. Abiotic stresses are defined as the harmful influence of non-living factors on the living organisms in a particular environment. There must be variation in the range of non-living factor beyond its normal range so as to to influence the environment and adversely affect the performance of population or physiology of the organism in a significant way. They are naturally occurring, often intangible and essentially unavoidable.

Important abiotic stresses which adversely affect environment are drought, submergence, salinity and acidic conditions, low or high temperature, light intensity and nutrient deficiency. Of the total global land area, 64% of the land is affected by drought, 13% by flood, 6% by salinity, 15% soil alkalinity, 9% by mineral imbalance and 57% by cold (Meena et al. 2017). Among them, moisture deficit induced stress is one of the most important and predominant abiotic stress, causing hindrance in crop production worldwide (Vejan et al. 2016). Moisture availability is the chief component that limits plant productivity and stress due to water deficiency and may lead to more than 50% yield losses for majority of crops globally (Boyer 1982). Salinity stress is another main environmental stress affecting crop stand and productivity. Generally, lack of sufficient moisture causes soil to become more saline and alkaline (Munns 2002). It is estimated that out of the world's 5.2 billion ha of dryland agriculture, nearly 3.6 billion ha of land is affected by moderate to high soil salinization, which subsequently may lead to soil degradation (Riadh et al. 2010).

In India, production constraints on nearly about 7.3 mha of arable land are triggered by the soil alkalinity and salinity along with water logging. Such soils are always imbalanced in soil nutrient availability and their uptake for plant growth hence causes nutrient stress like conditions (Maheshwari et al. 2012). According to estimates from Intergovernmental Panal on Climate Change, it is predicted that the average global temperature will rise by 1.5 °C by between 2030 and 2050 and is further likely to rise more than 3 °C to 4 °C by 2100 (Allen et al. 2018). Numerous studies have demonstrated the adverse effect of increased global mean temperature on crop growth and yield. Zhao et al. (2017) reported that for every 1 °C increase in global mean temperature there is a reduction in the average yield of wheat by 6.0%, maize by 7.4%, rice by 3.2% and soybean by 3.1%. Presence of heavy metal ions in environment also poses a serious stress factor for plant growth (Sangwan and Dukare 2018).

These abiotic stress factors are expected to significantly reduce crop growth and yields and impose severe pressure on our water resources and land. It is a major challenge to develop efficient, easily adaptable, cost-effective methods for management of abiotic stresses. To cope with abiotic stresses, worldwide extensive research is being undertaken, to develop strategies like developing tolerant varieties, resource management practices and shifting of crop calendars (Venkateswarlu and Shanker 2009). Responses of plants to abiotic stresses are multigenic and complex; the genetically complex mechanisms of abiotic stress tolerance make engineering of plants extremely difficult. Other disadvantages include high cost and long time for development. Recent studies have indicated that microorganisms can also help plants cope with abiotic stresses which are an ecofriendly and cost-effective method. It involves the application of multi-faceted characteristics of several microorganisms with an established role in growth promotion, disease control and nutrient management. The last two decades have witnessed many reports on the utilization of such microbes for induction of tolerance against abiotic stresses. In this chapter, we highlight the contemporary works on the role of plant growth promoting rhizobacteria (PGPR) in alleviating the impact of abiotic stresses in crop plants.

9.2 Impact of Abiotic Stresses on Plant Metabolism and Growth

Numerous abiotic stresses developed as a result of global warming and climate change pose a great risk to global agriculture. Such changes hinder plant growth and development leading to reduction in crop yield and productivity (Wang et al. 2000). Plants exposure to stresses causes numerous alterations and modifications at morphological, cellular, physiological and molecular level (Fig. 9.1). In general, most of the abiotic stresses induce osmotic imbalance and dehydration in crops. Nearly similar alterations in the physiological and biochemical status are observed when plants are exposed to various stress factors.

Abiotic stress triggered main effects at cellular level include increased synthesis and build-up of reactive oxygen species due to alteration in ion balance and osmosis. At biochemical and physiological level, the harmful impact of stresses includes oxidative damage, denaturation of proteins, nucleic acids, chlorophyll pigments and

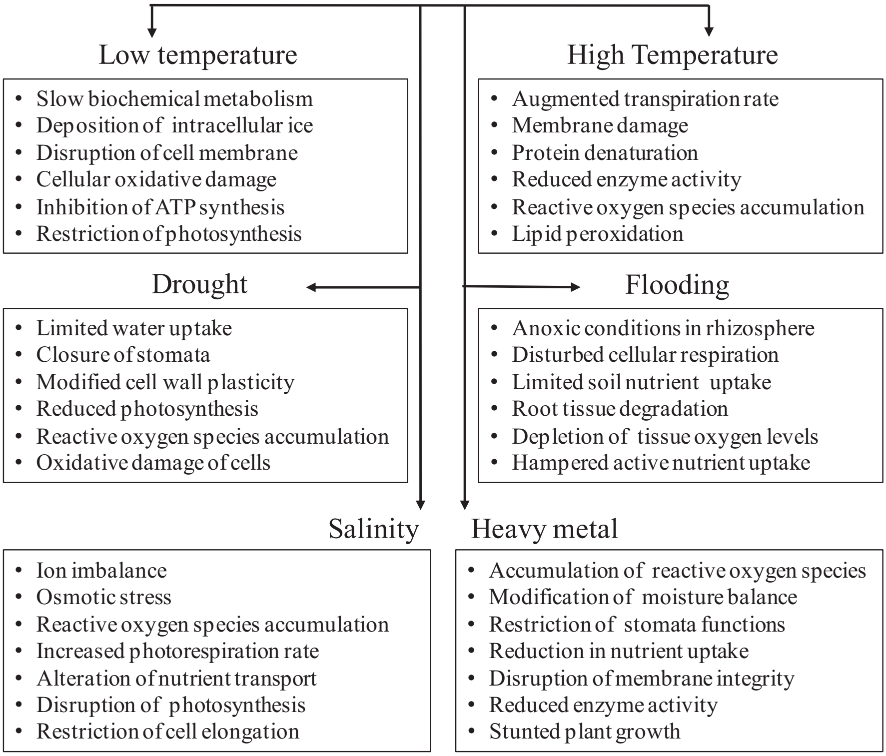

Fig. 9.1 Adverse impacts of abiotic stresses on cellular, biochemical and physiological attributes of plants

membrane lipids. All these changes significantly reduce photosynthetic activity and cause alteration in levels of cellular hormones. Hence, overall metabolic dysfunction limits plant growth and fertility, induces premature senescence and lowers yield.

9.3 Role of Microorganisms in Abiotic Stress Alleviation

Various stress factors such as temperature extremes, drought, salinity or alkalinity, acidic condition and heavy metals toxicity not only affect the plant growth but also the activities of rhizospheric microbes. This certainly may lead to change in the composition and biomass of a microbial community in the rhizosphere. Microbial adaptation to stress represents a multiplex regulative process in which numerous genes are involved (Srivastava et al. 2008). Certain microbial species are capable of surviving in extreme environments and they use different mechanisms to overcome the deleterious effects of stress. The mechanisms include production of exopolysaccharide, biofilm formation, osmolytes accumulation, production of antioxidative enzymes, improved antioxidant status, ion homeostasis and induction of stress responsive genes/proteins (Paul et al. 2015, 2017).

Several studies have reported the functional diversity and adaptation strategies of plant growth promoting microorganisms to adverse environmental conditions. In this regard, best known examples include *Rhizobium, Bradyrhizobium, Azotobacter, Azospirillum, Pseudomonas, Bacillus, Variovorax, Enterobacter, Burkholderia, Glomus* and *Trichoderma* (Paul et al. 2015, 2017; Alori et al. 2017; Meena et al. 2017). Enhanced production of exopolysaccharides by microbes under abiotic stress conditions has been well studied (Sandhya and Ali 2015; Bandeppa et al. 2018). Exopolysaccharide not only improves soil physicochemical properties; it also protects the microbes from stress probably by enhancing water retention and biofilm formation (Sandhya et al. 2009). Accumulation of osmolytes, like proline, glycine betaine and trehalose, is another adaptive mechanism observed in several microbes under abiotic stress conditions (Vendruscolo et al. 2007; Chen and Murata 2008; Rodríguez-Salazar et al. 2009). In response to osmotic stress condition, most bacteria increase the synthesis of osmolytes, thus leading to high osmotic potential within the cells and confer protection against the inhibitory effects of stress. Extremes of temperature negatively affect the activity of microbes in soil. Microorganisms respond to temperature stress by the induction of heat shock proteins (such as GroEL, DnaK, DnaJ, GroES, ClpB, ClpA, sHSP101 and sHSP70) (Münchbach et al. 1999; Koda et al. 2001; Ali et al. 2009).

The effect of plant growth promoting rhizobacteria on growth and yield of agronomically important crops has been well established (Biswas et al. 2000; Asghar et al. 2002; Basu et al. 2021). PGPR are beneficial microbes that actively colonize the rhizosphere/endorhizosphere of plants and, also possess unique traits by which they directly or indirectly facilitate plant growth (Kloepper et al. 1989). In general, PGPR promote the growth of plants directly either by regulating phytohormone production or facilitating uptake of nutrients from the soil especially nitrogen and phosphorous (Glick 1995). Moreover, PGPR also promote the growth of plants indirectly by acting as biocontrol agents, thus reducing the harmful effect of pathogens on the plant. This may be achieved by the production of certain lytic enzymes and inhibitory compounds, and induction of systematic resistance against a wide range of pathogens in plants (Glick 1995).

Gholami et al. (2009) studied the effect of inoculation with strains of *Pseudomonas putida, Pseudomonas fluorescens, Azospirillum lipoferum,* and *Azospirillum brasilense* on maize growth and development. They reported that inoculation significantly improved the rate of seed germination, seedling growth and yield over control. Improved root and shoot elongation were observed in different plants such as canola, wheat and potato upon inoculation with strains of *P. putida* and *P. fluorescens* (de Freitas and Germida 1992; Frommel et al. 1993; Glick et al. 1997). Similarly, seed inoculation with consortia of PGPR strains belonging to *Azotobacter chroococcum Mesorhizobium ciceri*, *P. fluorescens*, and *Azospirillum* spp. resulted in improved nutrient uptake, growth and yield of chickpea (Rokhzadi and Toashih 2011).

Besides facilitating plant growth, PGPR possessing unique stress tolerance traits could also be explored to promote plant growth under stressed environments. PGPR have the ability to modulate plant's physiology in response to various abiotic stress factors (Fig. 9.2). Following microbial inoculation, induction of systemic tolerance has been observed in crop at biochemical, physiological, and molecular levels. Microbial production of exopolysaccharides and biofilm formation influences soil physicochemical properties and improves plant tolerance to abiotic stress conditions. In addition, microbial inoculation also buildup several stress associated plant metabolites, such as proline, abscisic acid, glycine betaine, and synthesis of antioxidants including catalase, ascorbate peroxidase superoxide dismutase, glutathione, ascorbic acid and α- tocopherol (Agami et al. 2016).

Studies have been conducted on the role of PGPR improving overall growth performance of plants under different abiotic stress conditions (Chandra et al. 2021; Singh et al. 2021). Significant improvement in seedling vigour (in terms of radicle and plumule length and fresh weight) has been observed in pearl millet plants inoculated with rhizobacterial isolates MCL-1 and MKS-1 compared to uninoculated plants under drought stress (Manjunatha et al. 2017). Rathi et al. (2018a) have also made similar observations in guar using rhizobacterial isolates NSRSSS-1 and MKS-6. Niu et al. (2018) have also shown that inoculation of foxtail millets with three drought-tolerant rhizobacterial strains stimulated seed germination and

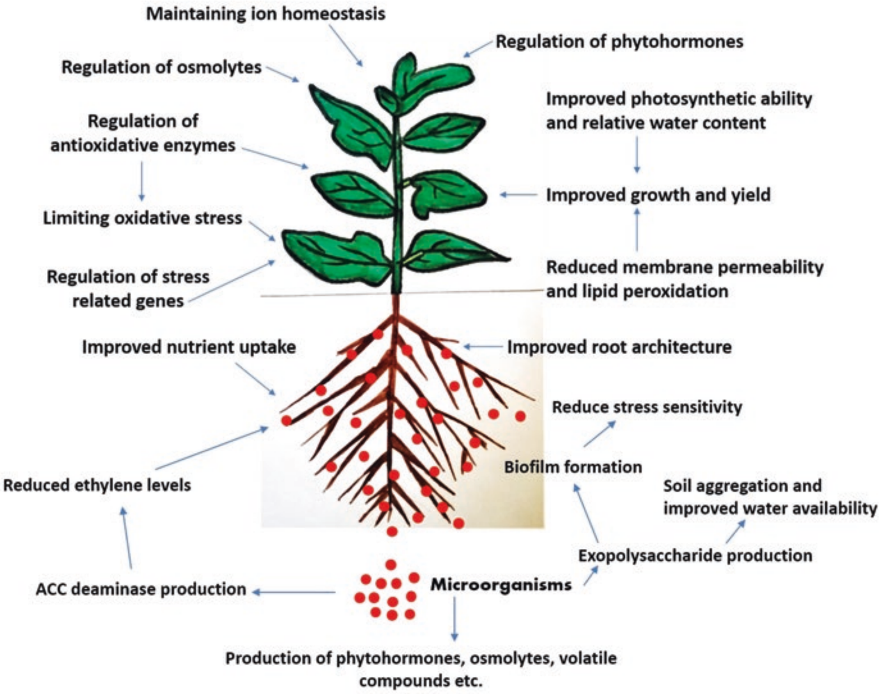

Fig. 9.2 Mechanisms of microbes-mediated alleviation of abiotic stresses in plants

seedling growth under stress thereby improving drought tolerance in plants. *Rhizobium ciceri* enhanced tolerance of wheat and chickpea to salt stress (Yilmaz and Kulaz 2019). Wheat plants subjected to heat stress showed increase in yield, grain size and quality upon inoculation with thermotolerant isolate *P. putida* AKMP7 (Ashraf and Foolad 2007). Similarly, inoculation of *Variovorax paradoxus* 5C-2 in pea resulted in better growth, water use efficiency and yield under drought stress (Belimov et al. 2009). Selvakumar et al. (2008a, b) reported that inoculation of *Burkholderia phytofirmans* PsJN helped in alleviating low-temperature stress in grapevine. Hence, utilizing such microorganisms for abiotic stresses alleviation in plants is a novel and promising technology for sustainable crop production under stressed environments.

9.4 Mechanisms of Abiotic Stress Alleviation by Microorganisms

9.4.1 Production and Regulation of Phytohormones

Phytohormones are the key regulators of plant growth which have a significant influence on plant metabolism and play an important role in triggering the plant defense responses against stresses. The exogenous phytohormone application for improving growth and metabolism of plants under stress conditions has already been in practice. However, recent studies have revealed that phytohormones produced by rhizobacteria may also prove to be an approach for inducing host tolerance to abiotic stresses (Egamberdieva et al. 2017b). Plant growth promoting microbes can provide stress tolerance either by production of phytohormones in low amounts which is taken up by plants or by triggering the plant machinery to fine tune the endogenous hormonal levels.

Abscisic acid is a vital stress hormone which regulates many osmotic responses under abiotic stress conditions. Accumulation of abscisic acid during stress condition leads to the regulation of ion channels and pumps involved in stomatal movements i.e., stomatal closure which restricts or prevents water loss from the leaves. Other responses include regulation of ion channel in guard cells, transcriptional levels of the calmodulin proteins and regulation of the expression of some abscisic acid responsive genes which are stimulated under drought and salinity stresses, involved in stress alleviating mechanisms. Calmodulin or calcium modulated proteins are activated in response to external stress stimulus involved in the activation of glutamate carboxylase for γ-Aminobutyric acid synthesis for plant development (Fang and Xiong 2015; Sah et al. 2016). Microbes take part in inducing stress tolerance in plants by producing abscisic acid or modulating its levels in plants.

Arabidopsis plants inoculated with the PGPR *Phyllobacterium brassicacearum* STM196 strain showed better resistance to drought with increased abscisic acid production (Bresson et al. 2013). The application of arbuscular mycorrhizal fungi can also alter the hormonal balance in plants under stressful environments. The stress tolerance mechanism of arbuscular mycorrhizal fungi is by regulating the stomatal conductance by closing stomata under drought conditions which may be due to the influence of abscisic acid (Jahromi et al. 2008). Inoculation of *Pseudomonas chlororaphis* to tomato (*Lycopersicum esculentum*) enhanced the abscisic acid level in leaves of water-stressed tomatoes allowing a more efficient modulation of stomatal closure that resulted in an improved water use efficiency and biomass accumulation (Brilli et al. 2019).

Auxin, indole-3-acetic acid is among the most important phytohormones which control several stages of plant growth including cell division and elongation, tissue differentiation and induction of apical dominance. Several plant growth-promoting rhizobacterial strains are also known to synthesize indole-3-acetic acid which aid in plant growth promotion. Plant-associated microbes synthesize indole-3-acetic acid via L-tryptophan dependent and independent pathways with three known dependent pathways. They utilize the L-tryptophan oozed out from roots as a precursor for the synthesis of indole-3-acetic acid (Goswami et al. 2016). The involvement of these microbes during abiotic stress in plants has proven to be vital. There was up-regulation of auxin biosynthetic genes and down-regulation of auxin efflux carrier genes in the roots of trifoliate orange inoculated with arbuscular mycorrhizal fungi *Funneliformis mosseae* leading to high density, diameter and length of root hairs and indole-3-acetic acid level in roots under drought stress (Liu et al. 2018). Elevated indole-3-acetic acid content and plant growth was reported in wheat plants when inoculated with rhizobacterial strains *Arthrobacter protophormiae* SA3 and *Dietzia natronolimnaea* STR1 under salinity stress and *Bacillus subtilis* LDR2 under water deficit stress (Barnawal et al. 2017). Indole-3-acetic acid produced by heavy metal tolerant plant growth promoting microorganisms alters the root morphology in a beneficial way to overcome heavy metal toxicity, thereby promoting plant growth (Mishra et al. 2017). Zn, Cu, Ni, and Co tolerant indole-3-acetic acid producing bacterial strains induced rapid root elongation in *Brassica juncea* in Cd contaminated soil (Belimov et al. 2005).

Cytokinins have a vital role in growth and development of plants but also have a significant role in alleviating abiotic stress in plants such as drought, salinity, heavy metal and temperature stress (Pavlu et al. 2018). The effect of cytokinin producing PGPR in inducing abiotic stress tolerance to plants is less studied and understood, however, few reports are available. Selvakumar et al. (2018) reported that tomato plants, under water deficit stress conditions, inoculated with cytokinin-producing bacteria *Citrococcus zhacaiensis* and *Bacillus amyloliquefaciens* showed enhanced photosynthesis, transpiration, relative water content and yield. *Pseudomonas* strains (*P. extremorientalis* TSAU6, *P. aurantiaca* TSAU22 and *P. extremorientalis* TSAU20) enhanced the growth of plants at high salt concentrations and also alleviated salinity induced wheat seed dormancy (Egamberdieva 2009). The relative leaf water content was increased in the seedlings of *Platycladus orientalis* under drought stress condition when inoculated with cytokine producing bacterium *Bacillus subtilis* and the higher concentration of cytokinin in the leaves was correlated with the higher abscisic acid levels but the stomatal conductance was affected possibly by the combined action of elevated cytokinin and abscisic acid levels (Liu et al. 2013).

The gibberellins are a group of tetracyclic diterpenoid carboxylic acids, of which only some function as plant growth hormones with gibberellin 1 and gibberellin 4 being the predominant bioactive forms. They stimulate the growth of plant through enhanced cell division and cell elongation in most of the organs and also promote developmental phase transitions. Gibberellins are prime targets in plants for stress-induced modulation of growth, and the involvement of gibberellins signaling may result in either suppression or promotion of plant growth that depends on the response to a specific abiotic stress (Colebrook et al. 2014). In bacteria, gibberellin was first characterized in the gnotobiotic cultures of *Rhizobium meliloti* by Atzorn et al. (1988) who demonstrated the presence of gibberellins.

The bioactive gibberellins producing PGPR *Serratia nematodiphila* PEJ1011 alleviated deleterious effects of low temperature (5 °C) in pepper plants (*Capsicum annum*. L) by increasing abscisic acid levels and decreasing salicylic acid and jasmonic acid (Kang et al. 2015). The endogenous gibberellins level was increased along with indole-3-acetic acid and abscisic acid and cytokinins in water stressed *Arabidopsis* plants inoculated with *P. putida* strain GAP-45 which was similar to the levels in uninoculated unstressed plants (Ghosh et al. 2018). The endophytic fungi *Phoma glomerata* LWL2 and *Penicillium* sp. LWL3 producing bioactive GAs significantly increased plant growth under saline and drought stress condition (Waqas et al. 2012).

Salicylic acid is a signaling compound under abiotic stress which induces genes coding for antioxidant enzymes, chaperones and heat shock proteins, as well as genes responsible for secondary metabolites synthesis (Jumali et al. 2011). It has been reported in several articles that microbes induce the expression of salicylic acid in plants under stress conditions. The responsiveness to drought stress was abated by *Piriformospora indica* where the fungus stimulated salicylic acid related genes in roots along with increased oxidative potential (Zhang et al. 2018). Inoculation of *Achromobacter xylosoxidans* (SF2) and *Bacillus pumilus* SF3 and *Bacillus pumilus* SF4 bacterial strains increased salicylic acid content in shoots of sunflower along with increased root and shoot dry matter under water stressed condition (Castillo et al. 2013). The salinity tolerance with improved physiology was attributing to increased salicylic acid along with abscisic acid concentration in poplar roots colonized by the ectomycorrhizal fungus *Paxillus involutus* (Luo et al. 2011). Examples on the effect of phytohormone production and 1- aminocyclopropane- 1-carboxylic acid deaminase activity in abiotic stress alleviation are given in Table 9.1.

Table 9.1 Alleviation of abiotic stresses in plants by microbe-mediated regulation of phytohormones

Microorganisms	Abiotic stresses	Host plants	References
Regulation and production of phytohormones			
Achromobacter xylosoxidans (SF2) and *Bacillus pumilus* (SF3 and SF4)	Drought	Sunflower	Castillo et al. (2013)
Arthrobacter protophormiae SA3, *Dietzia natronolimnaea* STR1	Salinity	Wheat	Barnawal et al. (2017)
Azospirillum lipoferum	Drought	Maize	Cohen et al. (2009)
Enterobacter cloacae HSNJ4	Salinity	Canola	Li et al. (2017)
Funneliformis mosseae	Drought	Trifoliate orange	Liu et al. (2018)
Pantoea dispersa 1A and *Serratia marcescens* SRM	Low temperature	Wheat	Selvakumar et al. (2008a, b)
Paxillus involutus	Salinity	Poplar	Luo et al. (2011)
Phyllobacterium brassicacearum STM196	Drought	*Arabidopsis*	Bresson et al. (2013)
Pseudomonas fluorescens	Drought	**Maize**	Ansary et al. (2012)
Pseudomonas putida GAP-45	Drought	*Arabidopsis*	Ghosh et al. (2018)
Pseudomonas putida **H-2-3**	Drought	**Soybean**	Kang et al. (2014)
Pseudomonas putida Rs-198	Salinity	Cotton	Yao et al. (2010)
Pseudomonas sp. PGERs17 and NARs9	Low temperature	Wheat	Mishra et al. (2008, 2009)
Sinorhizobium meliloti	Salinity	Medicago	Palma et al. (2013)
ACC deaminase activity			
Bacillus 23-B, *Pseudomonas* 6-P and *Mesorhizobium ciceris*	Drought	**Chickpea**	Sharma et al. (2013)
Bacillus sp.	Salinity	Rice	Misra et al. (2017)
Bacillus subtilis Rhizo SF 48	Drought	Tomato	Gowtham et al. (2020)
Enterobacter HS9 and *Bacillus* G9	Drought	Velvet bean	Saleem et al. (2018)
Enterobacter sp. SA187	Salinity	*Arabidopsis*	de Zélicourt et al. (2018)
Hartmannibacter diazotrophicus	Salinity	Barley	Suarez et al. (2015)
Ochrobactrum pseudogrignonense RJ12, *Pseudomonas* sp. RJ15 and *Bacillus subtilis* RJ46	Drought	Black gram and Pea	Saikia et al. (2018)
Pseudomonas fluorescens	Salinity	Wheat	Safari et al. (2018)

(continued)

Table 9.1 (continued)

Microorganisms	Abiotic stresses	Host plants	References
Pseudomonas putida UW4	Low temperature	Canola	Cheng et al. (2007)
Variovorax paradoxus 5C-2	Drought	Pea	Belimov et al. (2009)
Variovorax paradoxus RAA3*;* Consortium of *Pseudomonas* spp. DPC9, DPB13, DPB15, DPB16	Drought	Wheat	Chandra et al. (2019)

9.4.2 Aminocyclopropane-Carboxylic Acid Deaminase Activity

Ethylene is a gaseous plant hormone synthesized endogenously by plants from the precursor 1- aminocyclopropane- 1-carboxylic acid (ACC). Ethylene enhances the plant growth at lower concentrations but their levels get increased under stress conditions due to the enhanced production of ACC. ACC is an immediate precursor of ethylene in its biosynthetic pathway and ethylene is known to have deleterious effect on plant growth, particularly, on root elongation and thereby, affecting overall plant process (Glick 2014). Under submerged conditions, ethylene causes accumulation of reactive oxygen species in the roots that can be mitigated by bacterial ACC deaminase (Ali and Kim 2018). Many PGPR bacteria are known to produce an enzyme ACC deaminase which can enhance plant growth by reducing the ethylene levels in plants by hydrolyzing ACC to α-ketobutyrate and ammonia (Etesami et al. 2014; Ali and Kim 2018).

ACC deaminase enzymes have been widely reported to be produced by many species of PGPR such as *Achromobacter, Alcaligenes, Agrobacterium, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia* and *Variovorax* (Belimov et al. 2001; Blaha et al. 2006; Govindasamy et al. 2015; Bandeppa et al. 2018). It has been suggested that a significant portion of ACC is exuded by the plant tissues, usually from seeds or roots (Hontzeas et al. 2004), later sequestered and degraded by ACC deaminase producing rhizobacteria into α-ketobutyrate and ammonia (Glick et al. 1998), and this in turn lowers the ethylene levels in spermosphere and rhizoplane. As a result, the inhibitory effects of ethylene on plant growth are reduced, thus improving plant's tolerance under stresses. The role of ACC deaminase producing microorganisms in improving plant growth performance under stressed environments has been well documented (Saleem et al. 2007; Glick 2014).

Inoculation of wheat plants with rhizobacteria producing ACC deaminase significantly improved plant growth under water deficit stress conditions (Chandra et al. 2019; Danish et al. 2020). It is reported that inoculation of wheat seed with *P. fluorescens* strains possessing ACC deaminase activity exhibited a substantial improvement in the germination rate of a plant under salt stress (Safari et al. 2018). Inoculating plants with ACC deaminase producing rhizobacteria also induced more root proliferation and thereby, enhanced the water and nutrients uptake from soil under drought stress conditions (Zahir et al. 2008; Ngumbi and Kloepper 2016). Significant improvement in nodulation rates has been reported through the treatments of plants with ACC deaminase containing PGPR under adverse environmental conditions (Shahzad et al. 2010; Glick 2014; Sepúlveda-Caamaño et al. 2018). Several studies have reported the expression of ACC deaminase genes (*acdS* genes) in transgenic plants which helped to alleviate the adverse effects of salt (Sergeeva et al. 2006), metals (Grichko et al. 2000) and water-logging (Grichko and Glick 2001) stresses.

Suppressed plant growth and reduced root proliferation were induced in heavy metal contaminated soils as a function of elevated ethylene levels in plants. This condition could be reversed by the microorganisms producing the enzyme ACC deaminase by regulating the ethylene levels (Mishra et al. 2017). *Pseudomonas putida* UW4, ACC deaminase-producing bacterium promoted the growth of canola by reducing the ethylene level under salt stress (Cheng et al. 2007). A recent investigation by de Zélicourt et al. (2018) has revealed a novel molecular communication process during microbe induced salt stress tolerance. They showed that an endophytic bacterium *Enterobacter* sp. SA187 induced salt tolerance in *Arabidopsis thaliana* by the production of 2-keto-4-methylthiobutyric acid (known to be an ethylene precursor) which modulates ethylene signaling pathway.

9.4.3 Osmolyte Compounds in Stress Alleviation

The synthesis and accumulation of osmolytes or compatible organic solutes is one of the most important responses observed in plants to protect the cellular machinery and to impart tolerance against abiotic stresses. Osmolytes are low-molecular weight, soluble organic compounds which do not intervene with normal metabolism, even at higher concentrations. Examples of common osmolytes found in plants are amino acids, proline and glutamate, low molecular weight sugars and sugar alcohols, methylated tertiary N compound glycine betaine and other compounds like γ-amino butyric acid (Chen and Jiang 2010). They play a major role in osmotic adjustment under conditions causing cellular dehydration to maintain cellular activity. They are also involved in the stabilization of proteins and membrane structures, re-establishing the cellular redox balance by acting as scavengers of reactive oxygen species and regulation of stress related gene expression (Vicente et al. 2016).

The accumulation of compatible organic solutes, such as proline, glycine betaine and trehalose, is the most frequent and common acclimatization response of plants and bacteria under abiotic stress condition (Vendruscolo et al. 2007; Chen and Murata 2008; Rodríguez-Salazar et al. 2009). PGPR exudate osmolyte compounds in response to drought stress that act synergistically with osmolytes produced by the plants and stimulate plant growth (Paul and Nair 2008). In agreement with this, inoculation of rice with osmolyte producing rhizobacteria showed more beneficial effects under more severe stress conditions. The benefits accrued due to inoculation in shoot and root dry weight and numbers of tillers as compared to uninoculated rice plants were significantly more prominent under severe drought condition (Yuwono et al. 2005).

Under osmotic stress, proline acts as a compatible solute, and protects plant cells. It acts as molecular chaperone to protect protein integrity and enzyme activity and is also involved in reactive oxygen species scavenging activity (Alia Mohanty and Matysik 2001; Matysik et al. 2002). Plants inoculated with *Bacillus* strains showed increased proline accumulation under drought stress condition. This was attributed to the upregulation of *P5CS* (i.e., *Pyrroline-5-carboxylate Synthase*) gene involved in proline biosynthesis and inhibition of *ProDH* (i.e., *Proline Dehydrogenase*) gene expression involved in proline metabolism (Yoshiba et al. 1997). Plants inoculated with *Burkholderia* showed increased accumulation of proline under osmotic stress condition (Barka et al. 2006). Acquisition of osmotic tolerance in transgenic *A. thaliana* plants were correlated with the introduction of *proBA* (i.e., *γ-glutamyl kinase and γ-glutamyl-phosphate reductase*) genes derived from *B. subtilis*, which are mainly involved in proline production (Chen et al. 2007).

Increased proline content was correlated with reduced salt-stress symptoms such as chlorosis, necrosis and drying in *Medicago truncatula* inoculated by *Sinorhizobium meliloti* in comparison with uninoculated plants under salt stress condition (Bianco and Defez 2009). Even though accumulation of proline was observed under drought, saline stress and freezing (Verslues et al. 2006), heavy metal stress (Sharma and Dietz 2006) and plant pathogen defense (Fabro et al. 2004); it did not occur in plants under heat stress and plants were rendered more sensitive to heat by inducing proline accumulation (Rizhsky et al. 2004; Dobra et al. 2010; Lv et al. 2011).

Glycine betaine is a tertiary ammonium compound, widely accumulated in plants and other organisms and plays a potential role in drought, salt and stress induced by extremes of temperature (Ashraf and Foolad 2007; Chen and Murata 2008, 2011; Giri 2011). *Arabidopsis* plants inoculated with *B. subtilis* strain GB03, showed increased accumulation of glycine betaine and its precursor choline, imparting them with drought tolerance. However, drought tolerance induced by GB03 was lost in xipotl mutant of *Arabidopsis* which also had reduced choline production (Zhang et al. 2010). *Oryza* plants inoculated with *P. pseudoalcaligenes* reported rapid accumulation of glycine betaine which rendered stress tolerance (Jha et al. 2011). The osmotolerant bacteria produced glycine betaine which probably acted synergistically with the plant produced glycine betaine in response to stress, resulting in increased drought tolerance. Accumulation of glycine betaine increased about two-fold in arbuscular mycorrhizal fungi inoculated plants under salt stress condition (Al-Garni 2006).

Sugars such as sucrose, trehalose, raffinose family oligosaccharides and fructans are not only functioning as osmoprotectants during stress but also as substrates for growth and regulators of gene expression (Keunen et al. 2013; Radomiljac et al. 2013). Increased soluble sugar levels were observed in the plants subjected to drought, salinity, low temperature and flooding, whereas under other stress conditions such as high light irradiance, nutrient deficiency and heavy metals low sugar levels were noted (Strand et al. 1999; Gill et al. 2001). PGPR have been noted to increase the accumulation of soluble sugars and amino acids and thereby, impart stress tolerance in inoculated stressed plants. Under drought stress, *A. lipoferum* has been reported to accumulate free amino acids and soluble sugars and consequently, increased maize growth and stress tolerance (Bano et al. 2013). *Bacillus* strains inoculated maize seedlings had higher sugar content probably due to starch degradation, thereby imparting tolerance to plants during drought stress (Mohammadkhani and Heidari 2008). Chickpea plants inoculated with *A. lipoferum* FK1 showed significant increase in soluble sugar and protein content under salt stress condition as a mechanism in imparting tolerance (El-Esawi et al. 2019).

Polyamines are small, positively charged organic molecules with simple structure found in all living organisms with diverse cellular functions ranging from structural stabilization of key macromolecules to cellular membranes. Putrescine, spermidine and spermine are the three common polyamines found in plants. They also play a vital role in mitigating plant abiotic stress and at the same, their catabolic products may cause stress damage (Minocha et al. 2014). Wide arrays of bacterial secondary metabolites and volatile organic compounds promoting plant growth and inducing stress tolerance in plants have been reported from recent studies and one such example is polyamines. Xie et al. (2014) have found that the PGPR strain *B. subtilis* OKB105 promoted plant growth specifically by the production of the polyamine spermidine which inhibited the expression of ethylene biosynthesis gene *Aconitase-1* thereby, lowering ethylene levels in tobacco seedlings.

Polyamines may act as cellular signals in intricate cross-talk with phytohormonal pathways, including abscisic acid regulation of abiotic stress responses or may be involved in direct interactions with different metabolic routes (Alcázar et al. 2010). Spermidine secreting bacterium *Bacillus megaterium* BOFC15 induced polyamine production in *Arabidopsis* providing tolerance to drought stress correlating with elevated abscisic acid content and altered root architecture. Higher drought tolerance and abscisic acid) content were exhibited by inoculated plants under polyethylene glycol induced water-deficit stress (Zhou et al. 2016). *A. brasilense* Az39 promoted root growth and helped to combat osmotic stress in rice seedlings by cadaverine production (Cassan et al. 2009). Table 9.2 represents some reported examples of abiotic stress alleviation in plants mediated by regulation of osmolyte production, improved antioxidant status, Ion homeostasis, production of volatiles organic compounds.

9.4.4 Induction of Antioxidative Enzymes and Improved Antioxidant Status

Plants are constantly exposed to various abiotic stress factors in the field that considerably limit their growth and productivity. These stresses generally lead to the accumulation of reactive oxygen species, which cause severe oxidative damage to plants (Rejeb et al. 2014). Reactive oxygen species are toxic molecules found in

Table 9.2 Alleviation of abiotic stress in plants by microbe-mediated regulation of antioxidative system and cellular biochemicals

Microorganism	Abiotic stress	Host plant	References
Regulation of osmolyte production			
Azospirillum brasilense Az39	Drought	Rice	Cassan et al. (2009)
Azospirillum lipoferum	Drought	Maize	Bano et al. (2013)
Bacillus megaterium BOFC15	Drought	*Arabidopsis*	Zhou et al. (2016)
Bacillus polymyxa	Drought	**Tomato**	Shintu and Jayaram (2015)
Burkholderia, Arthrobacter and *Bacillus*	Salinity	*Vitis vinifera, Capsicum annuum*	Barka et al. (2006)
Glomus etunicatum	Salinity	*Glycine max*	Sharifi et al. (2007)
Klebsiella variicola F2, *Pseudomonas fluorescens* YX2 and *Raoultellaplanticola* YL2	Drought	Maize	Gou et al. (2015)
Pseudomonas putida GAP-P45	Drought	Maize	Sandhya et al. (2010)
Rhizobium etli	Drought	**Common bean**	Suárez et al. (2008)
Induction of antioxidative enzymes and improved antioxidant status			
Enterobacter P-68, *Enterobacter* P-46, *Enterobacter* P-39 and *Bacillus* G-4	Drought	Tomato	Bindu et al. (2018)
Microbacterium oleivorans KNUC7074, *Brevibacteriumi odinum* KNUC7183 and *Rhizobium massiliae* KNUC7586	Salinity	Pepper	Hahm et al. (2017)
Piriformospora indica	Salinity	Barley	Baltruschat et al. (2008)
Piriformospora indica	Salinity	Tomato	Ghorbani et al. (2018)
Plant growth-promoting bacteria	Drought	Strawberry	Erdogan et al. (2016)
Pseudomonas jessenii, R62, *P. synxantha*, R81, *Arthrobacter nitroguajacolicus* YB3 and *Arthrobacter* YB5	Drought	Rice	Gusain et al. (2015)
Pseudomonas mendocina and *Glomus intraradices*	Drought	Lettuce	Kohler et al. (2008)
Pseudomonas PF1 and TDK1	Salinity	Rice	Sen and Chandrasekhar (2015)
Pseudomonas fluorescens and *Trichoderma asperellum*	Drought	Rice	Singh et al. (2020)
Rhizobium ciceri A-08, EB-80 and Isolate-30	Salinity	Chick pea	Yilmaz and Kulaz (2019)

(continued)

Table 9.2 (continued)

Microorganism	Abiotic stress	Host plant	References
Rhizobium leguminosarum and *Serratia proteamaculans*	Salinity	Lettuce	Han and Lee (2005)
Sinorhizobium meliloti	Salinity	*Medicago truncatula*	Bianco and Defez (2009)
Ion homeostasis			
Bacillus pumilus	Salinity	Rice	Khan et al. (2016a)
Pseudomonas koreensis AK-1	Salinity	*Glycine max*	Kasotia et al. (2015)
Rhizophagus irregularis Chryseobacterium humi ECP37 *Ochrobacterium haematophilum* ZR3-5	Salinity	Sunflower	Pereira et al. (2016)
Thalassobacillus denorans NCCP-58 *Oceanobacillus kapialis* NCCP-76	Salinity	Rice	Shah et al. (2017)
Production of volatile organic compounds			
Bacillus thuringiensis AZP2	Drought	Wheat	Timmusk et al. (2014)
Brevibacterium linens RS16	Salinity	Rice	Chatterjee et al. (2018)
Fusarium oxysporum and *Verticillium dahliae*	Salinity	*Arabidopsis*	Li and Kang (2018)
Paraburkholderia phytofirmans PsJN	Salinity	*Arabidopsis*	Ledger et al. (2016)
Pseudomonas chlororaphis O6	Drought	*Arabidopsis*	Cho et al. (2008)
Pseudomonas simiae AU	Salinity	Soyabean	Vaishnav et al. (2015)

various subcellular compartments and can occur during regular cellular metabolism as a by-product. However, under stress condition, their formation is exacerbated. Plants have evolved a complex network of scavenging pathways to prevent the deleterious effect of excessive reactive oxygen species. Reactive oxygen species scavenging system of plants constitutes both enzymatic and non-enzymatic components. Superoxide dismutase, ascorbate peroxidase, catalase, glutathione peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase, glutathione *S*-transferase, and peroxiredoxin are enzymatic components. Non-enzymatic components comprise ascorbic acid, cysteine and glutathione. Superoxide dismutase enzyme converts O_2^- into hydrogen peroxide. Catalase, ascorbate peroxidase and glutathione peroxidase then detoxify hydrogen peroxide (Meyer et al. 2012).

Studies have reported that there was increase in the activity of different antioxidative enzymes and also enhanced accumulation of antioxidants in PGPR inoculated plants, exposed to various abiotic stress factors, thereby reducing the adverse effect of stress on the plant (Vurukonda et al. 2016; Singh et al. 2020). In some cases, inoculation with PGPR strains resulted in the decrease in antioxidant enzymes activities. Maize plants inoculated with plant growth promoting *Pseudomonas* spp. strains under drought stress condition showed significant reduction in antioxidant enzymes activity as compared with uninoculated plants (Sandhya et al. 2010), indicating a lowering of stress in the plants. Increased level of free radicals like hydrogen peroxide, malondialdehde and superoxide anion in leaves and roots of tomato seedlings exposed to salinity stress has been observed in a study by Ghorbani et al. (2018). However, inoculation with *Piriformospora indica* showed enhancement in the activity of antioxidative enzymes and reduction of free radical levels in stressed tomato seedling, thereby improving the plant health under stress. In another study by Yilmaz and Kulaz (2019) on chick pea, they reported that PGPR helped to ameliorate salinity stress by improving the activities of superoxide dismutase, catalase and ascorbate peroxidase in stressed plants.

9.4.5 Ion Homeostasis

Salt stress causes damage to the plant cell membrane and therefore increases its permeability resulting in leakage and accumulation of electrolytes in the surrounding tissues (Sandhya et al. 2010). Salinity leads to osmotic stress in plants. The response was found to occur within a short span of time in plants. The major components of salinity stress are Na^+ and Cl^- ions. It leads to hypotonic condition within cells and affect plant water balance, protein and membrane stability. This affect may lead to disturbed hormonal status, transpiration, photosynthesis, translocation of nutrients and other metabolic processes of plant (Munns 2002). In order to withstand the salinity stress, plant must maintain or quickly adjust both osmotic and ionic homeostasis within the cells. Plants usually seek to avoid high saline environments by keeping sensitive plant tissues away from the high salinity region, excluding ions from the roots, compartmentalizing ions away from the cytoplasm of physiologically active cells (Silva et al. 2010).

Plant growth-promoting rhizobacteria can alter the plant root structure with extensive rhizosheaths, are capable of trapping the cations in their exopolysaccharide matrix, thereby limiting the plant's salt uptake. Rhizobacteria have also been noted to enhance mineral exchange including macro and micro nutrients and alleviate nutrient imbalances caused due to high Na^+ and Cl^- ion influx. Plant growth-promoting rhizobacteria can also regulate the expression of ion affinity transporters. PGPR have been reported to maintain ion homeostasis and high K^+/Na^+ ratios in shoots by decreasing Na^+ and Cl^- accumulation in leaves; there is also increased Na^+ exclusion through roots and increase in the activity of high affinity K^+ transporter. Improvement of K^+ uptake and Na^+ exclusion was observed by the inoculation of *Azotobacter* strains C5 and C9 in maize under salt stress. The chlorophyll, proline and polyphenol contents in leaf were elevated which enhanced the plant stress responses (Rojas-Tapias et al. 2012).

In a study carried out to understand the spatiotemporal regulation of short and long-term salt stress, plants colonized with *A. thaliana* and *Burkholderia* *phytofirmans* PsJN showed greater tolerance to sustained salt stress. There were changes in the expression of genes involved in ion homeostasis such as *KT1* (i.e., *K^+Transporter 1*), *HKT1* (i.e., *High-Affinity K^+Transporter 1*), *NHX2* (i.e., *Sodium Hydrogen Exchanger 2*) and *SOS1* (i.e., *Salt Overly Sensitive 1*) after exposure to stress. The observed salt tolerance was presumably due to the rapid molecular changes induced by PsJN (Pinedo et al. 2015). Decreased Na^+ accumulation was noticed in a halophyte grass, *Puccinellia tenuiflora* inoculated with *B. subtilis* GB03. The gene expression analysis showed upregulation of *PtHKT1* and *PtSOS1* genes and down regulation of *PtHKT2* gene in roots under high salt concentrations (200 mM NaCl) (Niu et al. 2016).

Microbes are capable of altering host physiology for reducing foliar accumulation of toxic ions (Na^+ and Cl^-) by modifying their uptake by roots, along with improving the macro (N, P, K) and micro (Zn, Fe, Cu and Mn) nutritional status (Hamdia et al. 2004; Bano and Fatima 2009; Kohler et al. 2009). Zhang et al. (2008) reported that *B. subtilis* GB03 inoculation in *A. thaliana* could mediate the level of salt tolerance through regulation of the potassium transporter gene, *HKT1*. Certain volatiles produced by *B. subtilis* GB03 down regulated *HKT1* gene expression in roots and upregulated it in shoots, leading to lower Na^+ levels and recirculation of Na^+ in the whole plant under salt stress. There was increase in K^+ concentration in PGPR-inoculated plants leading to a high K^+/Na^+ ratio, resulting in effectiveness for salinity tolerance (Rojas-Tapias et al. 2012; Nadeem et al. 2013). Electrolyte leakage was lower in *Rhizobium* and *Pseudomonas* inoculated *Zea mays* plants (Sandhya et al. 2010). Shukla et al. (2012) reported similar finding in *Arachis hypogaea,* indicating that plant growth-promoting rhizobacteria help in maintaining the plant cell membrane integrity and protect from the harmful effects of salts. Inoculation with *Serratia liquefaciens* KM4 in maize has been reported to induce salt stress tolerance and improved plant growth by regulating ion homeostasis, stress-related genes expression, leaf gas exchange and redox potential (El-Esawi et al. 2018a).

The mechanism of beneficial soil microbial role in combating soil-water-plant relations and signalling cues are not completely revealed. Plant growth-promoting rhizobacteria have been proven to improve plant growth under stresses via several direct and indirect mechanisms. They also have the ability to modulate plant's physiology, thereby facilitating stress tolerance in plants (Yang et al. 2009; Dodd and Pérez-Alfocea 2012). The studies conducted by Smith et al. (2017) showed that plant-microbe interactions towards ensuing stress alleviation are regulated by complex network of signalling events occurring in metabolically active cells.

9.4.6 Volatile Compounds

A wide variety of microorganisms have the common property to produce volatile compounds. Among the PGPR, bacteria have been found to produce more than 1000 different volatile compounds and non-organic compounds, such as HCN and NH_3 (Audrain et al. 2015). Peñuelas et al. (2014) reported that the alkenes, ketones and alcohols are the major groups of bacterial volatile organic compounds. Treatment with volatile compounds from *B. subtilis* GB03 enhanced plant growth in *Arabidopsis* was for the first time reported by Ryu et al. (2004). The identity and quantity of volatile compounds produced by diverse microbes vary among species which seems to affect plant growth, development and/or stress tolerance (Effmert et al. 2012; Kanchiswamy et al. 2015). The interactive effects of soil salinity and inoculation with *Brevibacterium linens* RS16 in the moderately salt resistant (FL478) and the salt-sensitive (IR29) rice (*Oryza sativa*) cultivars were addressed in a study by Chatterjee et al. (2018). It was concluded that the salt stress reduced foliage photosynthetic rate, but induced foliage ACC accumulation, foliage ACC oxidase activity. During stress conditions emission of major classes of volatile organic compounds like lipoxygenase pathway volatiles, light-weight oxygenated volatiles, long-chained saturated aldehydes, benzenoids, geranylgeranyl diphosphate pathway products and mono and sesquiterpenes were also observed in the study.

Under salt stress, volatile compounds released from both the fungi *Fusarium oxysporum* and *Verticillium dahliae* helped *A. thaliana* growth and increased chlorophyll content and auxin accumulation in root apex (Li and Kang 2018). Volatile compounds released by *Pseudomonas simiae* strain AU significantly enhanced K^+ and P and reduced Na^+ content in the roots of soyabean seedlings under saline condition (Vaishnav et al. 2015). Root colonization of *A. thaliana* with *P. chlororaphis* O6 induced drought tolerance through the production of a bacterial volatile metabolite, 2R, 3R-butanediol (Cho et al. 2008). Reduced water loss indicated by the size of stomatal aperture and percentage of closed stomata was observed in *P. chlororaphis* O6 colonized plants. Chatterjee et al. (2018) carried out a study by inoculating *Brevibacterium linens* RS16 to rice (*Oryza sativa*) cultivars. It was noted that the inoculation reduced the temporal regulation of volatile compounds emissions and there was reduced accumulation of ACC (an ethylene precursor) and ACC oxidase activity leading to increased plant physiological activity, under saline condition.

Ledger et al. (2016) studied the *Paraburkholderia phytofirmans* PsJN volatile mediated effects in *A. thaliana* under salt stress. The plant exposed to a blend of volatile compounds (2-undecanone, 7-hexanol, 3-methylbutanol and dimethyl disulfide) was found to have similar effects as of strain PsJN on both inducing plant growth and salt stress tolerance. Similarly, the volatile compounds from *Alcaligens faecalis* strain JBCS1294 was found to regulate growth and induce salt stress tolerance in *Arabidopsis* (Bhattacharyya et al. 2015). A study was conducted by Jalali et al. (2017) in *A. thaliana* under salt stress (100 mM NaCl) inoculated with *Trichoderma* spp. The plant exposed to volatile compounds showed less hydrogen peroxide accumulation. This result may reflect the possible role of volatile compounds of this strain in plant protection against oxidative damage under salt stress.

9.4.7 Production of Exopolysaccharides

Exopolysaccharide production is found to have several roles in microbes such as protection against biotic and abiotic stresses, protection from antibiotics, providing ideal environment for chemical reactions, surface attachment, bacterial aggregation, biofilm formation and in plant-microbe interactions (Flemming and Wingender 2010; Sanchez-Garcia et al. 2010; Ates 2015). Studies have been reported that exopolysaccharides production by several plant growth-promoting rhizobacteria increased under abiotic stress conditions (Sandhya and Ali 2015; Bandeppa et al. 2018; Rathi et al. 2018b). The cellular response in *E. coli* strains included production of exopolysaccharide as a survival mechanism under desiccation stress (Zhang and Yan 2012).

Plant growth-promoting rhizobacteria producing exopolysaccharide for survivability can also provide growth promotion and impart tolerance to plants under stressful conditions such as drought, salinity and temperature stress. Exopolysaccharide producing *Pseudomonas aeruginosa* having a trait of high salt tolerance increased the tolerance of plants to salinity but the plants showed sensitivity to salinity when exopolysaccharide mutants were inoculated (Tewari and Arora 2014). Exopolysaccharide producing bacteria in the root zone sequester cations including Na^+ and reducing its availability to the plants causing tolerance to salinity (Upadhyay et al. 2011).

Microbial exopolysaccharide enhances soil particles aggregation and benefit plants by trapping nutrients and retaining moisture around the roots (Costa et al. 2018). Small amount of exopolysaccharide produced by *Sinorhizobium meliloti* has its impact on soil microstructure and inhibits the evaporation of pore water (Deng et al. 2015) which is due to the surface hydrophobicity of the exopolysachharides (Cruz et al. 2017). *P. fluorescens* DR7 isolated from the rhizosphere of foxtail millet which could efficiently colonize the root adhering soil had a role in enhancing the soil moisture and root adhering soil to root ratio (Niu et al. 2018). Mycorrhizae along with other soil organisms also play a significant role in improving physical properties of the soil by forming stable aggregates thereby, improving soil aggregation and water retention capacity of the soil. This is believed to be due to the production of insoluble glycoprotein glomalin (Nadeem et al. 2014).

Khanna et al. (2019) observed that heavy metal cd generated toxicity in tomato seedlings was reduced which enhanced upon inoculations of heavy metal resistant and exopolysaccharide producing plant growth-promoting rhizobacterial strains *P. aeruginosa* and *Burkholderia gladioli.* The production of exopolysaccharide from the rhizospheric microbes induce the formation of biofilms under high concentrations of toxic heavy metals where the transformation of toxic metal ions to non-toxic form takes place after adsorption (Mishra et al. 2017). Some reported examples of abiotic stress alleviation in plants mediated by exopolysaccharide production, improved membrane stability, physiological attributes of plant, root architecture and nutrient uptake are provided in Table 9.3.

Table 9.3 Alleviation of abiotic stress in plants by microbe-mediated biochemical synthesis and regulation of physiological and growth traits

Microorganism	Abiotic stress	Host plant	References
Exopolysaccharide production			
Pseudomonas aeruginosa	Salinity	Sunflower	Tewari and Arora (2014)
Pseudomonas fluorescens DR7	Drought	Foxtail millet	Niu et al. (2018)
Pseudomonas putida GAP-P45	Drought	Sunflower	Sandhya et al. (2009)
Pseudomonas putida GAP-P45	Salinity	Sunflower	Sandhya et al. (2009)
Rhizobium sp.	Salinity	Sunflower	Alami et al. (2000)
Rhizobium tropici	Drought	Cowpea	Rodrigues et al. (2015)
Decline in the cell membrane injury/improved membrane stability			
Burkholderia phytofirmans PsJN	Low temperature	Grapevine	Theocharis et al. (2012)
Pseudomonas aeruginosa 2CpS1	High temperature	Wheat	Meena et al. (2015)
Pseudomonas frederiksbergensis OS211, *Flavobacterium glaciei* OB146, *P. vancouverensis* OB155, and *P. frederiksbergensis*OS261	Low temperature	Tomato	Subramanian et al. (2016)
Pseudomonas putida AKMP7	High temperature	Wheat	Ali et al. (2011)
Improved physiological attributes of plant			
AM Fungi	Drought	*Capsicum annum*	Krishna (2018)
AM fungi	Salinity	Jatropha	Kumar et al. (2015)
Bacillus fortis SSB	Salinity	*Capsicum annum*	Yasin et al. (2018)
Bacillus megaterium M3, *Bacillus subtilis* OSU142, *Azospirillum brasilense* Sp245 and *Raoultella terrigena*	Low temperature	Wheat	Turan et al. (2012)
Burkholderiaphytofirmans PsJN	Low temperature	Grapevine	Barka et al. (2006)
Funeliformis mosseae and *Rhizophagus intraradices*	Drought	Flaxseed	Ansari et al. (2016)
Glomus claroideum	Drought	Wheat	Beltrano and Ronco (2008)
Paraburkholderia phytofirmans PsJN	High temperature	Tomato	Issa et al. (2018)
Piriformospora indica	Drought	Wheat	Hosseini et al. (2017)

(continued)

Table 9.3 (continued)

Microorganism	Abiotic stress	Host plant	References
Pseudomonas jessenii R62 and *P. synxantha* R81	Drought	Rice	Gusain et al. (2015)
Pseudomonas libanensis TR1 and *Pseudomonas reactans* Ph3R3	Drought	*Brassica oxyrrhina*	Ma et al. (2016)
Trichoderma atroviride ID20G	Drought	Maize	Guler et al. (2016)
Improved root architecture			
AM fungi	Drought	Banana	Srivastava and Singh (2019)
Azospirillum brasilense Sp 245	Drought	*Arabidopsis*	Cohen et al. (2015)
Bacillus amyloliquefaciens pb1	Salinity	Cotton	Irizarry and White (2017)
Bacillus firmus SW5	Salinity	Soybean	El-Esawi et al. (2018b)
Bradyrhizobium japonicum USDA 110 and *Pseudomonas putida* TSAU1	Salinity	Soybean	Egamberdieva et al. (2017a)
Streptomyces mutabilis	Drought	Rice	Suralta et al. (2018)
Improved nutrient uptake			
AM Fungi	Drought	Banana	Srivastava and Singh (2019)
AM Fungi	Salinity	Chick pea	Garg and Bhandari (2016)
AM Fungi	Salinity	Cowpea	Abeer et al. (2015)
Bacillus cereus Pb25	Salinity	Mungbean	Islam et al. (2016)
Burkholderia phytofirmans PsJN	Drought	Wheat	Naveed et al. (2014)
Effective microorganisms (photosynthetic bacteria + lactic acid bacteria + yeast + actinomycetes + fermenting fungi)	Salinity	Common bean	Talaat et al. (2015)
Funneliformis mossseae and *Rhizophagus irregularis*	Salinity	Pigeonpea	Garg and Pandey (2015)

9.4.8 Improving Plants Physiological Properties

Abiotic stress is associated with several types of physiological disorders in plants depending on the level of stress. The physiological, disorders include reduced water content, membrane stability, chlorophyll content and increased ethylene production and lipid peroxidation (Lata and Prasad 2011). The additional effects include the accumulation of free radicals that induce defective membrane function, protein conformation, lipid peroxidation and finally cell death (Tiwari et al. 2016). A study indicated that *Pseudomonas putida* inoculation conferred drought tolerance in *Cicer arietinum* by improving various physiological and biochemical traits such as water status, membrane stabilty, reactive oxygen species scavenging, osmolyte accumulation, and stress-responsive gene expression (Tiwari et al. 2016). Another study in capsicum showed that inoculation with halotolerant rhizobacteria *Bacillus fortis* strain SSB improved salinity tolerance and growth by activating physiological and biochemical processes (Yasin et al. 2018).

9.4.8.1 Relative Water Content

Relative water content is considered as an important marker to assess the water balance of plants (González and González-Vilar 2001). Limited water content reduced cell size, decreased cell membrane integrity and promoted leaf senescence that leads to decreased crop productivity (Gontia-Mishra et al. 2016). Inoculation of plant with beneficial microorganisms led to better maintenance of plant water status (Bandeppa and Kandpal 2015). Krishna (2018) reported that the inoculation of hot pepper (*Capsicum annuum*) plants with arbuscular mycorrhizal fungi led to change in morphological and biochemical indices, alleviated drought by maintaining relatively higher water status in plants. *Pseudomonas libanensis* TR1 and *Pseudomonas reactans* Ph3R3 were inoculated to *Brassica oxyrrhina* for improvement of heavy metal phytoremediation under drought conditions. The results indicated that inoculation significantly increased plant growth, leaf relative water and metal uptake (Ma et al. 2016). Hosseini et al. (2017) conducted a study to evaluate the effect of inoculation of root-colonizing endophytic fungus *Piriformospora indica* on wheat growth under combined drought and mechanical stresses. The result showed that the colonized plants were better adapted; leaves had greater relative water content and leaf water potential.

9.4.8.2 Membrane Permeability and Lipid Peroxidation

Malondialdehyde is used to explicate the extent of damage due to peroxidation and is induced during several abiotic stresses and leads to membrane leakage (Savicka and Škute 2010). Beltrano and Ronco (2008) reported that the inoculation of *Glomus claroideum* to wheat showed tolerance to drought stress with increased total dry weight, leaf chlorophyll concentration and improved cell permeability. Endophytic fungus *Trichoderma atroviride* ID20G treated maize (*Zea mays*) seedlings under drought stress prevented an increase in lipid peroxidation and reversed the changes caused by drought in pigment contents and photosystem efficiency (Guler et al. 2016). A study conducted by Kumar et al. (2015) on *Jatropha curcas* plant under different levels of Na_2SO_4 salt stress confirmed that arbuscular mycorrhiza inoculation was effective in alleviating the damage imposed by salinity stress on jatropha by reducing membrane lipid peroxidation and membrane permeability; and increasing the osmotic solute accumulation and antioxidant enzyme activity.

9.4.8.3 Photosynthetic Activity

The exposure of plants to various abiotic stresses leads to serious damage to photosynthetic machinery, resulting in huge yield losses. A major response of stress in plants is the degradation of photosynthetic pigments which is caused by chlorosis, reduced photosynthesis and oxidative damage (Lata and Prasad 2011). The the decrease in photosynthesis is mainly due to reduction in leaf area, chlorophyll content and stomatal conductance. From the measurement of chlorophyll fluorescence, the decrease in photosystem-II efficiency can be quantified (Netondo et al. 2004). Aquaporins are water conducting channels that regulate the water flux rates in plants that also indirectly influence mesophyll conductance and CO_2 assimilation and photosynthesis rates (Sade et al. 2014).

Studies of Hahm et al. (2017) reported that the inoculation of three plant growth-promoting rhizobacterial strains (*Microbacterium oleivorans* KNUC7074, *Brevibacterium iodinum* KNUC7183, and *Rhizobium massiliae* KNUC7586) in salt-stressed pepper plants exhibited significantly greater plant height, fresh weight, dry weight and total chlorophyll content. Mondani et al. (2019) showed that the inoculation of *B. subtilis* and *B. licheniformis* to soybean under water deficit stress showed increase in yield via rising of photosynthesis and radiation use efficiency. *Bacillus* sp. inoculated potato plants on exposure to drought, salt and heavy stresses exhibited positive influence of inoculation on Photosystem-II photochemistry of the plants Gururani et al. (2013). Another study using arbuscular mycorrhizal fungi in two different sweet potato genotypes showed improved water deficit tolerance. An increased content of chlorophyll pigments, Photosystem-II efficiency, photon yield of Photosystem-II, net photosynthetic rate and growth characteristics was recorded (Yooyongwech et al. 2016).

9.4.9 Improving Root Sytem Architecture

Improving the root system architecture is an adaptive trait for nutrient and water deficit stress endurance (Khan et al. 2016b; Koevoets et al. 2016). It integrates the topology of root system, primary and lateral root distribution and the length, number and diameter of roots (Vacheron et al. 2013). Stresses cause phytohormonal imbalance in plants which result in reduction or cessation of root growth. The major hormones that are reduced include auxins, gibberellins, jasmonic acid and salicylic acid (Iqbal et al. 2016; Egamberdieva et al. 2017b). Roots traits determine the acquisition of mineral elements and often compromise yields in input limited agricultural systems (White et al. 2013).

Inoculation of auxin producing bacteria in rhizosphere of wheat, pea, lettuce and maize led to increased root growth with a greater number of lateral roots and roots hairs (Dimkpa et al. 2009). This response led to increased water and nutrient uptake (Mantelin and Touraine 2004). Suralta et al. (2018) conducted a drought stress experiment in rice inoculated with rhizobacteria *Streptomyces mutabilis* and concluded that inoculation improved total root length, increased seminal root length as well as root hair lengths. Similarly, in a study by El-Esawi et al. (2018b) improvement in the root architecture of soybean (*Glycine max*) inoculated with *Bacillus firmus* SW5 was noted, leading to enhanced nutrient uptake under salt stress. Srivastava and Singh (2019) studied the effect of inoculation of mycorrhizal fungi in micropropagated banana under water stress and improved growth as number of lateral roots increased the surface area for nutrient and water absorption. A similar study carried out by Boris and Tomáš (2018) in wheat inoculated with mycorrhizal fungi under P deficit stress alleviated total root length, increased fine roots length and alleviated phosphorus deficit stress. Cotton seeds inoculated with the salt tolerant endophytic bacterium *Bacillus amyloliquefaciens* strain pb1 showed that the inoculation promoted plant growth, alleviated salt stress and altered root development of cotton seedlings with greater shoot height, primary root length and the number of lateral roots (Irizarry and White 2017).

9.4.10 Nutrient Uptake

Nutrients are so crucial for plants that they have evolved a number of signaling cascades for the uptake of the elements. Mineral nutrients are essential to carry out several plant processes particularly in photosynthesis, cell division, protein synthesis, disease resistance and for the primary and secondary growth of the plants. All the important physiological functions are disrupted in the deficiency or the excess of such requisite elements (Kerry et al. 2018). Abiotic stresses disturb the nutritional status of the plants. There is decreased nutritional transport from root to shoot due to reduction in transpiration rate and alteration in concentrations in tissues (Duman 2012). Microorganisms play an important role in the acquisition of nutrients and to stimulate the dissolution of insoluble minerals from the soil (Chikkanna and Ghosh 2018). Phytohormones produced by the microbes promote proliferation of root cells, which results in profuse root system and increases the uptake of important nutrients from the soil (Egamberdieva 2009).

Gómez-Muñoz et al. (2018) reported that addition of Mn/Zn and inoculation with *Penicillium* strains as seed treatment were able to reduce the adverse effects of cold stress in maize plants by improving the phosphorous uptake. Egamberdieva et al. (2017c) stated that inoculation of chickpea plants with *Bacillus cereus* NUU1, *Achromobacter xylosoxidans* NUU2, *Bacillus thuringiensis* NUU3 and *B. subtilis* NUU4 improved the uptake of nitrogen, phosphorous, potassium and magnesium minerals under salt stress condition. Srivastava and Singh (2019) reported that nutrient uptake by mycorrhizal plants was faster than non-mycorrhized roots under water deficit stress in banana. They observed that N, P and K levels in the leaves of mycorrhized plantlets were significantly higher as compared to the uninoculated plantlets after 60 days of acclimatization. Similar observations were also noted in inoculated plants under salinity, drought and nutrient deficiency stresses.

9.4.11 Induction of Stress-Related Genes Expression

Microbial inoculation is known to modify plant responses at gene level under stress conditions. The elicitors and various biomolecules released by microbes drastically change physiology and modulate plant growth and development. The hormone abscisic acid is the major hormonal player of stress responses in plants. The response signaling pathways are categorized into two as abscisic acid-dependent and independent. The promoters of genes involved in abscisic acid dependent pathway contain a conserved sequence namely 'Abscissic acid response element'. Several classes of transcription factors are found to bind Abscissic acid response elements and regulate gene expression. The major ones are basic leucine Zipper, (Fujita et al. 2013), Myeloblatosis related C and Myeloblatosis related B transcription factors (Nakashima and Yamaguchi-Shinozaki 2013). Timmusk and Wagner (1999) were the first to report plant growth-promoting rhizobacteria-mediated modulation in the expression of drought stress responsive genes.

The abscisic acid independent pathway is primarily regulated by *DREB2* (i.e., Dehydration Response Element Binding Factor 2) family transcription factor (Yamaguchi-Shinozaki and Shinozaki 2005). Overexpression of transcription factor *TaAREB3* (i.e., ABA Response Element Binding 3) in wheat plants led to enhanced abscisic acid sensitivity and drought tolerance (Wang et al. 2016). Induction of drought stress responsive gene *ERD15* (i.e., Early Response to Dehydration 15) was reported in *A. thaliana* inoculated with *Paenibacillis polymyxa* under water deficit stress condition. Vaishnav and Choudhary (2019) emphasized the effect of inoculation with *Pseudomonas simiae* strain AU, in soybean for water stress tolerance. The upregulation of transcription factors (e.g., *DREB/EREB*), water transporters belonging to aquaporin family (e.g., *PIP, Plasma Membrane Intrinsic Proteins*; *TIP, Tonoplast Intrinsic Proteins*) and osmoprotectants (e.g., *P5CS, Pyrroline-5-carboxylate synthase; GolS, Galactinol Synthase*) in inoculated plants, confirmed their involvement in imparting drought tolerance to the soybean plants.

Ethylene is another stress response hormone imparting tolerance. It's signaling pathway genes were differentially expressed by inoculation with beneficial endophytes in sugarcane (Vargas et al. 2014). In *Arabidopsis*, the inoculation of *P. indica* led to upregulation of a diverse set of stress-related genes and this resulted in increased tolerance to drought (Sherameti et al. 2008).

Arabidopsis thaliana seedlings inoculated with *Pseudomonas* PS01 survived under salt stress conditions up to 225 mM NaCl, while all non-inoculated plants were dead above 200 mM NaCl. The RT-PCR analysis showed that jasmonic acid synthesis gene (*LOX2, Lipoxygenase 2*) was up-regulated and genes related to reactive oxygen species scavenging (*APX2, Ascorbate Peroxidase 2*) and detoxification (*GLYI7, Glyoxylase 17*) were down-regulated in inoculated plants, indicating reduced stress upon inoculation in comparison to the uninoculated controls (Chu et al. 2019). Poupin et al. (2013) reported that in *Arabidopsis B. phytofirmans* PsJN inoculation modified the regulation of several genes in inoculated plants. It included genes involved in defence or against biotic or abiotic stresses. In grapevine also the strain led to upregulated *CBF* (*C-Repeat Binding Factor*) gene expression to improve cold tolerance (Theocharis et al. 2012). Tiwari et al. (2017) observed positively modulated stress responsive gene expression in rice inoculated with *B. amyloliquefaciens* SN13 under drought, salinity, cold, high temperature and phytohormone treatments. Modulation in expression of genes involved in osmolyte synthesis (e.g., *DHN, Dehydrin* and *LEA, Late Embryogenesis Abundant*), antioxidant pathway (e.g., *GST, Glutathione S-transferase*), membrane associated processes (e.g., *GRAM, Glycosyltransferase, Rab-like GTPase Activators, Myotubularins*) and genes encoding intracellular metal transporters (e.g., *NRAMP6, Natural Resistance-Associated Macrophase Protein 6*) were observed in the inoculated plants. These results suggested its multifaceted role in cross-talk among stresses and phytohormones in response to *B. amyloliquefaciens* SN13 inoculation. Other examples of abiotic stress alleviation in plants mediated by induction of stress responsive genes and proteins are given in Table 9.4.

Table 9.4 Alleviation of abiotic stress in plants by microbe-mediated induction of stress responsive genes

Microorganisms	Abiotic stresses	Host plants	References
Arthrobacter nitroguajacolicus	Salinity	Wheat	Safdarian et al. (2019)
Bacillus amyloliquefaciens 5113 and *Azospirillum brasilense* NO40	Drought	Wheat	Kasim et al. (2013)
Bacillus amyloliquefaciens 5113 and *Azospirillumbrasilense* NO40	High temperature	Wheat	El-Daim et al. (2014)
Bacillus amyloliquefaciens SN13	High temperature	Rice	Tiwari et al. (2017)
Bacillus amyloliquefaciens SQR9	Salinity	Maize	Chen et al. (2016)
Bacillus licheformis K11	Drought	*Capsicum*	Lim and Kim (2013)
Bacillus subtilis	Salinity	*Arabidopsis*	Zhang et al. (2008)
Burkholderia phytofirmans PsJN	High temperature	*Arabidopsis*	Su et al. (2015)
Dietzia natronolimnaea STR1	Salinity	Wheat	Bharti et al. (2016)
Gluconacetobacter diazotrophicus PAL5	Drought	Sugarcane	Vargas et al. (2014)
Klebsiella sp., *Enterobacter ludwigii* and *Flavobacterium* sp.	Drought	Wheat	Gontia-Mishra et al. (2016)
Paenibacillus polymyxa B2	Drought	*Arabidopsis*	Timmusk and Wagner (1999)
Paraphaeosphaeria quadriseptata	High temperature	*Arabidopsis*	McLellan et al. (2007)

(continued)

Table 9.4 (continued)

Microorganisms	Abiotic stresses	Host plants	References
PGPR	Salinity	Rice	Jha et al. (2011)
Pseudomonas chlororaphis O6	Drought	*Arabidopsis*	Cho et al. (2013)
Pseudomonas PS01	Salinity	*Arabidopsis*	Chu et al. (2019)
Pseudomonas putida MTCC5279	Drought	Chickpea	Tiwari et al. (2016)
Pseudomonas simiae AU	Salinity	Soybean	Vaishnav et al. (2015)
Pseudomonas simiae strain AU	Drought	Soybean	Vaishnav and Choudhary (2019)
Pseudomonas sp. AMKP6	High temperature	Sorghum	Ali et al. (2009)
Rhizobium tropici and *Paenibacillus polymyxa*	Drought	*Phaseolus vulgaris*	Figueiredo et al. (2008)

9.5 Conclusion

Agricultural productivity is being adversely affected by several environmental stresses globally. Different kinds of PGPR are useful in the mitigation of stress induced constraints in the crop plants and reduction of negative impact on crop yield and productivity. Plant associated microbes and their different metabolites modulate and change cellular physiology, hormonal homeostasis and activate stress-responsive genes in host plants. Application of PGPR in stressed agriculture is an encouraging, cheaper and efficient approach for abiotic stress alleviationin crops. Based on the available informations, future studies should be focussedon the exploration of potential PGPR under field conditions in a variety of crops.

In future, more extensive studies related to trait characterization, compatibility assessment, delivery methods, identification and impact of microbes obtained from the stressed ecosystem for the abiotic stress alleviation should be done. Further, there is a need to identify the functions of different microbial metabolites, produced under stressed environment. Currently, many authors have established shreds of evidence for microbe-mediated plant interactions and stress alleviationunder adverse edaphic and climatic conditions. Though, detail application of omics-based technologies such as genomics, proteomics, metagenomics and metabolomics in specific plant–microbe-interaction under abiotic stress is required in order to elucidate the precise mechanisms of stress tolerance in the crop plants. These studies may give detail insights on new directions and approaches in the arena of plant–microbe interactions under stressed environments and application of such microbes and their metabolite or molecules for alleviating adverse impacts of various stresses on crop health.

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Chapter 10
Microbial Alleviation of Abiotic and Biotic Stresses in Rice

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Abstract More than 90% of the cultivated area is affected globally by environmental constraints. For instance, abiotic and biotic stresses are major processes that decline agricultural production. Drought, salinity, heat, cold, acidity, and sodicity are major abiotic factors, while insects and pathogens are biotic factors. Rice, a staple food for more than half of the world's population, is highly susceptible to abiotic and biotic stresses. Here, we review stresses in rice and mitigation strategies, with focus on microbes to alleviate stresses. Abiotic stresses in rice are alleviated by microbes belonging to genus *Bacillus, Pseudomonas, Enterobacter, Ochrobactrum, Alcaligens, Paecilomyces, Burkholderia, Achromobacter, Azospirillum,* and *Glomus.* This alleviation proceeds through an accumulation of ascorbate, proline, ethylene, auxin, and stomata conductance of leaf, and by producing antioxidant enzymes, 1-aminocyclopropane-1-carboxylate deaminase, β-aminobutyric acid, salicylic acid and siderophores. Biotic stresses in rice include brown spot, leaf blast, blunt, leaf blight, sheath blight, sheath rot, root rot and seedling disease. They are suppressed by *Pseudomonas*, *Streptomyces, Bacillus, Trichoderma*, *Aspergillus* by inhibiting mycelia growth, iron competition, producing antibiotics, phytohormones, metabolites, and enzymes.

Keywords Rice · Microbial interventions · PGPMs · Abiotic stress · ACC deaminase · Rice diseases · Biocontrol · Induced systemic tolerance · Siderophore · Stress enzymes

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ACS	1-aminocyclopropane-1-carboxylate synthase
IAA	Indole acetic acid

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PGPMs	Plant growth-promoting microorganisms
PGPR	Plant growth-promoting rhizobacteria

10.1 Introduction

Presently, the world population is about 7.6 billion which is expected to increase by 20.8% to 9.6 billion in 2050 (UN Report 2013). Most of this increase (93%) will occur in developing countries, whose share of population is projected to increase from 78% in 1990s to 83% in 2020. Rice is a staple crop for over half of the world's population and is prone to a variety of abiotic and biotic stresses (Lafitte et al. 2004; Kumar et al. 2016, 2018a). High salinity, submergence, cold and drought stresses are the major abiotic factors, whereas insects and pathogens are the major biotic factors causing threat to rice crop thereby reducing food security for growing human population (Sanghera et al. 2011; Shanker and Venkateswarlu 2011; Wani et al. 2013; Kumar et al. 2018b). According to various estimates, we have to produce 40% more rice by 2030 and 70% more by 2050 to satisfy the growing demand without affecting the resource base adversely (FAO 2009; Tilman et al. 2011). We have to achieve this demand from less land, labour, water and fewer chemicals.

To meet the challenge of producing more rice from affected lands, a wide range of adaptations and mitigation strategies are required. Efficient resource management and rice crop improvement for evolving transgenic may be one of the alternatives to overcome abiotic and biotic stresses to some extent. However, such strategies being long drawn and cost intensive, there is a need to develop simple and low-cost biological methods for the management of abiotic stress and it can be used on short term basis (Kumar et al. 2017a, 2019). Plant growth-promoting microorganisms (PGPMs) are one of the best options to alleviate abiotic and biotic stresses in agricultural crops including rice with higher yield potential and greater yield stability, if we can exploit their unique properties of tolerance to extremities, ubiquity, genetic diversity, and their interaction with agricultural crops (Kumar et al. 2016). Researchers from all over the world have made great efforts in understanding the mechanisms of PGPM responses to abiotic and biotic stresses in rice (Sarkar et al. 2018; Pandey et al. 2013; Khan et al. 2016; Kakar et al. 2016; Reddy et al. 2007; Law et al. 2017; Saravanakumar et al. 2007). In this chapter, we emphasized a different abiotic and biotic stress mitigation strategy through microbial intervention particularly for rice crop and its mechanistic understanding is represented in Fig. 10.1.

10.2 Plant Stress

Stress can be defined as any unfavorable condition or substance affecting or blocking the metabolism, growth or development of a plant (Lichtenthaler 1996). Accordingly, climate and environmental factors regulate the geographical

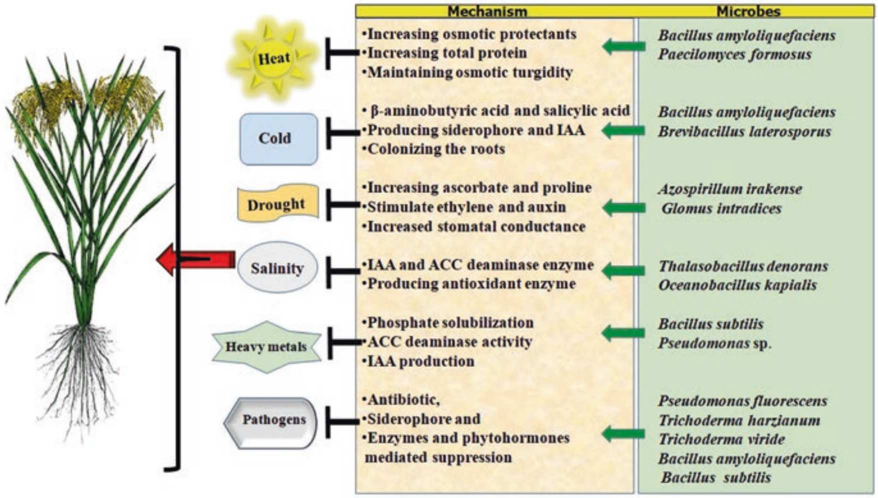

Fig. 10.1 Mode of action of plant growth-promoting microorganisms in rice under biotic and abiotic stresses. Lines with bar indicates inhibition of those environmental stresses by means of plant growth-promoting microorganisms and arrows represent secreted compounds and elicitors by plant growth-promoting microorganisms. IAA: Indole acetic acid; ACC: 1-aminocyclopropane-1-carboxylate

distribution of plants (Walther et al. 2002). Thus, unfavorable environmental changes can affect plant growth and crop yield (Duque et al. 2013). Reactive oxygen species molecules are generally formed in response of oxidative stress (Kumar et al. 2019). Drought, heat shock and salinity are the major oxidative stresses responsible to release reactive oxygen species in the system. Some of the well-known reactive oxygen species molecules that result in membrane and macromolecular damage include hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-) and superoxide anion (O_2^-) (Kumar et al. 2019; Blokhina et al. 2003; Karim 2007; Farnese et al. 2016). In order to increase rice stress tolerance and decrease the detrimental effect of toxic reactive oxygen species compounds, they utilize several antioxidant defense mechanisms in order to scavenge reactive oxygen species.

Several antioxidants that plant mainly uses are namely, ascorbate peroxidase, superoxide dismutase, glutathione reductase and catalase (Kumar et al. 2019) and non-enzymatic antioxidants such as carotenoids, glutathione, ascorbate and anthocyanin (Karim 2007; Mittler 2002; Blokhina et al. 2003; Gould et al. 2002). Whereas biotic stress includes parasitic organisms that are pathogenic and causes plant diseases; this involves a wide spectrum of microbes (fungi, bacteria, viruses, nematodes, protozoa and insects) (Adhya et al. 2018). Every year pathogenic diseases cause significant crop losses all over the world (Agrios 2005; Karim 2007). As we know, the nature of the parasitic organisms is to utilize the host plant for feeding, sheltering, multiplying and growing that causes significant host damage and ultimately leads to death. In these conditions, Plant growth-promoting microorganisms may act as biocontrol agents and mitigates the biotic stress in the plant (Kumar et al. 2013).

10.3 Plant Growth-Promoting Microorganisms

Plant growth-promoting microorganisms (PGPMs) are beneficial microbes that have the distinctive ability to support plant development directly and indirectly. They live in the rhizosphere zone which is rich with plant exudates such as sugars and amino acids or some microbes establish themselves as endophytes within the plants in order to survive in the root rhizosphere by means of penetrating/burrowing tissues of plants, that contributes to plant's nutrition, environment adaptability and survivability. These microbes extend their biological activities in order to survive in the rhizosphere, influencing plant survival and development (Kumari et al. 2015; Khan et al. 2016; Babalola 2010; Kumar et al. 2013). The process in which PGPMs play a role in stimulating variety of abiotic stress tolerance in plants is referred to as induced systemic tolerance (Kumar et al. 2012; Yang et al. 2009).

These PGPMs include multiple bacterial determinants such as *Bacillus amyloliquefaciens* and *Brevibacillus laterosporus*, *Azospirillum brasilense* that are involved in induced systemic tolerance by means of production of indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase, phosphate solubilization, and volatile, exo-polysaccharides, siderophores production (Farag et al. 2013; Kumari et al. 2015; Nadeem et al. 2016).

These traits help the plants to overcome stress. Certain PGPMs function is to synthesize ACC deaminase that catalyzes the transformation of ACC (ethylene biosynthesis precursor) to ammonia and α-ketobutyrate. Thus, plants with decreased concentrations of ethylene would finally overcome the inhibition of abiotic stress by associating with ACC deaminase-producing bacteria such as *Pleosporalean ascomycete, Alcaligenes*, *Rhodococcus* and *Variovorax* (Barnawal et al. 2014; Nadeem et al. 2010a; Senthilkumar et al. 2009; Glick et al. 2007; Mayak et al. 2004). Considerable attention has been made to the isolation of ACC deaminase-producing microbes for their utilization in direct plant growth promotion under unfavourable environments (Ali et al. 2016; Hardoim et al. 2008; Nadeem et al. 2010b).

In addition to ACC deaminase enzyme, they also produce a variety of substances such as plant hormone–indole acetic acid (Enebe and Babalola 2018), siderophore (Stajkovic-Srbinovic et al. 2014), PO_4^{2-} solubilizing enzyme, salicylic acid (Ekinci et al. 2014) and microbiocidal/biostatic enzyme (Moustaine et al. 2017). By trapping and integrating nitrogen into the plant *via* nitrogen fixation, some of these microbes contribute to plant nutrition (Kumar et al. 2017b; Richardson et al. 2009). The subsequent impacts of this specific form of plant-bacterial association would provide plants with a source of nitrogen (ammonia) (Hardoim et al. 2008). PGPMs also help to sustain the plant's inherent resistance to pathogenic and environmental problems. Some of these organisms are excellent in secretion of polysaccharide substances and formation of biofilm that helps to maintain stability during stress conditions (Kumar et al. 2013; Kasim et al. 2016).

The presence of microbes as bio-inoculant decreases metal stress in plants as they can produce metal rich solution through the biological oxidation of sulfur containing ore and plays a crucial role in metal immobilization and make them unavailable using polymeric substances and other chemicals such as siderophore production (Fashola et al. 2015) and have a significant contribution to bio-hydrometallurgy.

10.4 Role of Microbes in Alleviating Abiotic Stresses in Rice

10.4.1 Drought Stress

World's 64% of the total land area has been affected by water deficit/drought stress (Mittler 2006; Cramer et al. 2011). It has a major impact on soil nutrients availability and transportation through water to the roots. Thus, drought stress reduces the movement of nutrients and water-soluble supplements, such as, NO^{3-}, SO_4^{2-}, Ca, Si, and Mg which are considered essential for growth (Nasim et al. 2017). It also forms free radicals and reactive oxygen species that can further damage the rice plant by membrane lipid peroxidation or degradation of important structural and functional proteins (Kumar et al. 2019; Nair et al. 2008).

Drought stress have direct effects on plant physiology in rice as it ceases the cell growth because of altering the cellular turgidity and regular growth processes (Hsiao and Xu 2000; Rahdari and Hoseini 2012; Jabran et al. 2017). Among the various crops, rice is likely to be more vulnerable to drought stress (Showler 2016). Drought stress restricts the plants growth and development by interrupting biochemical processes such as low nitrate uptake from dry soils, which further reduces the rate of photosynthetic pigmentation, is an indication of photo-oxidation. It also influences some enzymatic activities such as nitrate reductase activity, due of low uptake of NO_3^- from dry soils which restricts plant growth and development (Ali et al. 2016; Awais et al. 2017a, b).

Furthermore, the grain filling stage of rice is adversely affected due to water shortage that favors the remobilization of stored carbohydrates into grains (Nasim et al. 2016a; Yang et al. 2012). Four components are assumed to be mainly involved in this procedure: (1) starch formation; (2) ADP-glucose-pyrophosphorylase; (3) sucrose formation; (4) starch branched compound (Taiz and Zeiger 2002). Under drought stress, decreased sucrose synthase activity lower the rate of grain filling and it also leads to inactivation of ADP-glucose-pyrophosphorylase which in turn causes developmental losses (Ahmadi and Baker 2001; Nasim et al. 2016b). Thus, drought conditions result in diminished photosynthesis, stomata closure and disturb cellular ionic balance because of low water content of soil (Flexas et al. 2004), consequently, reducing plant growth and development, obstructing grain filling and ultimately reducing grain yield.

One of the major weapons to mitigate this abiotic stress is beneficial microbes and some examples of these are presented in Table 10.1. Bacterial inoculation in rice enhanced the production of plant hormones such as IAA that improved lateral

Table 10.1 Beneficial microbes for alleviation of abiotic stress and plant growth-promotion in rice

Beneficial microbes	Abiotic stresses	Impact on plant-growth promotion in rice	Reference
Azospirillum brasilense, *Glomus intraradices*	Drought	Increased stomata conductance, photosynthesis, shoots fresh weight and plant vigor	Ruíz-Sánchez et al. (2011)
Bacillus amyloliquefaciens Bk7, *Brevibacillus laterosporus* B4	Drought, cold	Improved the seedling height and shoot number; alleviate chlorosis, wilting, necrosis and rolling of leaves	Kakar et al. (2016)
Bacillus pumilus	Salt	Improved growth and nutrient uptake	Khan et al. (2016)
Pseudomonas sp., *Burkholderia caryophylli, Achromobacter piechaudii*	Salt, drought	Reduce endogenous ethylene levels in plants and promotes root growth	Wu et al. (2009)
Ochrobactrum sp., *Bacillus* sp. (CdSP9, PbSP6, and AsSP9)	Heavy metals (cadmium, lead, arsenic)	Increase in germination percentage, relative root elongation, amylase and protease activities	Pandey et al. (2013)
Pseudomonas strain (TDK1)	Salt	Increases plant height, root length and leaf area	Sen and Chandrasekhar (2014)
Bacillus amyloliquefaciens	Salt, drought, desiccation, heat, cold	Increased accumulation of osmolytes (proline, soluble sugars, glycine betaine, trehalose, etc.); helped plant to overcome abiotic stresses by maintaining osmotic turgor	Tiwari et al. (2017)
Pseudomonas fluorescens	Drought	Encouraged the expression of abscisic acid synthetic genes particularly at the stage of reproduction by the plant	Saakre et al. (2017)
Thalassobacillus devorans (NCCP-58), *Oceanobacillus kapialis* (NCCP-76)	Salt	Increased germination ability, root and shoot growth, protein, and chlorophyll contents as well as nutrient contents with reduced sodium ion accumulation in the plant	Shah et al. (2017)
Bacillus sp.	Salt	Aided the alleviation of salt stress by increasing the biomass and growth of rice seedling via production of indole acetic acid and ACC deaminase enzyme	Misra et al. (2017)
Bacillus thuringiensis	Salt	Promotes plant-growth	Raheem and Ali (2015)

(continued)

Table 10.1 (continued)

Beneficial microbes	Abiotic stresses	Impact on plant-growth promotion in rice	Reference
Alcaligenes faecalis, Bacillus pumilus, Ochrobactrum sp.	Salt	Results in shoot and root elongation	Bal et al. (2013)
Pseudomonas pseudoalcaligenes, Bacillus pumilus	Salt	Increase in root length and promotes growth and yield	Jha et al. (2013)
Burkholderia pyrrocinia, Pseudomonas fluorescens	Water	Induces increased production of carotenoids and chlorophyll b and promotes plant growth by maintaining the integrity of enzymes and proteins of cell wall	Rêgo et al. (2018)
Azospirillum brasilense	Osmotic	Increases the root elongation, root surface area, root dry matter, and development of lateral roots and root hairs	Cassan et al. (2009)
Bacillus subtilis, Bacillus megaterium, Bacillus sp.	Heavy metals	Promotes plant growth and development along with increased dry matter, grain yield and phosphorus uptake	Asch and Padham (2005) and Becker and Asch (2005)
Enterobacter sp.	Salt	Promoted the growth of rice seedling and reduced ethylene production and antioxidant enzyme activities in the plant	Sarkar et al. (2018)
Pseudomonas fluorescence, P. jessenii, P. synxantha, Bacillus cereus, Arthrobacter nitroguajacolicus	Drought	Enhances plant growth by induction of stress related enzymes and activation of antioxidant defense systems and improves stability of membranes of plant cells	Gusain et al. (2015)
Bacillus amyloliquefaciens NBRISN13	Salt	Enhanced proline accumulation and upregulation or repression of set of stress responsive genes in leaf blade.	Nautiyal et al. (2013)

roots formation and root growth which ultimately increased leaf water content and decreased leaf water potential by increasing water uptake (Dossa et al. 2017). IAA produced by *Azospirillum* enhances tolerance of rice under drought stress, resulting in higher mineral quality and better grain yield (Dimkpa et al. 2009). Inoculation with arbuscular mycorrhizal fungus, *Azospirillum brasilense*, considerably enhances rice growth by increase stomatal conductance that improved growth parameter by 80% under water deficit condition (Ruíz-Sánchez et al. 2011).

During water stress conditions, lipid peroxidation increases with decrease in glutathione contents in plants; while inoculation with arbuscular mycorrhizal fungus, ascorbate and proline contents (as protective components) increase to bypass the deleterious effect of water limitation (Ruíz-Sánchez et al. 2011). Inoculation of rice plants with *Bacillus amyloliquefaciens* Bk7 and *Brevibacillus laterosporus* B4 in water deficit conditions, improve shoot number, seedling height and showed least symptoms of chlorosis, necrosis, wilting and rolling of leaves (Kakar et al. 2016). The endophytic Plant growth-promoting rhizobacteria (PGPR), *Azospirillum irakense* under drought stress trigger the expression of polygalacturonase encoding genes in rice inoculated roots (Sekar et al. 2000). Rice roots inoculated with endophytic PGPR, *Herbaspirillum seropedicae* stimulate the gene expression receptive to ethylene and auxin and results in suppression of defense-related thionins and proteins PBZ1 (Brusamarello-Santos et al. 2012). Therefore, above reports suggest that drought stress in rice might be mitigated through different microorganisms by modulating plant defense responses.

10.4.2 Cold Stress

One of the most significant environmental factors that hamper agricultural production by affecting plant growth is cold stress which affects 57% of the total land area of the world (Mittler 2006; Cramer et al. 2011; Hashimoto and Komatsu 2007). Low temperature impacts the agronomic development of crops including rice. The survivability of plants at extreme low temperature relies upon it’s adaptability to cold stress (McKhann et al. 2008). Plants exposed to low temperatures showed increased penetrability that is correlated with the injury of the plasma layer, a major problem for maintaining ionic equilibrium and reversing the damage caused due to cold stress. The unsaturated or saturated fatty acids tend to rearrange themselves that causes a change in the plasma layer *viz.* thickness alterations that result in declining turgidity of the cell (Hughes and Dunn 1996). This plasma layer modification tends to be cold sensitive in several rice varieties that initiates a response by specific gene expression during cold stress (Chinnusamy et al. 2006). Thus, cold stress directly or indirectly hampers geographical distribution of rice that overall reduces the rate of harvest (Pearce and Fuller 2001). Microbes mediated stress responses are one of the best ways to cope up with this cold stress (Table 10.1).

It was reported that, PGPR consortium of two different bacterial strains *Brevibacillus laterosporus* B4 and *Bacillus amyloliquefaciens* Bk7 attributed to the production of high amount of siderophore and IAA and effectively colonized the roots of the plant under cold stress (Kakar et al. 2016). They also induced systemic tolerance in rice under chilling stress and enhanced growth and development. This strain is also well known for the biofilm formation and the production of biochemical elicitors (β-aminobutyric acid and Salicylic acid) in rice for cold stress tolerance. Catalase and superoxide dismutase activities in plants increased by 3.6- and 3.0-fold respectively, after inoculation of Bk7. *Bacillus amyloliquefaciens* NBRI-SN13 (SN13) improved growth of rice seedling under cold stress by increasing proline content (Tiwari et al. 2017). It has been reported that, some phytohormones like abscisic acid, jasmonates, salicylic acid, and ethylene play a key role in cold, salt, heat and drought stresses response in several plants including rice to sustain a balanced and healthy growth of plant (Lata et al. 2011; Kohli et al. 2013).

10.4.3 Heat Stress

Most of the cereal crops especially rice, are at major risk due to annual increase in temperature and its deleterious effect on overall growth, development and productivity (Fahad et al. 2015a, b, 2016a, b, c, d, 2018; Watanabe and Kume 2009; Mahmood et al. 2007). It is expected that the rate of rice yield will decrease by 41% before the end of twenty-first century due to drastic increase in temperature (Ceccarelli et al. 2010). Even it is predicted that all the cropping zone of rice could completely wipe out if the temperature continues to be this extreme (Aghamolki et al. 2014).

The ideal temperature for appropriate rice growth and development ranges between 27 °C to 32 °C (Yin et al. 1996). Further higher temperatures than the given range could have severe impact on all the stages of rice; from growth stage to maturation and then till harvesting. Heat tolerance ability of rice plant is very sensitive at different growth stages. It is highly temperature sensitive particularly during generation and blossoming which could lead to permanent damage and reduced yield (Porter 2005). Heat stress also widely influences both vegetative as well as reproductive stages of rice; like at vegetative stage, a prolonged exposure to high day temperature can damage leaf properties, while a short time period of warmth could cause premature birth of botanical buds and open blooms in the middle of conceptive stage (Guilioni et al. 1997). Blooming and booting stages of rice are found to be more sensitive to high temperatures *i.e.,* conceptive stage is more susceptible to temperatures than the vegetative stage (Ali et al. 2016; Shah et al. 2011; Peng et al. 2004).

Microbes mediated mitigation strategy is one of the alternate ways to alleviating the heat stress. Tiwari et al. (2017) reported that inoculation of *Bacillus amyloliquefaciens* in rice increased accumulation of osmotic protectants such as proline, soluble sugars, glycine betaine, trehalose under heat stress conditions which helps rice plant to overcome inert stresses by maintaining osmotic turgidity. Inoculation with endophytic fungus, *Paecilomyces formosus* LWL1 in rice grown under no stress and high heat stress conditions, improved growth attributes *viz.* plant fresh weight, height, chlorophyll content and dry weight. Additionally, it also effectively mitigated heat stress by minimizing the endogenous level of stress-indicating components such as jasmonic acid, abscisic acid and increasing total proteins content by 18.76%–33.22% (Waqas et al. 2015). Such beneficial microbes might be very useful at high environmental temperature stresses to maintain an effective and sustainable production of rice.

10.4.4 Salinity Stress

Globally 6% of the total land area has been affected by salinity (Mittler 2006; Cramer et al. 2011). Salinity affected area has been increased by almost 34 million ha of irrigated land (FAO 2009). Increased annual loss of crop production in irrigated lands is due to land degradation by salinity (Qadir et al. 2014). Saline soils have a number of soluble salts such as Ca^{2+}, Na^{+}, $Mg^{2+}$and anions SO_4^{2-}, HCO_3^{-}, Cl^{-} with large amounts of K^{+}, NO_3^{-}, CO_3^{-}. A soil can be referred as saline if it has an osmotic pressure of approximately 0.2 M Pa (~40 mM NaCl) or the EC 4 dS m^{-1} or more (USDA-ARS 2008). The pH of saline soils ranges between 7–8.5 (Mengel et al. 2001; Ghosh et al. 2016). Increased salt accumulation is very common in arid and semi-arid zones, where high evaporation and low precipitation occurs. Moreover, this process of salt deposition has been also favored by weathering of the parental rocks (Rengasamy 2002).

Rice is considered to be one of the most sensitive crops to salinity (Rahnama et al. 2010). Salt stress causes change in plants physiological processes by suppressing seed germination (Shannon and Grieve 1998). The damage caused by Cl^{-} initialization in rice can be figured by broad leaf cutting, indicates burning whereas Na^{+} accumulation can be characterized by rolling and molting of leaves (Acosta-motos et al. 2017). Salt stress reduces the rice leaf development, which leads to stomatal closure and in turn decreases the rate of photosynthesis (Rahnama et al. 2010). The major components that regulate salt accumulation are reduced salt uptake, improved Na^{+}/K^{+} proportion, antioxidant regulation system, tissue resistance, proficiency of water utilization to minimize the concentration of NaCl in plants (Ismail et al. 2007; Hashmi et al. 2017). During the whole life cycle of plants, several phytohormones play a crucial role as they regulate the key processes of response in plants under abiotic stresses, including plant responses to salinity stress.

Salinity stress responses involve the synthesis of ethylene, as stress hormone, which also regulates the plant growth and development (Hardoim et al. 2008). Biosynthetic pathway of ethylene involves the conversion of S-adenosyl-methionine by the enzyme ACS (1-aminocyclopropane-1-carboxylate synthase) into ACC (1-aminocyclopropane-1-carboxylate), which is the immediate precursor of ethylene to α-ketobutyrate and ammonia. However, in rice plants, under salt stress conditions, ethylene involves in endogenous regulation of plants stable equilibrium which results in reduced growth of root and shoot, which finally impacts on yield productivity.

In plants, ACC is degraded and sequestered by bacteria producing ACC deaminase in order to supply energy and nitrogen under salt stress (Glick 2005). Further, by eliminating ACC, the harmful effect of ethylene is reduced by the bacteria that improves plants stress tolerance and promotes growth by inhibiting salt-induced growth. Soil microbes belonging to genera *Bacillus*, *Alcaligenes*, *Rhodococcus* and *Variovorax* have ACC deaminase producing activity which is effective to confer salt stress in rice (Belimov et al. 2005). *Ochrobactrum* sp. was also previously reported to have ACC deaminase producing ability (Jia et al. 2013). It was reported that under salt stress, the rice seedlings showed improved plant biomass and salt tolerance capability by inoculation with class 2 endophyte Ascomycota (*Fusarium culmorum* FcRed1) (Redman et al. 2011). Rice root inoculated with *Pleosporalean ascomycete*, isolated from the roots of halophyte *Suaeda salsa* belongs to family Amaranthaceae, significantly increased the proline accumulation followed by increased photosynthetic pigment (chlorophyll and carotenoids) levels under salt stress condition (Jogawat et al. 2013; Kumar et al. 2012). The fungal isolate from roots of halophyte *Suaeda salsa* could endophytically colonize rice roots and improved plant health under salt stress (Qin et al. 2016). A report also showed that, inoculation of strain *Pseudomonas fluorescens* MSP-393 in rice under salt stress, favored root colonization, the potential strain also synthesizes complex osmolytes such as glycine, alanine, serine, glutamine, asparagine and threonine in their cytosol along with increased production of salt stress protein for effective nullification of the negative impact of high osmolarity (Paul and Nair 2008).

Three promising isolates with multiple plant growth promoting traits *viz.* *Bacillus*, *Alcaligenes* and *Ochrobactrum* sp. promoted rice growth at 150 mM NaCl under axenic conditions and showed increased root elongation assay (Bal et al. 2013). Inoculation of *Bacillus pumilus* in rice seedlings under salt stress showed a progressive potential for the limitation of Na^+ concentration in rice leaves that favored several antioxidant enzyme activities *viz.* superoxide dismutase, catalase, peroxidase that reversed the effect of salinity stress and enhanced plant growth (Khan et al. 2016). Furthermore, it was reported that inoculation of the strain *Enterobacter* sp. P23 in rice seedling showed potential traits of IAA production, siderophore production, phosphate solubilization, ACC and NH_3 production, which decreases stress-induced ethylene and promoted growth and development (Sarkar et al. 2018). Inoculation of two more promising strains of *Bacillus i.e., Oceanobacillus kapialis* (NCCP-76) and *Thalassobacillus devorans* (NCCP-58) in rice, improved root elongation and shoot length under NaCl stress (Shah et al. 2017).

10.4.5 Heavy Metal Stress

Metal industries, agrochemical industries mainly pesticides, sewage sludge and other various sources discharge metalloids and heavy metals, which causes a critical threat to the environment as well as human health (Kumar et al. 2017a). The concentration of the toxic metals in soil results in absorption by the roots which is then transported to different parts of the plant leading to diminished plant metabolism, impaired growth and reduced yield production in rice (John et al. 2012). In rice plants, some of the heavy metals play a major role in supplement of micronutrients (Prasad 2013); although presence of some heavy metals (Cd, Pb, Ni, Cu, Al, Zn) in small quantities have harmful impact on rice crop (Kovács et al. 2009; Lakho et al. 2017). Plants exposed to heavy metal stress have shown penetrability expansion in plasma layer, as metal ions bind to OH^-group of phospholipids and SH-group of proteins and further replaces Ca^{+2} at the initial cell growth level. Altogether these conditions lead to imbalance in ionic homeostasis of cell and disturb the integrity layer of the cell (Lakho et al. 2017).

Among all the heavy metals present in soils, cadmium (Cd) is considered as the toxic one, as it reduces root and shoot growth of the plant and directly hamper productivity by reducing essential nutrient uptake and disrupting homeostasis as well (Hashmi et al. 2017). Increased accumulation of cadmium in the soil causes impaired growth and development of root, nutrients disruption as well as low metabolism of carbohydrate which result in reduced yield and biomass (Akram et al. 2019). Among the metals, lead (Pb) is considered as one of the abundant metals on earth and its ingestion also results in severe health issues in humans. Even its minimal concentration in rice soil leads to yield loss by disturbing seed germination, rate of photosynthesis, nutrition uptake, plant-water balance, activity of enzyme as well as cells proliferation (Patra et al. 2004). Several reports focused on the activity of enzymes under heavy metal stress and it is observed that metal stresses (Cd, Pb, Ni, Cu, Al) altered enzymatic activities. During seed germination the presence of heavy metals such as Cd, Pb, Zn and Cu severely impacts on the ratio of root/shoot length as well as height of young seedlings (Mahmood et al. 2007). Moreover, increased concentration of heavy metals has a major impact on vegetative growth, seed germination and rice yield.

In such circumstances, PGPR plays an important role in removal of metal toxicity and improve plant nutrition and development (Table 10.1). Many previous reports on bacteria in soil play a major role in mobilization and immobilization of metals for metals tolerant (Glick et al. 1998). PGPR helps in reduction of metal toxicity by two ways: (i) decrease in plants ethylene stress level in metal toxic soil by ACC deaminase activity resulting in longer roots development that allows better plant establishment during initial growth stages (Glick 2005), (ii) release of siderophores, an iron chelating compound that causes the increased accumulation of iron in roots of the plant in the metal polluted conditions (Fig. 10.1). The rice variety 'Satabdi' inoculated with cadmium resistant *Ochrobactrum* sp. CdSP9, arsenic resistant *Bacillus* sp. AsSP9 and lead resistant *Bacillus* sp. PbSP9, increased percent germination, overall biomass, relative root elongation, protease and amylase activity. It was also observed that all the three bacterial strains were positive to catalase and ACC deaminase activity (Pandey et al. 2013).

Several plant growth-promoting rhizobacteria, *Pseudomonas* spp., *Bacillus* spp., *Azotobacter* spp., *Phosphobacteria* spp., *Azospirillum* spp., *Aspergillus niger, Penicillium* spp. and *Gluconacetobacter* spp., isolated from rice roots rhizosphere were investigated for their role in heavy metal stress mitigation by production of IAA and catalase as well as growth enhancement in rice under heavy metal stress (Samuel and Muthukkaruppan 2011). Potent plant growth-promoting rhizobacterial strain *Enterobacter aerogenes,* isolated from heavy metal contaminated rice rhizosphere found to be resistant to high degree of Pb^{2+}, Cd^{2+}, As^{3+} up to 3800 μg mL^{-1}, 4000 μg $mL^{-1}$and 1500 μg mL^{-1}, respectively. Upon screening of the strains, it was found that they had different plant growth-promoting rhizobacterial attributes like ACC deaminase activity, phosphate solubilization, IAA production and nitrogen fixation which helped in enhancement of rice growth and development (Pramanik et al. 2018). Han et al. (2015) reported that inoculation of rice plants exposed to heavy metal stress (*viz*. 0.3 mM Cu^{2+}, Zn^{2+}, Co^{2+} or Ni^{2+}) with strain of wild type *Pseudomonas stutzeri* A1501 resulted in increased plant biomass, root length, fresh and dry weight of root and plant height of rice plant. Seed inoculation of two basmati rice cultivars (B-385 and KSK-282) grown in different concentration of nickel contaminated soil (0, 100, 250, 500, and 1000 ppm), with *Bacillus licheniformis* NCCP-59 showed enhanced seed germination and biochemical traits which reverses the effect of nickel toxicity; such strains can be used for the phytoremediation of Ni contaminated soil (Jamil et al. 2014).

10.5 Biotic Stresses in Rice

The term biotic stress described as "interactions between living organisms and plants that leads to partial plant damage which can cost upon plants survivability". Plants are utilized as host by the parasitic organisms for their feeding, sheltering, multiplying and growing purpose; which ultimately leads to senescence of the plants. Plant pathogens obtain nutrients by feeding on host plant organs and causes physical damage to the plant (Kumar et al. 2016). Biotic stresses can hence be referred to as external biological factors affecting plants by damaging the cells, tissues, organs, organelles or even whole plant. Biotic stresses generally include pathogenic-organisms *viz*., bacteria, fungi, viruses or even nematodes as well as insects (Kranner et al. 2010). Every year the reason behind major crop losses is due to attack of these disease-causing pathogens (Karim 2007). This is an interaction between pathogen-host at molecular and biochemical levels that causes certain physiological and metabolic changes which further leads to morphological disorders and even death of the plant host (Karim 2007). The stress responsive mechanisms for pathogen suppression can be categorized as (i) antibiotic mediated suppression, (ii) siderophore mediated suppression, (iii) enzymes and phytohormones mediated suppression (Dreher and Callis 2007).

10.5.1 Antibiotic-Mediated Suppression

Pseudomonas fluorescens can produce several antibiotic compounds *viz*. phenazine, 2, 4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin etc. (Kumar et al. 2018a; Mageshwaran et al. 2012; Meera and Balabaskar 2012). Balasubramanian (1994) reported that leaf and neck blast of rice can be controlled by *P. fluorescens* through production of Phenazine-1-Carboxylic acid. By producing these compounds, *P. fluorescens* not only enhances its own growth but also play a major role in protection of crops from pathogens. It inhibited the growth of *Xanthomonas oryzae pv. oryzae,* the causative agent of bacterial leaf blight disease of rice thereby maintains soil health (Kumar and Mishra 2014; Vasudevan et al. 2002; Velusamy et al. 2006). It was also reported that strain *P. aeruginosa* PUPa3 successfully suppressed the disease caused by *Sarocladium oryzae* and *Rhizoctonia solani* by producing Phenazine-1- Carboxamide antibiotics in rice (Megha et al. 2007). The causative agent of rice sheath blight, *S. oryzae* was found highly susceptible to the antibiotics produced by *P. fluorescence* (Nathan et al. 2011). *P. fluorescens* isolated from the rice rhizosphere showed effective antifungal activity and suppressing mycelial growth by 62–85% against *Rhizoctonia solani*, *Sarocladium oryzae*, *Magnaporthe grisea* and *Drechslera oryzae* (Reddy et al. 2007).

Streptomyces vinaceusdrappus is reported to inhibit the growth of rice blast disease causing agent, *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*), by inhibiting mycelial growth up to 88.73% (Law et al. 2017). Besides these, *Streptomyces* are well known producers of prolific and bioactive antibiotic compounds. Blasticidin-S and Kasugamycin isolated from *Streptomyces griseochromogenes* and *Streptomyces kasugaensis*, respectively; are often used as active fungicides for controlling rice blast (Fukunaga et al. 1955; Tapadar and Jha 2013; Copping and Duke 2007). *Streptomyces* sp. PM5 isolated from rice rhizosphere having the ability to produce two aliphatic compounds SPM5C-1 and SPM5C-2 with a ketone and lactone carbonyl unit, which was effective against rice disease causing pathogen *R. solani* and *P. oryzae* as they showed active antifungal activity and suppressed the growth of these pathogens at concentrations of 25, 50, 75 and 100 μg mL^{-1} (Prabavathy et al. 2006). Omura et al. (1984) found that, 20 membered macrolides produced by *Streptomyces flavus* subsp. *irumaensis* showed potent activity against *P. oryzae*, however, an antifungal metabolite dapiramycin, obtained from *Micromonospora* sp. found to be effective against *R. solani* (Nishizawa et al. 1984). Three isolates namely *Enterobacter agglomerans*, *Xanthomonas luminescens* and *Serratia liquefaciens* were isolated from rice rhizosphere grown in Bali, effectively inhibited the growth of *P. oryzae* cv. that causes rice blast (Suprapta 2012) (Table 10.2).

10.5.2 Siderophore-Mediated Suppression

Siderophores are extracellular iron binding compounds having low molecular weights and higher ferric iron affinity, produced by microbes for the uptake of iron from the environment (Saha et al. 2016). This iron sequestration ability of microorganisms offers them a competitive advantage over pathogens. Siderophores serve as vehicle for transportation of ferric ions by chelating the ions into the microbial cell with a high specific activity (Neilands 1981). The ferric siderophore complex formed is particularly recognized by a membrane receptor that mediates the transportation of iron into the cell (Mercado-Blanco and Bakker 2007). In various oxido-reductive enzymatic reactions, iron acts as a co-factor and a crucial element in binding with siderophore. Thus, binding of iron with siderophores creates an

Table 10.2 Microbial biocontrol agents for disease suppression and growth promotion of rice

Microbial biocontrol agents	Pathogens	Diseases	References
Pseudomonas fluorescens	*Cnaphalocrocis medinalis*	Brown spot	Saravanakumar et al. (2007)
P. fluorescens, Trichoderma spp.	*Pyricularia oryzae*	Blast	Singh (2014)
Trichoderma harzianum, T. viride, T. virens, T. deliquescens	*Neovossia indica*	Blunt	Singh (2014)
Pseudomonas fluorescens, P. putida, T. harzianum, T. viride, T. virens, Aspergillus niger	*Rhizoctonia solani*	Sheath blight	Kumar and Mishra (2014) and Singh (2014)
T. viride	*Drechslera oryzae*	Brown spot	Singh (2014)
Bacillus spp.	*Xanthomonas oryzae*	Bacterial leaf blight	Singh (2014)
T. viride (Tv2), *T. harzianum* (Th5), *T. reesei* (Tr3)	*Cochliobolus miyabeanus*	Brown spot	Harish et al. (2008)
Streptomyces sp. PM5	*P. oryzae*, *Rhizoctonia solani*	Blast & sheath blight	Prabavathy et al. (2006)
P. fluorescens	*Magnaporthe grisea*	Blast	Reddy et al. (2007)
	Drechslera oryzae	Brown leaf spot	
	Rhizoctonia solani	Sheath blight	
	Sarocladium oryzae	Sheath rot	
Streptomyces vinaceusdrappus	*Magnaporthe oryzae*	Blast	Law et al. (2017)
P. fluorescens	*Magnaporthe oryzae*	Leaf blast	De Vleesschauwer et al. (2008)
Bacillus amyloliquefaciens RWL-1	*Fusarium spp.*	Root rot	Shahzad et al. (2016)
P. fluorescens (S3), *P. tolaasii* (S20), *P. veronii* (S21), *Sphingomonas trueperi*	*Achlya klebsiana, Pythium spinosum*	Seedling disease	Adhikari et al. (2001)

artificial deficiency of iron in the soil, which results in disease suppression through iron competition with the pathogen of rice (Bakker et al. 2007; Duiff et al. 1997). Siderophore production by *P. fluorescens* was initially reported by Kloepper and Schroth (1981) and its plant pathogenic suppression was reported by Becker and Cook (1988). *Fusarium oxysporum*, causative agent of wilt diseases in rice was effectively controlled by *P. fluorescens* through iron competition (Shahzad et al. 2016). Root application of *P. fluorescens* WCS374r in rice successfully controlled *M. oryzae,* a causative agent of leaf blast in rice, through triggering the ISR, siderophore and pseudobactin production, which accelerated the complex defense system. Thus, by generating multiple blast-effective pathways *P. fluorescens* successfully induced resistance (De Vleesschauwer et al. 2008).

10.5.3 Enzymes and Phytohormones-Mediated Suppression

Several defense enzymes *viz.*, cellulase, chitinase, β-1,3 glucanase also play a key role in pathogenic (fungal pathogens) disease suppression in rice by means of cell wall degradation through breakdown of glycosidic bonds, chitin and β −1,3 glucan (Chet et al. 1990; Lorito et al. 1996; Schroth and Hancock 1981). Microbes involved in excretion of chitinase are categorized as effective biocontrol agents (Inbar and Chet 1991; Ordentlich et al. 1988). Chitinase produced by *P. fluorescens* suppressed the phytopathogenic fungi by breaking and fragmenting cell wall of fungus (Narayanan et al. 2009). *P. oryzae* causing blast disease was inhibited by *P. fluorescens* (AUPF25) through production of proteases and phytohormones such as IAA and siderophore, which inhibited mycelial growth (Shyamala and Sivakumaar 2012; Antoun and Prévost 2005). The endophytic bacterial strain, *Bacillus amyloliquefaciens* RWL-1 isolated from rice seed suppressed the pathogenic effect of *Fusarium* spp. by producing phytohormones such as gibberellic acids GA4, GA12, and GA20. Two pathogenic rice seedling diseases caused by *Pythium spinosum* and *Achlya klebsiana* was inhibited by *P. tolaasii* (S20), *P. fluorescens* (S3), *Sphingomonas trueperi* (S12) and *P. veronii* (S21). However, other biocontrol agents such as *Trichoderma virens*, *B. subtilis* and *P. fluorescens*, respectively showed 80%, 63% and 93% reduction of the pathogenic fungi *Aspergillus flavus* (Reddy et al. 2009). Thus, several beneficial microbes along with active plant growth promoting traits in rice also give an immense contribution in the field of biocontrol through modulation of enzymes and endogenous hormones.

10.6 Conclusion

Seven decades ago, there was a drastic increase in global agricultural production which was possible because of the green revolution era that saved billions of people from undernourishment and starvation. This triggered the introduction of chemical fertilizers and pesticides by human that marked the dawn of environmental damage. This injury further extended to the dome of abiotic and biotic stresses that added to environmental disturbances. These stresses are of a major threat and concern to rice productivity. The present chapter concludes a positive trend that could be set by the use of plant growth-promotion microorganisms in terms of conferring abiotic stresses to alleviate different stress effect on rice. Additionally, several researchers strongly advocated the use of bio-control agents to manage insect and diseases in rice without affecting the soil health. Moreover, their use in sustainable production for rice exists but more efforts are required to explore and spread awareness of these eco-friendly, non-harmful and omnipotent use of microbes. Thus, the use of these beneficial stress mitigating microbes will become safeguard for the stability and productivity of agro-ecosystem, which could uplift the global agricultural sustainability and lead us towards to become one of the ideal agricultural producing nations.

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Chapter 11
Nutritional Biofortification of Crops by Microbes

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Abstract Malnutrition is a major challenge for food security. In malnutrition, an individual does not get enough nutrients from food intake and thus is unable to perform normal functions efficiently, eventually leading to serious health issues in humans, particularly in children, adolescent and lactating women. This hidden hunger involves micronutrient deficiencies, particularly iron, zinc, iodine and vitamin A, and can occur without a deficit in energy intake as a result of consuming an energy-dense, yet nutrient-poor diet. More than two billion people suffer from micronutrient deficiency. Here we review the use of microorganisms for the nutritional biofortification of crops by marker-assisted breeding, back-crossing, transgenic breeding, genome editing, and microbes-mediated nutrient fortification. Microbes-mediated fortification is one of the fastest and easiest way for increasing nutrients into food crops and food products. We exemplify microbe-mediated bioaccumulation of micronutrients such as metal ions, vitamins, and fatty acids, in the plant biomass. We also discuss the role of arbuscular mycorrhiza alone or in combination with biofertilizer agents for increasing the bioavailability of minerals into food.

Keywords Siderophores · Biofortification · Deficiency · Micronutrients · Arbuscular mycorrhiza · Carotenoids · Lysine · Harvest plus · *Spirulina*

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11.1 Introduction

The ever increasing human population is mainly dependent on the staple crops for their quota of daily nutrients (Díaz-Gómez et al. 2017; Gómez-Galera et al. 2010; Gregorio 2002; Meena et al. 2018; Zikankuba et al. 2019). Although, multiple crops are produced extensively due to the Borlaug-Swaminathan green revolution (Borlaug et al. 1969; Swaminathan 2010; Davis et al. 2019; Stone 2019; Taylor 2019), they are deficient in nutrients which eventually may lead to serious, chronic health issues in humans (Chen et al. 2018b; Hambidge 2000; Mann and Truswell 2017; Saedisomeolia and Ashoori 2018; Spedding 2019). This is also supported by a report of the United Nations Children's Fund which indicates that 39% of adults are overweight, 20 million babies are underweight and every third reproductive women are anemic across the globe (https://www.unicef.org/). The World Health Organization also accounted that globally more than 2 billion people suffer from micronutrient deficiency (WHO 2015).

One way to tackle this nutrition deprivation is biofortification, a process of enhancing the fatty acids, amino acids, vitamins, minerals, and secondary metabolites content in crops using conventional breeding or genetic engineering includes transgenic breeding and genome editing (Champagne et al. 2013; Connorton and Balk 2019; Khush et al. 2012; Mayer et al. 2008; Mehta et al. 2020; Mene-Saffrane and Pellaud 2017; Nestel et al. 2006; Poletti and Sautter 2005; White and Broadley 2005; Zunjare et al. 2018). Earlier, minerals and vitamins supplemented tablets, powders, drinks, and energy bars were provided to the people (Chen et al. 2006; Kattumuri 2011; Ojaghi et al. 2016; Sinha et al. 1993; Stella et al. 1997). However, the scheme failed due to the steep decline in productivity, non-availability of materials, interested organizations and distributors, unaffordable prices, and lack of awareness of its non-achievable targets (Garg 2018).

Few organizations are working relentlessly for "nutrient biofortification of crops". One such organization is 'Harvest plus' which a part of the International Food Policy Research Institute, and the Consultative Group on International Agricultural Research Program that provides fortified food with the help of conventional breeding and fertilizers (HarvestPlus 2020). This organization has a vision of making the world free of hidden hunger and bridging the gap between nutrition and agriculture. It focuses primarily on micronutrients like Zn, Fe, and vitamins. They have released several crop varieties like rice, wheat, and maize with 40%, 50%, and 70% increased zinc content respectively. Additionally, they have also distributed sweet potato, cassava which is enriched invitamin A content, pearl millet with 80% zinc and maize with 70% increased zinc content.

For the last two decades, the use of plant growth-promoting microbes regarding fortification has come into consideration tremendously (Beneduzi et al. 2012; Gutiérrez-Mañero et al. 2001; Lata and Gond 2019; Lugtenberg and Kamilova 2009; Tyagi et al. 2018). Plant growth-promoting microbes can be distinguished into two types which are plant growth-promoting rhizobacteria and plant growth-promoting fungi (Jahagirdar et al. 2019; Lata and Gond 2019; Mehmood et al. 2018). The former class of plant growth promoting rhizobacteria includes multiple bacteria like *Azospirillum, Azotobactor, Bacillus, Pseudomonas,* and *Rhizobium sp.* which resides in the rhizosphere region and help in the acquisition of nutrients such as N, P, Zn, Fe, and Se (Ali and Vidhale 2013; Anitha et al. 2016; Aras et al. 2018; Lata and Gond 2019; Mehmood et al. 2018; Yasin et al. 2015). Consortia of different bacterial strains also increase the uptake of nutrients (Kumar et al. 2016a, b, 2017a; Rana et al. 2015; Verma and Yadav 2018; Singh et al. 2019; Xia et al. 2020).

Additionally, siderophores are low molecular weight compounds excreted from plant growth-promoting microbes and show high affinity towards Fe^{3+} ions and result in the transfer of iron in plants (Aguado-Santacruz et al. 2012; Crowley et al. 1988; Jin et al. 2006; Johnstone and Nolan 2015; Kumar et al. 2018; Leventhal et al. 2019; Radzki et al. 2013; Sah et al. 2017). Furthermore, these plant growth-promoting microbes help in the phytoremediation of waste and toxic compounds (Dotaniya et al. 2018; Ma et al. 2019; Sapre et al. 2019). With the help of a genetic engineering approach, scientists have exploited the bacterial genes of many plant growth-promoting rhizobacteria to improve the vitamins, oils, flavonoids, and anthocyanin content in many crops (Fujisawa et al. 2008; Kumar et al. 2019; McClements 2019; Rahman et al. 2019). As a result, this chapter emphasizes the microbe-assisted biofortification of micronutrients like iron, zinc, selenium, fatty acids, carotenoids, and amino acids in multiple plant varieties (Fig. 11.1).

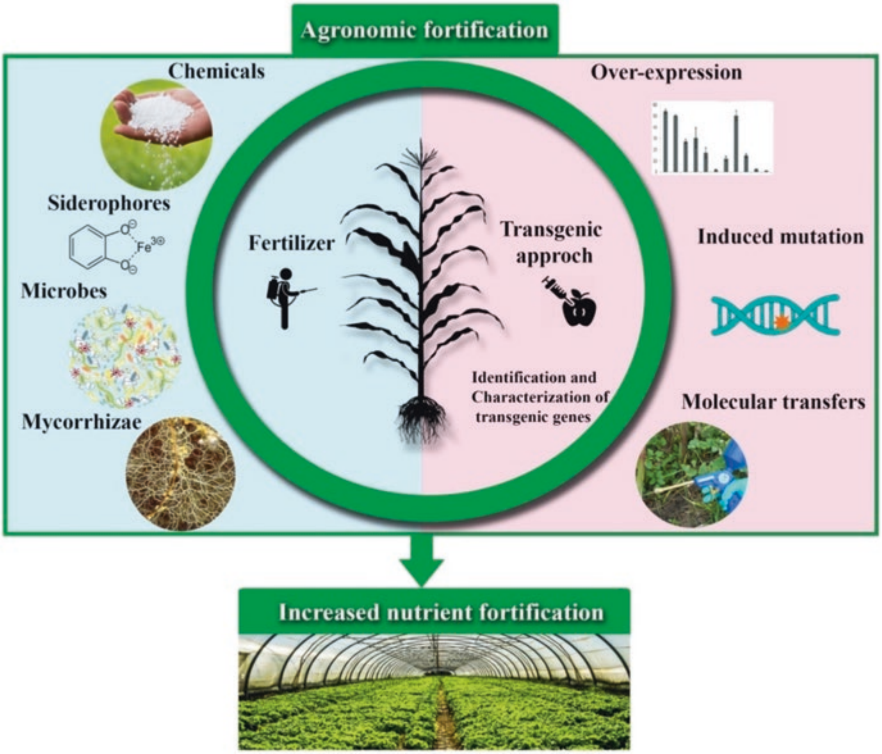

Fig. 11.1 Approaches for nutrient fortification of plants

11.2 Biofortification of Crops with Micronutrients

11.2.1 Iron

Iron (Fe) is an important micronutrient in the human diet (Briat et al. 2015; Finkelstein et al. 2017). It is a central part of the biological molecules like hemoglobin and cytochrome and its insufficiency leads primarily to anemia (Gangania et al. 2017; Lopez et al. 2016). Furthermore, iron deficiency causes loss in the ability to maintain and regulate body temperature during cold weather, impairment of the nervous system, weight loss, increased maternal mortality rate, and loss of muscle function (Lopez et al. 2016; UNICEF/WHO 1999). According to the World Health Organization, 43% of total pre-school children age less than 5 years, 29% of non-pregnant women, and 38% of pregnant women suffer from anemia (WHO 2015).

Various plants belonging to different angiosperm families adopt two types of strategies to uptake Fe from the soil (Banakar et al. 2017; Garnica et al. 2018; Suzuki et al. 2016; Zanin et al. 2019). Monocotyledons like rice, wheat, and other grasses use a chelating strategy where the plants produce small molecular weight compounds known as mugineic acid belonging to the family phytosiderophores (Banakar et al. 2017; Garnica et al. 2018; Suzuki et al. 2016). Phytosiderophores have a high affinity towards Fe^{3+} and form complex followed by transport into plants through both apoplast and symplast pathways (Banakar et al. 2017; Duck and Connor 2016; Garnica et al. 2018; Suzuki et al. 2016). The second strategy is a reduction based strategy in which the plants release protons (H^+) into the rhizosphere and ultimately decrease the pH of soil and increases the solubility of Fe^{3+} ions, which is further readily transferred into the plants through ATPase transporters (Gao and Chao 2016; Kim and Guerinot 2007; Zhang et al. 2019).

Plants interact with different types of microbes that aid in the transfer of nutrients (especially Fe^{3+}) to plants and protect plants from pathogens (Freitas et al. 2015; Khalid et al. 2015; Maheshwari et al. 2019; Mehmood et al. 2018; Prasad et al. 2019). Siderophores are low molecular weight organic compounds released by microorganisms (*Azotobacter, Azosprillum, Bacillus, Rhizobium, Pseudomonas,* and *Trichoderma) i*n an extracellular medium and chelate Fe^{3+} ions (Ali and Vidhale 2013; Kumar et al. 2018; Leventhal et al. 2019; Sah et al. 2017). Besides this, siderophores also improves soil fertility and increases the concentration of nutrients (Ali and Vidhale 2013; Khan et al. 2019; Kumar et al. 2018; Leventhal et al. 2019; Sah et al. 2017).

Trichoderma, a fungal biocontrol agent that resides in the plant vicinity, primarily aids in plant growth and total yield (Ghazanfar et al. 2018; Herrera-Téllez et al. 2019; Kottb et al. 2015; Nawrocka et al. 2017, 2018; Srivastava et al. 2013). It has been observed that *Trichoderma* strain MPPLUNS1 produced higher number of siderophores (*in vitro*) and also helped in chelating the ferric ions (Srivastava et al. 2013; Akter et al. 2019; Angel et al. 2016; Li et al. 2015; Mukherjee et al. 2018).

Similarly, the bacteria also secrete a variety of siderophores which acts as fertilizers and their direct application to the plants showed greater Fe^{3+} acquisition (Akter et al. 2019; Dimkpa et al. 2009; Jin et al. 2006; Sah et al. 2017). Wheat seeds treated with combination of bacteria *Bacillus sp. (strain* AW1*)* and *Providencia sp*. (strain AW5) resulted in enhancement of 14–34% in plant biometric parameters and 28–60% in micronutrient content, as compared to full dose of fertilizer application together with 143.6% higher level of Fe and 63.7% higher level of than the control plants (Rana et al. 2012a). The direct application of *Rhizobium* sp. also elevated the Fe content up to 45.2% in the rice leaves (Adak et al. 2016).

The potential of plant growth-promoting actinobacteria in increasing seed mineral density of chickpea (*Cicer aretinum* L.) has also been investigated under field conditions (Sathya et al. 2016). They independently inoculated the chickpea seeds with 19 different *Actinobacteria* strains for about 50 minutes and then sown the seeds. They also treated chickpea with the same *Actinobacteria strains* for every 15 days until flowering. All the 19 strains of *Actinobacteria*-inoculated seeds showed 10–38% higher Fe content than the control plants. Additionally, 17 isolates treated plant seeds showed higher level of Zn, 16 for Ca, 9 for Cu, and 10 for Mn and Mg which respectively showed higher mineral content in the range of 13–30%, 14–26%, 11–54%, 18–35% and 14–21% over control plants. Further, two bacterial strains *Pantoea dispersa* MPJ9 and *Pseudomonas putida* MPJ6 positively impacted greater Fe^{3+} chelation activity in mungbean which leads to 3.4-fold increase in Fe accumulation activity (Patel et al. 2018). The siderophore activity of MPJ9 and MPJ6 was found to be 88.7% and 82.3%, respectively. Moreover, the seed germination capacity was reported to be 93.3% (in MPJ9) and 90% (in MPJ6). Additionally, there was an increase in shoot and root girth, seed vigor, and fruit weight.

11.2.2 Selenium

Selenium (Se) is a vital nutrient in the human diet and is present in a variety of states on earth such as selenium, selenide and selenite (Dhakate et al. 2019; Jablonska and Vinceti 2015; Li et al. 2017; Schomburg 2017; Vinceti et al. 2016). In humans, 25 different selenoproteins have selenocysteine amino acid residues and show antioxidant activity, hence play a significant role in the human central nervous system, cardiovascular, hypothyroidism, mental retardation, immunity, and prevent colorectal and liver cancer and keshan disease (Avery and Hoffmann 2018; Carlson et al. 2012; Khan et al. 2017; Roman et al. 2014; Schoenmakers et al. 2016; Schomburg et al. 2019; Schweizer and Fradejas-Villar 2016).

Food and Nutrition Board and National Academy of Medicine has recommended the intake of selenium for males, females, and pregnant women respectively as 25, 55, and 60 micrograms (https://www.nap.edu/). Due to its importance in the diet, people are exploiting many strategies to overcome the Se deficiency (Jablonska and Vinceti 2015; Li et al. 2017; Lidon et al. 2019; Schomburg 2017; Vinceti et al. 2016). In the field, farmers are spraying Se upgraded fertilizers which ultimately translate into the higher content of selenium in the crops (de Abreu et al. 2019; Ekanayake et al. 2015; Hu et al. 2018; Lidon et al. 2019; Ngigi et al. 2019; Ros et al.

2016). One of the basic worldwide high nutritive value staple cereal crops is wheat which is illustrated by the fact that 2.5 billion human population are dependent directly on wheat. It has a high content of carbohydrate, gluten protein, and is rich in fiber (Shewry and Hey 2015).

It has been estimated that only 19% of calories and 21% of protein are met solely by wheat. With this concern, researchers are trying to enhance selenium content in wheat. Yasin et al. (2015) tested the effect of Se-tolerant bacterial strains of *Bacillus (*YAM6 and YAM7) on *Triticum aestivum*. Plant samples treated with bacterial strains after the reproductive stage accumulated good quantity of biomass as compared to the untreated plants and showed an increase of 180–375% in Se concentration. This was also followed by the 8–104% of iron content in the stem region. Additionally, in the wheat kernel, the concentration of Se and Fe was enhanced to 134–154% and 172–240% for *Bacillus* strains YAM6 and YAM7, respectively. This inoculation also resulted in enhanced selenium content in fruits than in the shoots (Yasin et al. 2015). Other nutrients like calcium and sulfur status were also found to increase in stem, fruit, and kernel by at least 25%. Similarly, wheat inoculated with two bacterial strains *Pseudomonas* sp. R8 and *Stenotrophomonas* B19 also showed a remarkably higher Se content in both roots and leaves as compared to the uninoculated plants (Acuña et al. 2013).

Use of bacterial consortium has also resulted in increased accumulation of minerals in crop plants. *Brassica juncea* plants treated with G1 consortium consisting of *Bacillus thuringiensis, B. licheniformis, B. endophyticus, Cellulosimicrobium cellulans*, and *Exiguobacterium* sp. showed respectively 11% and 15% higher Se concentration in leaf tissue and pods as compared to the control plants. The Se accumulation was found to be 711 mg kg^{-1} in leaf dry weight, 358 mg kg^{-1} in seeds, and 276 mg kg^{-1} in pod husks. However, consortium (G2) treatment consisting of *B. licheniformis, B. cereus, and Bacillus pumilus* showed no significant increase as compared to the control plants (Yasin et al. 2015). Similarly, the endophytic selenobacteria strain *Acinetobacter inoculation* increased the Se content in wheat at the tillering and mature stage (Durán et al. 2018).

11.2.3 Zinc

Zinc (Zn), the most abundant element after Iron, is a basic micronutrient and is present in normal oxidation state of Zn^{2+} (Broadley et al. 2007; Cakmak and Kutman 2018; Yasuda and Tsutsui 2016). Zn has very important and ubiquitous biological role in signal transduction, apoptosis, gene expression, cell growth, differentiation, and metabolism (Cakmak and Kutman 2018; Prakash et al. 2015; Prasad 1991; Yasuda and Tsutsui 2016). The deficiency symptoms include the impaired immune system, short stature, hypogonadism, and renal disease (Grüngreiff et al. 2016; Maret 2013; Ohashi et al. 2019; Prasad 2008; Sapkota and Knoell 2018). As per World Health Organization estimates (WHO 2015) 17.3% of the total world population is at risk of zinc deficiency.

Spirulina is a cyanobacterium that plays a beneficial role in the Zn biofortification in many crops consumed by humans and animals (Ali and Saleh 2012; Baghestani et al. 2013; Zlateva et al. 2019). Treatment of *Spirulina* aids in agriculture by increasing the nutrient content as well as yield or biomass which ultimately prevents malnutrition and is important in nucleic acid metabolism (Anitha et al. 2016; Tuhy et al. 2015; Zlateva et al. 2019). Foliar spraying of the different concentrations of *Spirulina* filtrate (5%, 7%, 10%, 15%, 20%, and 25%) were done on radish plants, and the micronutrient content in plants were evaluated. It was observed that Zn concentration increased by 5.5 fold at 20% filtrate in radish (Godlewska et al. 2019). Tuhy et al. (2015) performed a field-scale experiment on maize to check the effect of *Spirulina platensis* inoculation on Zn content. Inductively coupled plasma-optical emission spectrometry estimated the Zn content as 2126 mg kg^{-1} in a bioformulation of *Spirulina*. Additionally, the content of other micronutrients like manganese and copper was also significantly increased and the recorded values were 1118 and 3991 mg kg^{-1}, respectively.

Besides, the European space agency also created a dietary product made up of *Spirulina gnocchis* for long space flights. This dietary product made up of *Spirulina* was a good source of micronutrients. Anitha et al. (2016) conducted field trials for evaluating the effect of *Spirulina* on three plant species viz., *Amaranthus gangeticus*, *Phaseolus aureus,* and tomato. In the *Amaranthus gangeticus,* Zn concentration was found to increase significantly i.e., 77.23 ± 0.02 ppm when treated with *Spirulina* and fertilizers in a ratio of 75:25 as compared to the control. In *Phaseolus aureus*, the Zn content was 54.4 ± 1.69 ppm when treated with *Spirulina* and organic manure in 50:50 ratios and in tomato, Zn concentration was observed as 5.28 ± 0.09 ppm as compared to control.

Wheat seeds inoculated with *Providencia sp*. (PW5 strain) showed an increase in Zn accumulation upto ~42 mg kg^{-1} (Rana et al. 2012b). In rice, the Zn concentration also increased by 14% when treated with bacterial consortia consisting of *Providencia* sp. PR3, *Brevundimonas diminuta* PR7, and *Ochrobactrum anthropi* PR10 (Rana et al. 2015). Okra was also found to have increased Zn concentration status by 60–70% when incubated with *Azotobacter* and *Calothrix* together as compared to the untreated plants. Besides, the Zn concentration was also observed higher as compared to control plants at harvest time. Other micronutrients such as Fe were also found to be hiked by 7–8 folds as compared to the uninoculated plants (Manjunath et al. 2016). Similar studies were also done on different rice varieties with single bacteria as well as bacterial consortia. High Zn translocation index was observed in rice treated with bacterial consortia as compared to the single bacteria and control plants (Shakeel et al. 2015).

11.2.4 Vitamins

Vitamins are required in small quantities by humans but are not synthesized by themselves, therefore, their need is fulfilled only from external sources. If not taken in the required amount, vitamin deficiency may lead to multiple diseases like night blindness, anemia, beriberi, scurvy etc. (Basu and Dickerson 1996; Combs Jr and McClung 2016). The occurrence of vitamin A deficiency is more in countries of Africa and South Asia ranging from 5–20% in women and 60–70% among children (https://www.who.int). One of the most engineered and popular vitamins is carotenoid. Carotenoids are defined as basic pigments composed of two molecules geranylgeranyl diphosphate which further gives rise to phytoene, lycopene, and beta-carotene. This class of molecules has a positive effect on reactive oxygen species and cancer prevention (Amengual 2019; Eggersdorfer and Wyss 2018; Johnson 2002; Rao and Rao 2007).

The power of genetic engineering involves the transfer of useful genes between unrelated organisms which has been exploited for enhancing the carotenoid content in many crops (Aluru et al. 2008; D'Ambrosio et al. 2018; Kang et al. 2017; Sankari et al. 2018). The most prominent example is Golden rice, a genetically modified crop that was produced to enhance the Vitamin A content by adding a phytoene desaturase gene (*crt*I) from the soil bacteria *Erwinia uredovora* under the control of *CaMV 35S* promoter and *Psy* (phytoene synthase) gene from daffodil to the genome of rice crop (Burkhardt et al. 1997). The field trials of golden rice cultivars were conducted in the USA, Philippines, Taiwan and Bangladesh. Successful transformation and cultivation showed that it produces 4–5 times more beta-carotene than the control plants. In the year 2005, a USA based company (Syngenta) produced another version of golden rice variety namely, Golden Rice 2, by introducing phytoene synthase gene from maize and phytoene desaturase gene (*crt*I) from *E. uredovora* and reported an increased level of β-carotene up to 23-folds when compared with the golden rice 1 (Stein et al. 2006).

Transgenic tomato plants were produced by introducing the *crtI* gene from *E. uredovora* under the control of constitutive *CaMV 35S* promoter through the *Agrobacterium* transformation technique (Römer et al. 2000). The results showed that β-carotene and lutein increased two-fold. In another instance, the tomato plant was again transformed with fruit-specific tomato polygalacturonase promoter along with the *crtI* gene and making the lycopene, carotene, and lutein levels increased by two-fold (Fraser et al. 2002). Canola seeds were also transformed by over-expressing the bacterial gene (*crtB*) and resulted in a 50-fold increase in total carotenoids, with phytoene content raised to 11–29%. Moreover, three bacterial genes *crtB, crtI, and crtY* were expressed with 50% increase in the β-carotene (Ravanello et al. 2003). Wheat Cv. Bobwhite transformed with *E. coli* phytoene synthase genes *crtB and crtI* resulted in darker red/yellow endosperm than the control endosperm. The level of provitamin-A was found to be increased even upto T3 transgenic lines ranging from 0.18 to 3.86 μg g^{-1} of seed dry weight, and the β-carotene level was 52.7–56 fold high in transgenic lines (Wang et al. 2014).

Linum usitatissimum L. is mainly used in the food and oil industries and is primarily famous for linseed oil due to the high omega-3-fatty acids enrichment. Using the *Agrobacterium* transformation technique, flax seeds were genetically modified using the bacterial phytoene synthase gene (*crtB*) under the control of the *CaMV35S* promoter. The seven T1 transgenic lines were assayed for total carotenoid content and accumulation of phytoene, α-carotene, and β-carotene. As a result of transformation, higher α-carotene, β-carotene, phytoene, and lutein were detected in T1 seeds as compared to the control untransformed seeds. The amount of lutein, α-carotene, β-carotene, and phytoene were respectively observed as 12.2–24.0, 12.5–26.8, 30.9–85.2, and 12.1–28.0 μg/g fresh weight (Fujisawa et al. 2008).

Further, transformation of maize embryos by expressing two bacterial genes *crtB* and *crtI* under the control of the maize ubiquitin '*ZmUbq*' promoter using the biolistic transformation technique was done by Aluru et al. (2008) for vitamin biofortification of maize. The T1 maize endosperm showed significantly increased levels of provitamin-A content. Moreover, T1 seeds were self-pollinated and the mean carotenoid contents in T2 seeds were higher than the T1 seeds and β-carotene content increased to ten-fold in the T2 transgenic maize lines. Strawberry is a very popular source of calories, folate, iodine, anthocyanin, Fe, Mn, vitamin-A, and vitamin-C worldwide. In most populous country, China, strawberry production is 40% over the other countries due to the increased demand and consumption. It contributes to preventing diseases and detoxifies reactive oxygen species. Strawberries inoculated with *B. amyloliquefaciens and P. fungorum* also showed enhanced content of anthocyanins, phenolics, and antioxidant in fruit (Rahman et al. 2019).

11.2.5 Amino Acids

Amino acids are organic compounds that build the proteins and contain an amine as well as carboxylic groups in their side chain that makes the amino acids important supplements in the human diet. Out of 22 amino acids, nine amino acids are considered essential as they are not synthesized by the human body and make humans dependent solely on dietary products. The deficiency may lead to digestive problems, slow growth, and infertility. Using agronomic and genetic manipulation techniques, scientists have tried to reduce the deficiency of amino acids (Ohnoutkova et al. 2012; Yang et al. 2016; Zhou et al. 2009). In rice, the AAT gene is responsible for the biosynthesis of amino acids. The same AAT gene was isolated from the bacterium *E.coli* and over-expressed in j*aponica* rice (Cv. Zhonghua-11) under the *CaMV 35S* promoter control. The amino acid content was estimated in both T1 and T2 line seeds. The total amino acid content was 166.80, 153.80, and 171.56 mg g^{-1} in T1 lines of *OsAAT1-OX*, *OsAAT2*-OX, and *EcAAT*-OX, respectively. In T2 seeds of *OsAAT1*-OX and *OsAAT2*-OX, various amino acid content hiked from 10.3 to 39.1% as compared to the control plants (Zhou et al. 2009).

Lysine content was also reported to be higher after the expression of bacterial genes *AK, DHPS*, and *LKR/SDH* in *japonica* rice (Cv. Wuxiangjing 9). Two transgenic lines namely GR-14 and GR-65 were found to be higher in lysine content (9.6–10.1 folds) as compared to the control (Yang et al. 2016). Likewise, the *dapA* gene encoding for dihydrodipicolinate synthase was cloned from the bacterium *E.coli* and expressed in the different barley cultivars. The total lysine, methionine, and threonine levels in the T1 leaf samples were observed to be significantly higher as compared to the control plants (Ohnoutkova et al. 2012). In another instance Falco et al. (1995) expressed the *Corynebacterium DHDPS* gene in canola which resulted in 100 fold higher accumulation of lysine in canola seeds. Soybean transgenic lines expressed with the stacked cassette having *Corynebacterium DHDPS* gene along with *E. coli AKIII-M4* gene showed a higher level of lysine in seeds as compared to the *corynebacterium DHDPS* gene alone.

11.2.6 Fatty Acids

A fatty acid is a micronutrient composed of a carboxylate head group attached to the hydrophilic tail which is either saturated or unsaturated based on their bonds. Fatty acids are important dietary sources as well as stored energy sources (Gunstone 2011). When glucose gets depleted in our body, the breakdown of the fatty acids acts as energy fuels to the cells. These fatty acids are essentially not made in enough quantity, so the intake quantity is fulfilled from an outer source. Linoleic acid is important in our diet as it involves maintain blood pressure, inflammation, and omega-3 fatty acid in protecting against fatal heart disease. In order to improve oleic acid (C18:1) and linolenic acid (C18:3) content in *Brassica napus,* inoculation with two bacterial isolates namely *Azospirillum brasilense and Azotobacter vinelandii were done on a field-scale plot* (Nosheen et al. 2011). The fatty acid content revealed that oleic acid was about 7% higher compared to control when treated with *A. vinelandii* whereas the linolenic acid content was higher when inoculated with *A. brasilense*.

Higher oil content was found in sunflowers when treated with *Azotobactor* in the presence of N (Soleimanzadeh et al. 2010). *Glycine max* is also an important source of vegetable oil as it contributes upto 25% of the world's edible oils. Transgenic soybean plants have been prepared by over-expressing the seed-specific bacterial phytoene synthase gene *(crtB*) from *Pantoea ananati* (Schmidt et al. 2015). They observed 45% increase in oleic acid content as compared to the control plants. Sunflower crop is an important and the third most widely grown oilseed in the world after groundnut and soybean. Sunflower oil is mainly composed of linoleic acid at 59% and oleic acid at 30%. Namvar et al. (2012) studied the effect of bacterial biofertilizers *Azotobacter sp.* and *Azospirillum sp.* along with chemical fertilizers on sunflower plants. Oil content in biofertilizer treated plot was about 10.84% more as compared to the control. Results observed by Akbari et al. (2011) showed higher seed oil yield (1141.5 kg oil ha^{-1}) as compared to the control. Various promising microorganisms and potential genes for increased nutrient mobilization and nutrient fortification in various crops are mention bed in the Table 11.1.

Table 11.1 Microbes and promising genes for nutrient fortification in crops

Microbes/ Genes	Nutrients	Crops	References
Corynebacteria DHDPS and *E. coli* AKIII-M4	K	Canola and Soybean	Falco et al. (1995)
Herbaspirillum	N	Rice	Baldani et al. (2000)
crtI	Pro-vitaminA	Rice	Potrykus (2000)
*crt*I	β-carotene and lutein	Tomato	Römer et al. (2000)
crtB, crtI, and *crtY*	Carotenoids and phytoene	Canola	Ravanello et al. (2003)
Rhizobium	Fe	Red clove	Jin et al. (2006)
crtB and *crtI*	Carotenoid and β-carotene	Maize	Aluru et al. (2008)
crtB	Phytoene, α-carotene, and β-carotene	Flax	Fujisawa et al. (2008)
Streptomyces siderophore	Fe	Cowpea	Dimkpa et al. (2009)
AAT	AAs	*Japonica* rice	Zhou et al. (2009)
A. brasilense and *A. vinelandii*	Oleic acid and linolenic acid	Canola	Soleimanzadeh et al. (2010)
Azotobacter and *Azospirillum*	Linoleic acid and oleic acid	Sunflower	Namvar et al. (2012)
dapA gene	K, M, and T	Barley	Ohnoutkova et al. (2012)
Bacillus and *Providencia*	Fe	Wheat	Rana et al. (2012b)
Providencia	Zn	Wheat	Rana et al. (2012b)
Pseudomonas and *Stenotrophomonas*	Se	Wheat	Acuña et al. (2013)
AM fungi	Fe and Zn	Chickpea	Pellegrino and Bedini (2014)
crtB gene	Oleic acid	*Glycine max*	Schmidt et al. (2015)
Sirulina platensis	Zn	Maize	Tuhy et al. (2015)
Bacillus sp. and *B. thuringiensis*	Se	Wheat and *Brassica juncea*	Yasin et al. (2015)
Rhizobium	Fe	Rice	Adak et al. (2016)
Spirulina	Zn	*Amaranthus gangeticus*, *Phaseolus aureus* and tomato	Anitha et al. (2016)
Pseudomonas	P, K, Zn and Fe	Sorghum	Dhawi et al. (2016)
Anabaena and *Azotobacter*	Zn	Okra	Manjunath et al. (2016)

(continued)

Table 11.1 (continued)

Microbes/ Genes	Nutrients	Crops	References
Actinobacteria	Fe	Chickpea	Sathya et al. (2016)
Glomus mosseae	N and P	Okra and Pea	Kumar et al. (2017b)
Pseudomonas siderophore	Fe	Maize	Sah et al. (2017)
Selenobacteria (*Acinetobacter*)	Se	Wheat	Durán et al. (2018)
Pantoea dispersa and *Pseudomonas putida*	Fe	Mung bean	Patel et al. (2018)
Spirulina	Zn	Raddish	Godlewska et al. (2019)
Bacillus amyloliquefaciens and *Paraburkholderia fungorum*	Antioxidant, carotenoids, flavonoids, phenolics, and anthocyanins	Strawberry	Rahman et al. (2019)
*crt*I	Pro-vitaminA	Rice	Stein et al. (2006)

11.3 Arbuscular Mycorrhiza

Arbuscular mycorrhiza is a symbiotic association between fungi and plant roots. In this symbiotism, plants get the transferred nutrients (P, Zn, Fe, and K) and in return fungi get a place to live inside plant roots and nearly 20% of the photosynthetically fixed carbon in the form of sugar and lipids (Pellegrino and Bedini 2014; Frew 2019; Rasouli-Sadaghiani et al. 2010). In addition to this, arbuscular mycorrhiza provides resistance against abiotic and biotic stress (Augé 2001; Chen et al. 2018a; Durán et al. 2016; Hajiboland et al. 2019; Ma et al. 2018; Pozo and Azcón-Aguilar 2007) as well as increase the seed production (Bisen et al. 2015; Bethlenfalvay et al. 1997; Colla et al. 2015; Kavitha Mary et al. 2018; Koide 1991). The arbuscular mycorrhiza role in nutrient uptake is considered as one of the major strategies primarily employed to carry the nutrient fortification in crops (Frew 2019; Symanczik et al. 2018) which minimizes the use of fertilizers as well as chemicals even upto 35%.

Experiments were conducted with inoculation of arbuscular mycorrhiza fungi, *Glomus mosseae,* on okra plants. Fruits were harvested and the nutrient status was calculated. The uptake of N and P was found to increase even up to about 3% and 18% as compared to the uninoculated plants. In another experiment, respectively 4% and 20% higher N and P was observed in pea pods upon inoculation of pea with arbuscular mycorrhiza as compared to the un-inoculated plants. The arbuscular mycorrhiza inoculated pea also showed an increased concentration of Zn and Cu of about 14 and 39% respectively (Kumar et al. 2017b). Furthermore, the association between rice and *Herbaspirillum* sp. enhanced the N-uptake which was estimated as 31–54% of their total N-requirement by the *H. seropedicae (*Baldani et al. 2000).

Experiments performed on sorghum also showed higher nutrient uptake. Sorghum was inoculated with *Pseudomonas* and arbuscular mycorrhiza fungi (*Glomus intraradices, Glomus mosseae, Glomus aggregatum, and Glomus etunicatum)* together and in another treatment they treated sorghum with arbuscular mycorrhiza alone. After harvesting, the uptake of different elements was evaluated using inductively coupled plasma mass spectrometry in plant samples. The root samples showed higher P and K with a range upto two fold when treated with *Pseudomonas* only and 5.6 fold treated with arbuscular mycorrhiza only. Microelement Mn content in root was three fold higher in *Pseudomonas* treatment and ten-fold higher in plants treated with plant growth promoting microorganisms with arbuscular mycorrhiza. Fe and Zn concentrations increased by 5 and 5.6 fold in treatment with *Pseudomonas* and arbuscular mycorrhiza together (Dhawi et al. 2016). Maize treated with bioinoculants also yielded significantly higher uptake of Zn, Fe, Al, and Ca (Dhawi et al. 2015).

Rice inoculated with *H. seropedicae* and *G. mosseae* showed increase in the crop yield by 20% and 35%, respectively as compared with control. The concentration of Zn was 35.38 mg kg^{-1} and Fe was 35.66 mg kg^{-1} in rice grain when treated with N (75%) + bacteria and N (100%). It was further noted that application of *H. seropedicae* and *G. mosseae* significantly increased the yield by 20% and 35%, respectively, compared with the control treatment and by 33% and 40%, respectively, compared with the highest rate of chemical fertilizer (Hoseinzade et al. 2016). Endophytic *Selenobacteria* strain and *Acinetobacter coupled* inoculation increased the Se content in wheat at both tillering and maturation stages (Durán et al. 2018). In another experiment with chickpea; the seeds were inoculated with the different strains of arbuscular mycorrhizal fungi in autumn and spring seasons (Pellegrino and Bedini 2014). A trap-culture-enriched locally-sourced arbuscular mycorrhizal fungal community increased the Fe and Zn uptake by 4% and 21% respectively as compared to the control plants.

11.4 Conclusion

Treatment of plants with fertilizers is a fundamentally one of the easiest and cost-effective method for increasing yield and nutrient content. However, the limitation of using fertilizers is that over the time, it accumulates to the toxic level in the soil and eventually the adverse effects are seen on the plants, animals, marine life, and overall ecosystem. Moreover, if plants are treated solely with fertilizers, it is not sure that nutrients directly reach the edible parts and seed apart from stem and leaves. Additionally, nutrient fortification relies also on different plant species, nutrient mobility rates, and soil composition in different regions. For example, studies on 30 rice genotypes showed different phosphorous uptake among the varieties that differs upto 20 fold (Ismail et al. 2007).

Furthermore, conventional breeding is efficient and widely accepted, as a result, international and national organizations have released several crops and their cultivars. However, it is the slowest route to reach farmers and people as the new variety development takes a lot of years. As a result, it opens a window for enhancing nutrient uptake and level through advantageous plant growth promoting microorganisms. This methodology exploits a natural route to increase nutritive value in the crops as well as positively enhance soil fertility. In a long way to the future, with the help of fully covered transcriptome, proteomic, metabolomic, and phenomic data, the interaction of microbes and crops will be understood in a detailed manner. Deciphering of every possible transport channel in microbes meant for the nutrients accumulation, consecutive isolation of the corresponding genes from microbes, development of suitable bioinoculant and their application to the plants will help in nutrient acquisition by the plants. This will translate into the enhancement of nutrients uptake and maximum acquisition and will ultimately lead to the generation of promising fully biofortified plants and plant products for mitigating malnutrition among the population.

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Chapter 12
Microbial Rejuvenation of Soils for Sustainable Agriculture

Pankaj Sharma, Anupam Patra, Baljinder Singh, and Sahil Mehta

Abstract Agriculture is actually facing reduced production, increasing costs, and increasing resistance of plant pathogens and other pests. The extensive usage of agrochemicals, monoculture, and soil pollution have deteriorated soil health. Poor management practices have altered the soil biology, deteriorated the soil structure, and reduced the soil organic matter content, calling for more sustainable management such as the use of microorganisms for rejuvenating degraded soils. Here we review the improvement of soil health with microorganisms with focus on carbon sequestration, nutrient cycling, degradation of contaminants, reduction of plant pathogens, and reduced usage of fertilizers.

Keywords Agroecosystem · Tillage · Irrigation · Soil health · Soil structure · Carbon sequestration · Monocultures · Xenobiotic compounds

12.1 Introduction

Agriculture, in a wide range, is an act of integration of definite agro-ecological elements and the production inputs for optimal crop yield and livestock production. The traditional act of practicing agriculture confronts several issues like abridged production, amplified costs, deteriorated soil health, etc. Furthermore, the agricultural practices of monocultures on the same land have serious ill effects like depletion of top soil, lowering of groundwater quality, degradation of soil vitality, and

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reduction in the population of beneficial microbes which make the crops more vulnerable to the attack of various pathogens and parasites. The ever-increasing usage of pesticides and fertilizers along with the energy demands for ploughing to ventilate soils and the accelerating costs of irrigation are much-concerned issues (Singh et al. 2011, 2019a; Sharma et al. 2020a, b).

Moreover, the existing human practices of unrestrained application of chemicals escalated usage of non-renewable and conventional energy sources and unchecked generation of a vast array of left-over produce in every sort of industrial process has gallantly crumbled the environmental sustainability. Thus, the world now faces a galactic need as well as responsibility meant for the adoption of tenable measures for cleaner production and also the involvement of green technologies for preserving the ecology of this green planet for the coming generations (Akinsemolu 2018; Kumar et al. 2020).

Although, the green revolution has proved to be an act of paramount success of human efforts which has resulted in attaining worldwide food security especially in some developing countries like India which has travelled far enough to become food surplus from food deficient countries but the gradual and shocking rise in human population is again becoming a hindrance in the global food security thus sparking the need for another green revolution to endure the burden levied by increasing population (Vasil 1998; Leisinger 1999; Rani et al. 2019; Singh et al. 2021). Chemical fertilizers are usually recommended for overcoming the deficiencies of different nutrients but the perpetual involvement of such fertilizers for yield improvement is attaining saturation beyond which there is no further enhancement in the crop yields.

Thus, it has become limpid clear that the prevailing agricultural methods are not potent enough to nurture the production base and a healthy plant-soil system for a longer time. This promiscuous and lavish employment of chemical fertilizers has become a seedbed for several environmental and health-related hazards thus piquing the need for the development of potent alternatives that can warrant competitive yields along with the protection of soil health. Such an approach to farming frequently appertains as sustainable agriculture which necessitates the environment-friendly agricultural practices that are meant for upholding the enduring ecological balance of the soil ecosystem. In this context, the use of microbial inoculants represents an eco-friendly substitute for mineral fertilizers (Khan et al. 2007; Sharma et al. 2021).

The microorganisms of agricultural importance are a viable option for the environment-friendly management and regulation of the efficiency and the availability of nutrients to plants; thereby they enhance soil fertility by ameliorating the soil biodiversity and nutrient availability (Mahawar and Prasanna 2018). Microorganisms are tremendously expanded by their roles in various environmental processes (Mehta et al. 2019; Singh et al. 2019b; Rahman et al. 2019; Kapoor et al. 2020). Furthermore, these are crucial agents in several cleaner technologies and green processes which range from biogeochemical cycling to several industrial production processes. Thus, the judicial use of microorganisms can play a major role in sustainable development (Kuhad 2012).

Biopesticides and biofertilizers are formulations comprising of effective microorganisms that improve plant growth in many different ways as compared to synthetic fertilizers and consequently help in improving crop productivity by preserving the sustainability of the environment. The rhizospheric soils also encompass a distinctive array of efficacious microbes with salutary effects on the overall productivity of the crops. The cyanobacteria and the plant growth-promoting rhizobacteria are among the various dwellers of the rhizospheric soil and produce various bioactive substances that are responsible for plant growth promotion and protection against various pathogens which makes them effective agents for agriculture improvement and environment sustainability (Singh et al. 2011). The soil biodiversity and the interactions of different organisms in soil have experienced rigorous changes under the green revolution technology. The major downside of the green revolution technology appears to be the loss of functional diversity of the soil which has significantly disrupted the efficiency of the ecosystem (Srivastava et al. 2016); thus, also deteriorated the soil health significantly.

Soil health is the capacity of soil to function as a vital living system, within the ecosystem and land-use boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health. According to Doran and Parkin (1994), soil quality is “the capacity of a soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health”. Soil health is always related to the holistic management of the soil whereas soil quality is always described concerning the constituent parts of the soil *i.e*., physical, chemical, and biological parts of the soil. However, in this chapter both the terms are used in the same context. An important parameter for the determination of soil characteristics is the buffering capacity of the soil. The attribute of maintaining its productivity, despite facing several stresses like fluctuations in water availability, various kinds of soil disturbances including tillage, or different sorts of imbalances like outbreaks of pests is provided by the effect of buffering (Sherwood and Uphoff 2000).

The complexity of the soil systems is an undeniable fact. Soil structure is continuously modified by the soil microbes by aggregating both organic as well as mineral components via producing extracellular compounds which are endowed with adhesive properties. There is a consorted change pronounced in the structure of the soil as well as the topology of the network of pores which makes the microbial habitat and that affect the water accessibility and its distribution, gases and substrate delivery to organisms and also removal of the products of metabolism from their vicinity. Such kind of activities of microbes is strongly administered by the availability of organic carbon (Kibblewhite et al. 2008). Thus, soil health comprehends the living as well as the dynamic nature of the soil which distinguishes it from the soil quality.

The main focus of soil is on the capacity of the soil to meet distinct human requirements such as the growth of any specific crop, whereas soil health is mainly focused on the soil’s sustained capacity to withstand the growth of the plants along with the maintenance of its functions (Bünemann et al. 2018). People alter natural systems, however, because the existing maximum biological productivity is either insufficient or undesirable. Some soils are quite accommodating to human interventions while others have a low tolerance.

The organic matter content of healthy soil should be high as the organic matter acts as a pool of nutrients as well as moisture, and thus, maintain a vast diversity of organisms to flourish in the soil environment. Thus, it is necessary to add organic amendments regularly for maintenance as well as enhancement of the soil organic matter content to improve soil health (Turmel et al. 2015).

However, several human practices like, addition of municipal solid wastes impairs the soil quality as it contains heavy metals like lead, cadmium *etc*., in a very high concentration, which also leads to the contamination of the food chain. Thus, extreme addition of metals causes metal pollution which may affect the soil quality (Smith 2009). The soil microbial biomass is an important parameter for assessing soil health and is very sensitive to heavy metal concentrations in soil and water (Ouni et al. 2013). Soil pollution also significantly affects soil microbial biomass which disturbs various microbial processes in the soil; thus, deteriorates soil health (Romero-Freire et al. 2016). Agricultural practices and land use patterns also vigorously affect soil health. The practice of crop rotation favours the natural process of nutrient replenishment (especially nitrogen) and also pulverizes the possibilities of any kind of comprehensive pest burst, thus permits benefit to the farm system from the available biological diversity (Table 12.1).

12.2 Constituents of Healthy Soils

There are different vantage points for the assessment of soil health. The general perspective of health assessment is an evaluation by productivity which could range from biomass production to productivity indices corresponding to elementary properties of soil. Earlier studies about the soil-fitness were based only on yield increment targeting the profit outlook. As the various aspects of soil conditions are being realized, the quality of soil is gaining wider and worldwide attention. Soil is the bedrock of water security, food security, biodiversity protection, and climate change mitigation (McBratney et al. 2014). The high-quality soil also means a greatly fruitful soil with very small levels of soil degradation along with the high capability to resist extreme weather conditions and a diminished loss of nutrients (Karlen et al. 2013).

The different ways of classifying soil health usually involve multiple facets concerned with physical as well as chemical properties of the soil including some biological indicators. According to the Food and Agriculture Organization of the United Nations; soil health is defined as the “soil’s capability of functioning as a living structure, with the ecosystem and the land-use boundaries, to endure the productivity of the animals and the plants, preserving or improving the quality of air and water, and encouraging the health of plants as well as animals. The occurrence of a diverse community of microbes in the healthy soils check plant disease, controls insects and weeds and also form symbiotic associations with the roots of the

Table 12.1 Factors affecting soil properties and possible remedies for soil health improvement

Factors	Consequences	Remedies	Mechanisms	Remarks	References
Metal contamination and acidification	Reduced growth of lettuce plant	Alkaline amendments	Improvement in the enzymatic activities, and bacterial community structures in the soils	The amendments led to an enhancement in the levels of Proteobacteria and Gemmatimonadetes in the soil which are vital for phosphate dissolution, microbial nitrogen metabolism, and soil respiration	Lu et al. (2020b)
Tillage	Soil disturbance and depletion of soil organic carbon pools	No-tillage agriculture coupled with a cover crop	Improvement in soil organic carbon, total nitrogen, available phosphorus, exchangeable K-Mg, cation-exchange capacity, bulk density, soil penetration resistance, and substrate-induced respiration	The Z-score formula, a formula to calculate the value of certain variables that we observe with a specific treatment factor and compare it with the average value of certain variables in all treatments, for soil organic carbon, several soil characteristics, crop productivity, and biomass input, ensured the employment of the combination of no-till with cover crops as a tool to improve soil-health	Wulanningtyas et al. (2021)
Continuous cropping	Decline in soil organic carbon	Biochar addition	Increase in soil organic carbon, nitrogen pool, decline in bulk density of soil, and increase in microbial abundance	Biochar addition is a beneficial tool to improve soil health for ensuring food production in agricultural land facing degradation and climate change impacts	Lu et al. (2020a)
Reduced soil organic carbon	Reduced barley production	Organic amendment	Improvement in fertility status of soil, enhancement in soil enzymatic activities and improvement in physical properties of soil	Use of organic wastes is an efficient tool for improving health of nutrient-poor soils of the arid and semi-arid regions	Lal et al. (2020)

(continued)

Table 12.1 (continued)

Factors	Consequences	Remedies	Mechanisms	Remarks	References
Management practices	Deteriorated soil health	Higher crop diversity, fewer tillage operations, and higher number of organic amendments	Higher levels of aggregate stability, protein, active carbon, respiration, and organic matter	Soil management is the key for soil health, management practices comprising of crop diversity, zero tillage, and application of organic amendments promote soil health	Williams et al. (2020)
	Reduced plant productivity	Rice husk biochar application	Improved soil pH and carbon content	Biochar application is an effective tool for alpine meadow restoration	Rafiq et al. (2020)
	Deteriorated soil health	Wheat-sorghum rotation as cover crops	Change in microbial community structure and enzymatic activities	Cover crops increase the total microbial community size, fungal abundance, and enzyme activities associated with carbon and nutrient cycling	Thapa et al. (2021)
	Deteriorated soil health	Organic amendments	Overall improvement in the soil quality index by the interaction of soil properties	Addition of organic fertilizers improves soil quality and plant yield as compared to chemical fertilizer	Li et al. (2020)
Heavy metal contamination	Reduced plant productivity	Biochar application	Increase in pH, organic matter content, and available phosphorus in soil and reduced bioavailable vanadium	Biochar application is a cost-effective and ecofriendly amendment for both soil quality improvement and the vanadium contaminated soil remediation	Yu et al. (2020)
	Declined soil health	Compost application	Slow-release of nutrients and thus favoring long term soil fertility and immobilization of heavy metals	The soil health improves considerably even at the lowest compost application rate	Alburquerque et al. (2011)

plants; recycle vital plant nutrients; improve soil structure with positive repercussions for soil water and nutrient holding capacity thus eventually enhance the crop production" (FAO 2008). Furthermore, healthy soils generate healthy produce that sequentially nourishes humans and animals (FAO 2015).

The quality of soil cannot be determined directly, but it refers to evaluating the physical, chemical as well as biological properties of the soil (De Paul and Lal 2016). The agricultural point of view has mainly long focused purely on the physical as well as chemical properties of the soil whereas the innate biological constituents of the soil which contribute to the overall soil health were fiercely neglected. The soil mass present in 1m^3 of soil lies in the range of 1200–1700 kg of soil which contains around 2.3–2.6% of soil's total carbon in the form of microbial biomass (Haney et al. 2018).

The steady-state conditions of the soil are usually utilized by the soil researchers as an estimate of the microbial activity of the soil (*i.e.*, constant water, temperature, and oxygen content). This was the possible initial methodology for laboratories; nevertheless, the drying/rewetting cycle induced by the rainfall and events of the irrigation leads to the creation of a highly dynamic mechanism of nutrient cycling which is driven by microbes in the agricultural fields (Haney and Haney 2010). The researchers recognized CO_2 respiration as an indicator of soil fertility around 100 years ago (Gainey 1919; Lebedjantzev 1924). The microbial biomass has an important role to play in decomposing the organic matter as well as the cycling of nutrients, and thus, is extremely allied to the vigorous pools of probable carbon and nitrogen mineralization. The organic compounds from the soil organic matter are usually oxidized by the microbial population along with the release of CO_2 (Haney et al. 2018).

The concentration of soil organic carbon is usually deliberated as a representation of the soil quality as it optimally symbolizes the dynamics of the soil biota and also plays a crucial role in the fertility of the soil, water availability, and stability of aggregates in the croplands. There are various other soil attributes like bulk density, soil depth, respiration rate, pH, electrical conductivity which help to understand the soil processes thus incorporating evolving concerns on the assessment of the soil quality (De Paul and Lal 2016). However, it is also anticipated that the inputs of various elements like P, C, K, Mg, and Ca are found to be higher in low input or conventional and organic agricultural systems which are considered as a good sign of soil health.

Soil pH along with microbial biomass is also found to be somewhat higher in the soils which are organically managed. Such soils contain a vast diversity of bacteria, fungi, nematodes, earthworms, and arthropods as compared to other soils. Highly assorted ecosystems with various taxa that form a multifarious food web having several trophic levels are usually deliberated as healthy and thriving ecosystems. Consequently, the taxonomic and functional variety indices are frequently used as indicators of soil health status. The soils which are regularly cultivated possess a lower microbial diversity as possessed by them as the natural habitats. Thus, organically managed healthy soils appear to be healthier as well as natural compared to the other management strategies (van Bruggen et al. 2015).

There are several factors in organic farming like disparate crop rotation, utilization of carbon-based alterations which contribute to the overall soil fertility. Such exercises significantly upsurge the biologically accessible soil organic matter along with an increase in the number of beneficial soil microbe as well as invertebrate activities which further improves the physical properties of the soil, condense the disease potential, and ultimately escalate the plant health. Several studies have also proved that the fruits and vegetables grown in healthier and organic soils comprehend a greater level of health stimulating phytochemicals (Reeve et al. 2016). Thus, various unhidden aspects of soil fertility strongly affect the produce, plant, and animal health along with an ultimate effect on human health.

12.3 Importance of Soil Health

Soil health has an important role to play in agricultural production, quality of food, environmental resiliency, and sustainable management of the ecosystem. The recent concerns on food policy have progressively concentrated more on the notion of soil health which holistically measures the productivity of the soil along with its resilience and sustainability. The findings of various disciplines of science like agronomy, soil science, ecology, and plant biology have made it quite clear that soil has a great impact on food security and nutrition. In light of these findings, the year 2015 was declared as the international year of soils by Food and Agriculture Organization. The benefits derived from soils of superior health can be easily categorized into ecological/environmental benefits and agronomic benefits. The agronomic benefits of healthy soils mainly correspond to the elevated yields, better pest management, reduced usage of fertilizers, and less necessary irrigation practices; whereas the ecological benefits are mainly concerned with better environmental management principles. The main benefits derived are reduced rate of erosion, flood control, better carbon sequestration, cleaner water due to less flow of nitrates, and enhanced biodiversity (Stevens 2018). Thus, the importance of soil health can never be undermined.

The fundamental effect of the soils on human health is very well-acknowledged. There are several positive effects of soils on human health such as the supplement of vital nutrients for the production of nutritious food for the human diet and it also acts as a basis of various antibiotics (Brevik et al. 2018). Several nutrients having importance to human health find their origin in the soil. As plants grow in the soil these nutrients are absorbed by the plants and then these are passed on to human beings feeding on that plant material (Brevik et al. 2017). Plant fibers are a great source of clothing, various fibers like flax, cotton, and hemp are important sources of fibers for making clothes. Such plants also find optimal growth conditions in healthy soils.

Another important aspect related to healthy soils is the practice of water conservation. The practices that promote soil aggregation encourage many properties of the soil, comprising water infiltration as well as retention. The increase in the organic matter content of the soil has a direct effect on the water-holding capacity of the soil. An increase in soil organic matter by only one percentage point amplifies the water-holding capacity of soil beyond 252,556 Litre/hectare (Cano et al. 2018). The rate of water infiltration is significantly higher in healthy soils. Healthy soils can upsurge the water infiltration as well as storage from precipitation. It is of ample importance as conservation of water meant for irrigation purposes is strongly favored along with an increase in crop productivity and a decline in soil erosion (Lehman et al. 2015).

12.4 Indicators of Soil Health

The quality of soil is one of the three constituents of environmental quality in addition to the air and the water quality (Andrews et al. 2002). Air and water quality are usually defined largely by the extent of pollution they suffer that have various direct impacts on human as well as animal health, and also on natural ecosystems. Conversely, the definition of soil quality cannot just be inferred from the extent of pollution in the soil but is often defined from a broad range of parameters (Bünemann et al. 2018) (Fig. 12.1).

The site-specificity, as well as the complexity of the underground ecosystem along with the associations among soil-based ecosystem services and the soil functions, should also be reflected while defining soil health. The quality of soil is indeed much complex as compared to the water and air quality, not because soil constituents may be either of different states, but also because soils can be used for a larger variety of purposes (Nortcliff 2002). The changes in quality of soil can be evaluated by assessing suitable indicators and the obtained values can further be compared

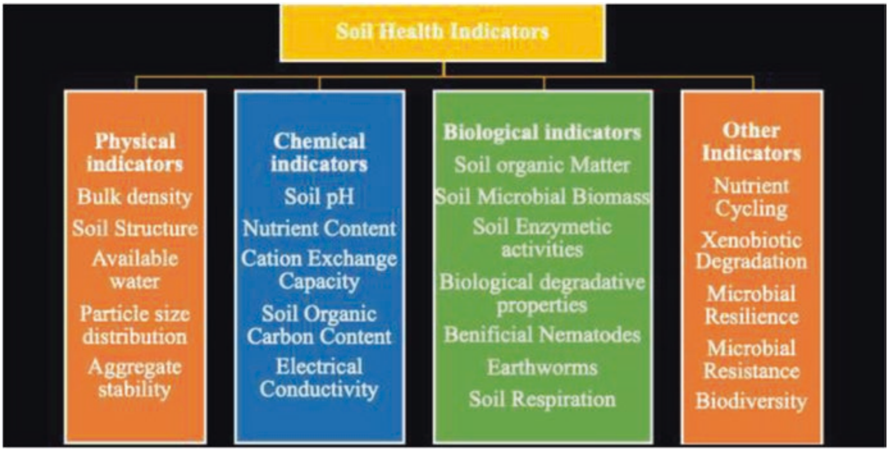

Fig. 12.1 Indicators governing soil health

with the expected values which are usually threshold levels or critical limits at different intervals of time (Arshad and Martin 2002).

12.4.1 Essential Characteristics of Soil Health Indicators

The indicators of soil health are referred to as assessable characteristics of the soil influencing the soil's capacity of conveying ecosystem services. The attributes of soil showing the most sensitivity towards the management is often considered desirable indicators. They are found to be responsive towards the management of agriculture thus reflecting changes in the functional properties of the soil and are finding an increased usage in the assessment of the current condition of the soil health (Takoutsing et al. 2016).

Presently, there is no unanimity among the scientific community concerning the finest indicators to be dignified in the health assessment of soil through diverse agroecosystems. Preferably, the indicators of soil health must be much sensitive enough for the detection of alterations in the soil ecosystems and thus signify functions appropriate to the agricultural systems and the soil management (Lehman et al. 2015). These indicators need to be penetrating towards the alterations which appear due to the management practices of the soil and should cover an extensive range of environments along with the integration of physical, chemical as well as biological properties (Doran et al. 2002).

Moreover, these indicators need to be economical, reproducible, and accessible to the producers through various laboratories and offered online to community shareholders for the collection of meta-data and supervision in practices of management as well as the development of databases, online repositories, and tools for future researchers (Cano et al. 2018; Anamika et al. 2019). The suitable methods for assessing soil health are still under controversy as a single indicator cannot comprehend all the facets of soil health and the measurement of all the possible indicators is not feasible (Takoutsing et al. 2016). There are several soil indicators and some of them can be easily observed by touching and viewing whereas others require various specialized apparatus as well as analytical skills. This is an undeniable fact that determining the various features of soil health can be a hellacious job even in the farming systems having technological advances at the peak.

12.4.2 Soil Health Parameters

Although there are myriad ways of systemizing soil health measures, a common framework is often shared by different authors. Soil health can easily be construed into three major constituents: physical, chemical, and biological characteristics. There is an existence of various possible attributes as well as indicators governing soil health within these three basic components (Stevens 2018). The chemical properties used for the assessment of soil health are often found to be very limited due to the heavy charges of sample analysis. Moreover, the trustworthy, as well as the precise methods for assessment, are still not available. There is also a great scarcity of various monitoring initiatives in this field along with the inadequacy of land management in a sustainable way (Shepherd et al. 2015).

12.4.2.1 Physical Parameters

There are various kinds of physical parameters which have been best argued for being used as soil health indicators like, bulk density, soil structure, available water, particle size distribution, aggregate stability etc. (Rabot et al. 2018). The soil organic carbon content can also be mapped by various high-resolution mapping devices such as remote sensing devices along with progressive geostatistical methods (Rinot et al. 2018). The soils of sound health are found to have a high organic matter which permits the growth of a great variety of soil organisms and also acts as a pool of soil nutrients along with adequate moisture. The regular addition of organic amendments is essential to upsurge or sustain the organic matter content of the soil and thus it subsidizes the soil health (FAO 2011). In common, the quality of soil is indistinguishably associated with the dynamics of soil organic matter and soil carbon, which have a direct influence on the physical, chemical, and biological function of the soil.

The soil organic matter leads to the stabilization of the aggregates, helps in the prevention of erosion, upturns the water-holding capacity of the soil, and also encourages slow-release of nutrients (Karlen et al. 1990). In general, the soil's physical possessions provide information related to water and air movement through soil, as well as conditions affecting germination, root growth, and erosion. For instance, the soil physical property of aggregate stability is a relative measure of confrontation of soil aggregates to exterior energy like heavy rainfall and cultivation, which is particularly governed by the soil structure. The soil structure represents an important health indicator as it also governs the accretion of organic carbon, penetration capability, movement as well as storage of water, and root and microbial activities. Furthermore, it also measures the soil resistance towards erosion as well as other management-induced changes (Allen et al. 2011).

The soil structure also governs the pore size of the soil systems which is further strongly linked to the soil physical quality as it has a direct influence on the soil physical indices comprising soil aeration capacity, plant available water capacity, and relative field capacity (Reynolds et al. 2009). The speed at which water comes into the soil surface and travels through soil depth is acknowledged as soil water infiltration. The water infiltration rate has also been accepted as a potent indicator of soil health owing to its ability to alter with soil use, management, and time (Arias et al. 2005; O'Farrell et al. 2010).

The soil bulk density, which happens to be a measure of soil compactness is also a useful indicator of soil health owing to its negative correlation with soil organic matter and soil organic carbon content (Weil and Magdoff 2004). Several other physical parameters like rooting depth and soil surface cover have also been found to affect the important ecological processes happening in the soil systems, thereby, have also been accepted as important soil health indicators (Allen et al. 2011).

12.4.2.2 Chemical Parameters

The chemical indicators like soil organic carbon content, nitrogen content, and pH have also found wide applications in the assessment of soil health (Rabot et al. 2018). Of them, the pH of soil along with several other factors like the salt concentration in soils, nutrient retention capacity, soil toxicity, and oxygen accessibility to plant roots also represent important chemical parameters governing soil health (Srivastava et al. 2020). The pH of soil systems is largely a function of soil parent material, time of weathering, vegetation, and climate, and is deliberated among the leading chemical indicators of soil health. The soil pH indicates the changing patterns of soil's biological as well as chemical functions comprising acidification, salinization, crop performance, nutrient obtainability, and cycling and biological activity (Dalal and Moloney 2000).

Another important chemical parameter is soil electrical conductivity, which is a measure of soil salt concentration, is deliberated an effortlessly measurable and trustworthy indicator of soil health owing to its ability to notify soil biological quality in response to crop management practices (Arnold et al. 2005; Gil et al. 2009). In addition to it, the cation exchange capacity governs the soil health owing to its traits of retaining the major cationic nutrients, like Ca, Mg, and K, coupled with the arrest of potentially toxic cations like Al and Mn. Furthermore, it also determines the soil's inherent capability of retaining toxic pesticidal particles and other chemicals. A lower value of soil cation exchange capacity denotes the augmented leaching of base cations in response to high and intense rainfall events (Allen et al. 2011). The accessibility of nutrients in the soil to the plant systems is also an imperative chemical indicator of soil health as the quantification of extractable nutrients gives a clear sign of a soil's aptitude to assist plant growth; along with a concomitant identification of the threshold values for environmental hazard assessment (Dalal and Moloney 2000).

12.4.2.3 Biological Parameters

The microbial section of soil signifies only 0.1–0.3% of the total volume of the soil, and yet is indispensable to the global soil quality, assisting 90% of the soil ecosystem occupations (Muñoz-Rojas 2018). The criteria for being useful indicators for soil quality assessment are also fulfilled by soil organisms and other biotic parameters (e.g., diversity, abundance, community stability, or food web structure). The organisms inhabiting the soil ecosystem react sensitively towards the climate and practices of land management. They are finely associated with beneficial and healthy soil and various ecosystem functions comprising decomposition and cycling of nutrients, water storage, and suppression of pathogenic and deleterious organisms along with detoxification of toxic compounds.

The richness and variety of soil organisms are found to be well allied with many constructive soil functions (Doran and Zeiss 2000). The largeness of the waves in microbial thicknesses, their occurrence, and the time needed to return to initial conditions before an organic amendment may be used as indicators for soil health. Soils with higher microbial diversity and variety are found to be more suppressive towards the pathogens. Thus, microbial resilience and resistance can serve as essential indicators of healthy soil (van Bruggen et al. 2015).

Major attribute defining soil fertility is soil basal respiration of microbial biomass (Niemeyer et al. 2012) which also serves as an indicator of the soil health or quality (ISO 2002). There are pronounced changes in the soil in response to any alteration in the soil affecting its quality (Romero-Freire et al. 2016). The microorganisms of the soil underneath stress can be metabolically less operative as they need more energy to invest for the maintenance of cell function, which results in an enhanced CO_2 carbon release per unit of the microbial biomass, this ratio is called a microbial metabolic quotient (qCO_2) (Niemeyer et al. 2012). The physical properties of the soil are also affected by microorganisms. Microbial maintenance of soil structure is done by producing various extracellular polysaccharides and other compounds or cellular debris which leads to the stabilization of soil aggregates by their functioning as cementing agents. Thus, the infiltration rate, erodibility, crusting, water holding capacity, and susceptibility to compaction are strongly affected. Therefore, indicators of soil health can be spotted by observing reactions of the microbial community of soil towards the application of diverse stress factors at numerous intensities.

The extent of a particular response and time required to come back to the same pre-stress situations can assist as measures of soil health (Gil and Gil 2011). Various members of soil fauna like nematodes, earthworms, collembolan, and predatory mites were also proposed as possible indicators of soil health whereas bacteria-feeding or predatory nematodes, soil algae, and basidiomycete fungi possibly will act as indicators defining soil facing industrial pollution (van Bruggen and Semenov 2000).

Nematodes are other members of soil fauna that can be easily traced in several marines, freshwater as well as terrestrial environments. They inhabit numerous trophic groups and fulfil significant roles in various ecosystem processes, and also respond quickly to environmental disturbances. These are habitually used as indicators of disturbances in various ecosystems that are induced by pollutants. Therefore, nematodes are also utilized as pointers of disturbances prompted by diverse agricultural practices (Du Preez et al. 2018).

Soil enzymes have also established much engrossment as long-standing biological indicators of soil health because of the correlation of enzyme activity levels to the organic matter, microbial biomass and soil physical properties, and microbial biomass. In addition to it, the enzymatic assays prove to be economical as well as easy to operate. Enzyme assays meant for the assessment of various nutrient transformations including N, P, C, and S cycles have been successfully developed.

Moreover, the changes occurring in soil health over a very little period of 1–2 years can be successfully measured by enzyme activities (Bandick and Dick 1999). Various enzymes, for instance, ammonia monooxygenase, nitrate reductase, urease, alkaline phosphatase, arylsulfatase, glucosidase, and hydrolysis of fluorescein diacetate (Dose et al. 2015) and dehydrogenase activity can be used as enzymatic indicators whereas β-glucosaminidase, β-glucosidase, arylsulfatase, and acid phosphomonoesterase are commonly assayed as indices of N, C, S, and P cycling, respectively (Acosta-Martinez et al. 2018).

Dehydrogenase is one of the important soil enzymes meant for the assessment of biological activities present in the soil. The dehydrogenase activity of the soil is a measure of total oxidative metabolic events of soil microbes; hence it is deliberated as a good indicator (Gu et al. 2009). The hydrolysis of fluorescein diacetate is used as another degree of quantifying total microbial activity because it can be easily hydrolyzed by both exoenzymes as well as membrane-bound enzymes (Schnurer and Rosswall 1982). It is often used for quantifying the amount of fungal and bacterial population positioned on the acetyl esterases in the alive protist cells (Dotaniya et al. 2019).

Due to increased human intervention and other biological processes, the quantity of xenobiotic compounds is steadily increasing in the soil. The degradation of xenobiotic compounds has also been proposed as an indicator of healthy ecosystems. The degradation of xenobiotic compounds is lower in soils facing heavy metal contamination (Kools et al. 2005).

12.5 Factors Affecting Soil Properties

The process of soil genesis or factors relating to soil formation is not the sole element affecting soil health. Soil health is also affected by the use and management practices of the soil (Moebius-Clune 2016) (Table 12.1). There is a significant decline in productivity with time which is further escorted by increasing requirements of fertilizers for attaining the desired levels of production (Dotaniya et al. 2013). Intending to assess soil degradation due to various anthropogenic activities, an appropriate understanding of various soil biological and physicochemical variables is very crucial (Tripathi et al. 2016). The community of farmers is often deceived with the myth of a proportionate hike in crop yields with the application of pesticides and fertilizers in huge amounts, which further deteriorates the biological quality of the soil.

The rate of decline of soil organic matter lessens the transformation rate of the nutrients as well as the accessibility of plant nutrients from the soil. In the case of sandy soils, the loss of nitrogen-containing fertilizers has been up surged due to the volatilization and leaching thus there is a requirement for a higher dose of nitrogen-containing fertilizers (Dotaniya et al. 2019). The pool of soil organic carbon is largely disturbed by agricultural practices which is a source of various greenhouse gases with great potential. Thus, the quality of soil is much degraded by the loss of soil organic carbon thereby laying more pressure on the sustainable production of crops and maintaining food security (Lal 2007). The over-application of fertilizers is found to be problematic as it leads to the accumulation of the fertilizers in the fields and their absorption is not permitted by the physiological mechanisms of the plants.

The key drivers behind the processes of nutrient transformations are microbes which mineralize the nutrients, thus promoting plant growth. The transformation and mineralization are conveyed by various microbial enzymes, that can either be endogenous enzymes or extracellular enzymes of microbes (Dotaniya et al. 2017). These enzymes are together called soil enzymes which are dynamic for sustaining fertility as well as the health of the soil along with the protection of the environment by the degradation of pollutants (Stirling et al. 2017). Thus, the activities of enzymes are well reflected in the respiration as well as the diversity of microbes. So, a low microbial count and lower enzymatic activity denote poor soil health. Sandy soils possess a lower microbial diversity and low population density. Although sandy soils get more aeration than clay soils, however the organic matter content of such soils is much low which restricts the growth of microbes (Dotaniya et al. 2019).

Likewise, the soils receiving much chemical treatment are also degraded easily and have poor soil fertility, and thus, abridge their potential of crop production (Dotaniya et al. 2016). The extent of global pesticide usage was 3.75 million Megagram in the year 2000 and it is further estimated to upsurge to 15.6 million Megagram by the year 2020 and up to 25.1 million Megagram by the year 2050 (Tilman et al. 2001). The technology of the green revolution has brought an adverse change in the biodiversity of soil and its interactions as well. The loss of functional biodiversity mediated by the green revolution has led to the destruction of the ecosystem's efficiency. The perpetual use of monoculture and automation, as well as enhanced usage of xenobiotic pesticides, has supposedly abridged the biodiversity of soil at each taxonomic level (Srivastava et al. 2016). There is a strong and dynamic effect of agricultural practices on soil health.

The process of crop rotation naturally replenishes the soil nutrients and maintains the biological diversity of the soil, and thus, protects the soil from pest outbreaks (Livingston et al. 2015). The traditional methods of soil management by rotating crops between nitrogen-fixing and nitrogen-leaching microbial species can solve this problem. Furthermore, tillage is another parameter and a dynamic managing judgment for farmers. The agricultural practices of no-tillage or low tillage can increase the organic matter content of the soil and lessen the erosion, but it can also promote the enlarged growth of weeds along with soil compaction.

Irrigation also plays a dynamic role in maintaining soil health. Soil properties are also influenced by irrigation patterns. The recent advancements of precise irrigation have permitted farmers the effective usage of water (Taylor and Zilberman 2017). Additionally, soil pollution is largely responsible for the reduction of microbial biomass of soil thus interfere with the capacity of performing key ecological functions. The presence of metals and metalloids is becoming an issue of environmental concern as these are not degraded and can accrue in the soils and sediment (van Gestel 2008). The increased anthropogenic activities have made the readiness of different metals like Pb, As, Cu, or Zn to the soil which are common pollutants of soil with a grave potential of degrading soil's ecosystems, and thus deteriorating soil fertility (Burgos et al. 2008). The presence of contaminant elements in the soils can ominously hinder the bacterial ability of decomposing complex substrates.

Furter, quick expansion of nanotechnology has augmented the usage of silver nanoparticles as antimicrobial additives in plastics, paints, washing machine liners, detergents, textiles, and food supplements (Impellitteri et al. 2009). They are progressively entering the environment, followed by their escalated production and usage, where the soil is anticipated to be the foremost sink (Gottschalk et al. 2013). Consequently, the silver nanoparticles may profoundly influence the soil ecosystem because of their great reactivity (Anjum et al. 2013). They have also been acknowledged for their inhibitory consequences on plant growth by causing discrepancies in the community composition of microbes, and further reducing the enzymatic activity of the soil. In addition, there is an enlarged accrual of silver in the plant tissues along with augmented antioxidant enzyme activity (Cao et al. 2017).

Human activities are also largely responsible for deeply modifying the soils and potentially worsening the soil features. Some of these activities are extremely alarming for example, during the infrastructure and the building construction. Others are less perceptible but are similarly as treacherous as regarding pedodiversity preservation (Lo Papa et al. 2011) and the protection of various environmental resources, concerning the formation of soil after the entombment of the wastes from numerous origins and nature. The creation of such new soils is always problematic (Lo Papa et al. 2018).

12.6 Soil Biology

The tiny sheet of earth's crust serving as the natural standard for growth as well as the development of plants is called soil. It is a natural body that consists of several layers called soil horizons. These horizons are different layers of mineral components of different thicknesses, which diverge from their native constituents in several ways. Thus, soil serves as a natural medium for growth, multiplication, and death for several life forms. There is a vast array of microorganisms that are present in the soil and are often designated as "black box" (Paul and Clark 1989). There is a tight association between soil microorganisms and the particles of soil. The conditions at the levels of microhabitats are not consistent and may keep on changing even at very small distances and such conditions strongly govern the activities of microorganisms present in the soil (Wieland et al. 2001). The microbial part of the soil is attracting much attention as the fertility of the soil is also governed by the quantitative as well as qualitative aspects of microflora inhabiting the soil. (Giri et al. 2005). The general classification divides microorganisms into five main taxonomic classes: Algae, Eubacteria, Fungi, Protists, and Viruses.

When the complexity level is taken into account eukaryotes are designated as more complex cells which further include Protists, Fungi, and Algae whereas prokaryotes are titled as structures with a lesser cellular complexity constituting Eubacteria and Archaebacteria (Bakshi and Varma 2011). There is great variation in the soil organisms and the discrepancy is observed from a few per hectare to countless per gram of soil. The supply of food, temperature, moisture, the soil reaction as well as the physical conditions of the soil strongly governs the population density of the biological part of the soil. The bacterial population is found to be dominating in the neutral soils whereas fungi dominate in the acidic soils and soils with high organic matter content. The moist and shady soils are usually found rich in algal content.

12.7 Microbes for Improvement of Soil Health

Soil is the definitive hub of nutrients and also a pool of various bioresources for diverse crops. There is a huge diversity of microbes that are harboured by the soil which assist as potent mediators for recycling, sequestration, and supply of different nutrients to plants. The soil microflora also performs a diverse array of tasks like mineral chelation, suppression of pathogens, enhancement of soil aggregation, aids plants in toleration of different kinds of stresses and bioremediation of the soils by producing various metabolites (Sahu et al. 2019; Sharma et al. 2019) which constitutively improves soil health (Fig. 12.2). The creation of microenvironments in the rhizosphere of plants is a hub for microbial diversity as well as different kinds of interactions that aids plant as well as soil health. Regular microbial populations of

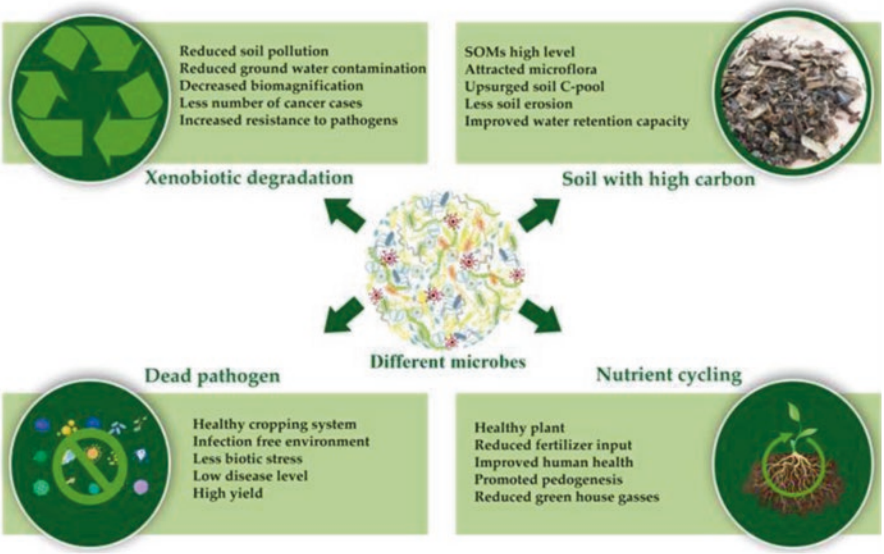

Fig. 12.2 Beneficial attributes of soil microbes for improving soil health

soils are crucial in the degradation of pollutants (Fierer 2017). If a soil face contamination, the native microbiome of the soil amends and acclimatizes to the environmental trepidation and therefore may metabolize the exterior pollutant (Tezel and Pavlostathis 2015).

The relation between soil health and microorganisms is an unhidden and undeniable fact. Therefore, the relation between microorganisms and soil health is very important. The amalgamation of the organic amendment along with abridged tillage residue administration is an emerging trend for the management of nutrients which is regarded as a sustainability-intensive agricultural practice. This practice of reduced tillage also improves the physical status of soil health as this agricultural practice precludes the fate of soil erosion by water and wind (Panagos et al. 2015). This practice increases the quantity of labile carbon; therefore, it acts as a bait for microbial dynamics as microbes are the prime utilizers of this labile carbon (Murphy et al. 2007).

There is a succession of enzymes for the decomposition of this organic matter which thereby increases the soil organic matter pool as well as microbial biomass of the soil systems (Nevins et al. 2018). Thus, this increase in microbial activity of the soil significantly improves soil health as well as soil quality.

12.8 Practices for Improving Soil Biology

The amendments of the soil with biochar are another important trait for improving soil biological status thus enhancing soil health. Biochar is the solid carbonaceous produce that originates from the pyrolysis involving waste biomass in an oxygen deficient environment. It finds great applications in the agroecosystems for the enhancement of soil carbon sequestration as well as soil fertility (Bamminger et al. 2017). The application of biochar modifies the copiousness of soil microflora in several ways. It modifies the environment which is inhabited by the microbes by supplying nutrients and altering the soil pH. It is a direct source of energy and carbon-rich substrate for the microbes and it also provides habitats for the proliferation of microbes (Dai et al. 2018).

Soil erosion is considered an important factor in determining soil health. Healthy soils suffer from minimum levels of erosion. The deterioration of soil health can be checked by the cyanobacterial content of the soil. Cyanobacteria help in maintaining soil integrity by binding the soil particles together. The filaments of cyanobacteria absorb water when they come in contact with the water and may swell up to ten times their original size. Thus, the moisture is stored in the upper soil layer where many plant root systems and various other organisms live. They also play an additional straight role in assisting plant endurance and growth.

Microbes have the inherent ability of nutrient cycling and assimilation. The microbial assimilated nutrients are supplemented to the soil's systems as cherished organic matter entrenching different mineral nutrients which are released slowly for the improvement of soil quality (van der Wal and de Boer 2017). Therefore, the practice of addition of different microbial inoculants significantly contributes to soil health. The addition of fungal hyphae and arbuscular mycorrhizae are vital for the development and constancy of soil macro-aggregates. Therefore, the infection of mycorrhiza in the soil is also considered as an indicator of decent soil aggregation. Therefore, it can be truly said that the precise use, as well as management of microbes, can significantly improve the physical, chemical as well as biological health of the soil.

12.9 Relation Between Soil Health, Microbes, and Sustainable Agriculture

It is a general estimate that a usual gram of soil comprehends a bacterial population of around 90–100 million and fungal population of nearly 2 lakhs. The majority of these organisms usually inhabit the roots of the plants. The high microbial population of microbes in the roots is the result of the secretion of root exudates by the roots of the plants. A large proportion of the carbon fixed by the process of photosynthesis is lost by the process of root exudation as bait for attracting microorganisms. The exudates are often utilized by the microorganisms as a source of food and the microbial interactions with the plants can be constructive, destructive, or unbiased for the plant. The microflora hired by the roots of plants favors the constructive interactions between the different rhizospheric components that indirectly improve soil health as well as plant growth.

12.9.1 Carbon Sequestration

Soil is deliberated to be the major carbon pool, attributable to its capability of holding carbon in an amount greater than the atmosphere and vegetation collectively. The organic matter sustaining the soil systems is mainly composed of organic portions represented by decayed animals and plants along with the microorganisms, besides inorganic forms for instance carbonates and lime. The organic portion of soil carbon is largely derived from atmospheric carbon dioxide which is fixed by plants and autotrophic microbiota by the process of photosynthesis where the inorganic form of carbon is transformed into organic carbon, for instance, sugar and cellulose, for the maintenance of cellular integrity in the form of cellular biomass. The amount of carbon sustaining in the soil systems is around 2344–2500 Gigatonne. A major portion of this, about 1550 Gigatonne of carbon is stored in organic forms and 950 Gigatonne is stored in inorganic forms.

The process which increases the soil organic carbon by removing carbon dioxide from the atmosphere coupled with its introduction into the soil is said to be carbon sequestration. The more carbon stored in the soil and less carbon lost specifies that the land is highly capable of carbon sequestration, and vice versa. The process of photosynthesis either by plants or microorganisms contributes to carbon sequestration process whereas respiration and decomposition add to carbon loss from the terrestrial ecosystems. Plants allot about 40% of photosynthetically fixed carbon to the soil by a process named as rhizodeposition. Plants secrete several organic compounds through their roots which are popularly known as root exudates and serve as nutrients for the soil biota thereby attract an enhanced level of microbial activity in the rhizospheric portion.

However, plants are not the sole contributors to soil organic carbon. The quest for the processes for enhancing carbon sequestration in the soil systems coupled with a reduction in the carbon losses and emissions has put forward the involvement of certain microbial inoculants for their paramount roles in this process. The involvement of appropriate microorganisms endowed with the veracious mechanism for specific soils is of great importance to upsurge carbon sequestration in soils as alterations in the succession of microbial communities strongly affect the soil organic matter cycling as well as storage due to the ability of soil microorganisms to regulate inputs of multiple pathways and loss of soil carbon (Ahmed et al. 2018).

When microbial carbon sequestration is taken into consideration it unveils a diverse array of mechanisms involved in upsurging soil carbon pool, such as, the aptitude to deposit carbonates, the formation of headstrong vegetative tissues and products, and the ability to form stable forms such as soil aggregates that protect carbon soil organic forms. In dryland ecosystems, the soil organic carbon pool is largely contributed by its microbial inhabitants by secreting exopolysaccharides secreting and by forming filaments networks. Such processes not only add to the soil organic carbon pool but also protect the soil from erosion and other factors targeting soil degradation.

Such possessions of soil microbes enhance the water retention capacity of the soil and also increase the nutrient soil fertility by accumulating other nutrients into the soil in the form of their biomass as well as metabolites. These processes of microbial systems can also lead to the creation of suitable soil conditions for the proliferation of other organisms such as mosses, lichens, and herbaceous and perennial plants thereby increasing the C storage potential (Kheirfam 2019). Therefore, it can also be said that microbes also play suitable roles for indirect sequestration of soil carbon by creating conditions and environments suitable for the growth of plants which in turn sequester more carbon from atmospheric carbon dioxide.

The functioning of microbial communities is directly affected by the elevated levels of atmospheric carbon dioxide, for instance, it has been noted that the plant interactions with arbuscular mycorrhizal fungi are greatly enhanced under higher carbon dioxide levels. The higher carbon dioxide levels increase the external as well as internal hyphae due to the enhanced root biomass and higher distribution of fixed carbon to the external hyphae. The microbial contribution of fixed carbon to the soil is largely governed by the microbial growth efficiency *i.e.*, the amount of new biomass carbon produced per unit substrate carbon metabolized, degree of protection of microbial biomass in the soil, and the rate at which microbial by-products are decomposed by other microorganisms. Thus, the fate of microbially sequestered carbon is largely governed by their rates of degradation and their extent of recalcitrance.

The bacterial communities prevailing in soil ecosystems are largely associated with decomposition and carbon dioxide respiration, therefore, they present a low carbon assimilation efficiency as compared to fungi-dominated microbial communities. The fungal cell walls are comprised of polymers of melanin and chitin and are resilient towards degradation while phospholipids are the main constituents of the bacterial cell wall which are energy-rich and are readily decomposable substrates and are easily accessible to a vast array of soil microbes. Therefore, the soil carbon pool is anticipated to be more tenacious when mediated by fungal biomass and more labile when facilitated by bacterial biomass (Grover et al. 2015).

12.9.2 Nutrient Cycling

Soil systems are greatly acknowledged for supporting a greater range of life and here the conception is analogous to human health; it is not difficult to understand or recognize when the system is viewed as a whole. The microbiological aspect of soil ecosystems, ranging from genes and species to communities, is largely responsible for the strength of healthy soils. This competent rhizospheric microbiota represents a vital constituent of soil habitat which is acknowledged for playing important roles in the functioning and possessions of soil-plant systems by controlling the nutrient cycling reactions crucial for supporting soil quality as well as for subsidizing the process of pedogenesis along with its maintenance. The process of nutrient cycling maintains the healthy status of soil and plant systems along with the regulation of the flow, root growth, and storage of nutrients. Surprisingly, the fertilizers added for the plant growth promotion and yield enhancement have to pass through the competent rhizospheric microorganisms before being utilized by the plant systems.

The soil organic matter which acts as a prime source of phosphorus, sulfur, and nitrogen is decomposed by the native soil microbes to its own components or subcomponents with the help of several enzymes like amylase, arylsulfatase, cellulase, chitinase, dehydrogenase, phosphatase, and urease. This process of mineralization of organic matter is a biological process of paramount significance where the organic compounds present in organic matter are biochemically transformed by the soil microbes to simpler organic compounds and mineralized nutrients. The residues of dead plants and animals represent a greater pool of nutrients that is added to biogeochemical cycling by the microbial activities targeting its decomposition to simpler forms. The residues are often classified as easily degradable, moderately degradable, and difficultly degradable which are utilized as substrates by different classes of microorganisms. The oxidation of numerous elements of biological importance at different rates like nitrogen, carbon, sulfur, phosphorus, etc. during mineralization of organic matter is of supreme prominence to the plants. Microbes are largely responsible for the conversion of such elements into usable forms for use by the plants (Sahu et al. 2017).

12.9.3 Degradation of Xenobiotic Substances

A large number of unusual organic compounds have been discovered and synthesized by human beings in the last century and many of these compounds are xenobiotic in nature. These synthetic chemicals have largely found applications as refrigerants, solvents, dyes, pesticides, and other compounds of importance in agricultural systems (Duong et al. 1997). Undoubtedly, pesticides play an important role in modern agriculture and have greatly contributed to combatting global hunger problem but these chemicals are not degraded easily by the biological processes (Villarreal-Chiu et al. 2017). The xenobiotic compounds also pose various other health hazards. According to *United States Environmental Protection Agenc*y, 90% of fungicides, 60% of herbicides, and 30% of insecticides are recognized as potentially cancer-causing (Grube et al. 2011). The pesticide production in India started in 1952 with the production of benzene hexachloride which was further followed by dichlorodiphenyltrichloroethane.

Subsequently, the patterns of production along with their consumption have amplified enormously and are attributable to their assorted applications. At present, India is the second leading producer of pesticides in Asia with a twelfth global rank (Gupta 2004). The extensive usage of xenobiotic compounds has significantly deteriorated the soil health along with several other harmful effects including dwindled soil fertility, nitrate leaching, soil acidification, increased resistance in flora and fauna, pollution of groundwater along with the surface water, and impurity of the agricultural soils (Kumar et al. 2018). Consequently, the degradation of such compounds by either physicochemical or biological processes has been greatly researched. Therefore, the microbial ability to degrade such contaminants seems to be a preferred method that can contribute to restoring soil health.

The native microbial diversity of the contaminated regions is usually explored by scientists in the quest for indigenous bacteria having the capability of utilization and degradation of an extensive variety of pollutants (Stroud et al. 2007). Several bacterial genera have the capability of biotransformation of such organic contaminants in their natural environments. The members of the microbial genera like *Cladosporium*, *Flavobacterium*, *Aspergillus*, *Arthrobacter*, *Flavobacterium*, *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, and *Burkholderia* etc. can be utilized for degrading the contaminants for restoring soil health (Kumar et al. 2018). The approach of rhizosphere engineering and microbial consortia having specialized functions like degradation of polycyclic aromatic hydrocarbons is evolving new tools and methodologies for amelioration of such problematic soil (De Roy et al. 2014).

12.9.4 Soil Suppressiveness

The capacity of soil to regulate the flow of soil-borne pathogens is called soil suppressiveness. The level of disease is found to be minimum in healthy soils thus they provide infection-free environments for the germination of seeds during an initial phase of development of the plants. Several soil systems are found to be inherently capable of suppressing the soil-borne pathogens to a definite magnitude, which proves to be a highly robust and anticipated tool for the development of healthy cropping systems with a decreased reliance on chemical inputs. This capability of the soil to suppress pathogens is a result of different physical, chemical as well as biological parameters of the soil. The biological properties of soil, especially microbiological, are known to play crucial roles in soil suppressiveness.

The general suppressiveness of soil systems is largely governed by the biomass, activity as well as the diversity of the microorganisms inhabiting the soil. The non-pathogenic microbial inhabitants of soil reward the suppressiveness to soil as a result of their capability to compete with the pathogenic microbes. Furthermore, there are different mechanisms by which the growth of a pathogenic organism is suppressed like the production of hydrogen cyanide, surfactins, salicylate and catechol-type siderophores, lipopeptide, iturin etc. (Arthee and Marimuthu 2016).

The other form of soil suppressive is called specific soil suppressiveness which targets the inhibition of specific pathogens. It occurs due to the presence of specific microbial taxa or groups in the soil which inhibits the growth of specific pathogens by their antagonistic behaviors. This type of suppressiveness is considered to be less persistent in the soil as compared to generalized suppressiveness. However, soil suppressiveness undoubtedly instigates from the combined effects of general and specific soil suppressiveness (Bongiorno et al. 2019).

12.10 Conclusion

The realm of agriculture has to confront an expansive gamut of challenges of climatic changes, stagnant crop yield, nutrient deficiency, deterioration of soil organic matter, availability of water, and dwindling cultivable land. Although green revolution proved to be an act of paramount success; the excessive usage of chemical fertilizers, pesticides, and other land-use practices has resulted in stagnant crop production. Furthermore, soil proves to be the ultimate sink for each type of chemical. As these chemicals are non-biodegradable, they prevail in the soil for a very long time and deteriorate the soil health. Besides soil health deterioration, they also enter the food chain and cause various health-related hazards. Therefore, the demand of the hour is cleaner and chemical-free food production along with the management of soil health. Soil health is also adversely affected by various human-mediated management practices.

The soil can be rejuvenated by the application of microorganisms as they possess various properties which improve the physical as well as chemical properties of the soil. In addition to it, microorganisms also possess the unique trait of carbon sequestration which adds to the organic carbon pool of the soil. The microbes also have the great potential to transform the normal soils into soil systems which are found to be suppressive for various pathogens. Such attributes of microbes decrease the reliance on chemical inputs thereby advocate sustainability.

Healthy soil is found to inhabit a vast variety of microbes in varying numbers and the status of such soil microbiota can be enhanced by various agricultural practices like no-tillage, amendments of organic matter, mulching, and crop rotation. The declining status of soil health needs a strong microbiological intervention for revitalizing it as all forms of life depend on the soil ecosystems directly or indirectly. Thus, it can be concluded that the tremendous potential of soil microbes than any other life form can be utilized effectively for uplifting the status of soil systems but more research is required in the field to combat the various problems. The ever-increasing problems can be solved by the judicious use of microorganisms by keeping in mind the fact that "the role of infinitely small is infinitely large in nature".

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Chapter 13
Microbial Remediation of Agricultural Residues

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Abstract The rising crop production generates a high quantity of agricultural residues that are not fully recycled, e.g. in bedding for animals and feed production, thus leaving large amounts of unused residues that induce environemental pollution. For instance, the residue excess is often set to fire by the farming communities. Since residues contain nutrients, microbes can be used to convert residue into valuable products. Here we review the microbial conversion of agricultural residues into fuels, food and feed materials. Biofuels include bioethanol, biodiesel, biobutanol, and biogas. Microbial systems transform residues into useful compost for plants, and into nutrient-enriched feed for animals. Solid-state fermentation of residues can be used to produce food such as mushrooms.

Keywords Microbes · Residues · Soil · Biohydrogen · *Clostridium* · Lignocelluloytic · Bioethanol · Biogas · Biobutanol · Mushroom production

13.1 Introduction

A major proportion of the Indian population still depends on agricultural systems for livelihood directly or indirectly which makes India an agrarian economy. A greater proportion of land is utilized for agronomic practices and an extensive range of crops are cultivated in its diverse agro-ecosystems (Rani et al. 2019; Singh et al. 2019, 2021; Sharma et al. 2020a, b, 2021; Kumar et al. 2020). With a production of 93.9 million tons of wheat, 104.6 million tons of rice, 21.6 million tons of maize, 20.7 million tons of millets, 357.7 million tons of sugarcane, 8.1 million tons of fiber crops (jute, cotton), 17.2 million tons of pulses, and 30.0 million tons of

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oilseeds crops, in the year 2011–12 (Ministry of Agriculture 2012), it is an undeniable fact that such a major crop production would generate an enormous volume of crop residues both on-farm and off-farm. The crop production leads to the generation of around 500–550 million tons of the crop remains on an annual basis in the country. These remains of harvest often find usage as feed for animals, for producing bio-manures, soil mulching, thatching for houses in rural areas, and as fuel for home as well as industrial purposes. Such residual crops are of great significance to the farming community.

Conversely, a major proportion of these residues are set to fire at the site predominantly for the purpose of field clearance to sow the subsequent crop. Surprisingly, this problem of burning the residual crops is escalating in the current years as a result of the unavailability of human labor, inefficacy of traditional practices of residue removal, as well as the employment of high-tech machinery to harvest the crops. The remains of maize, cotton, rice, millet, jute, wheat, sugarcane, rapeseed-mustard, and groundnut are usually set to fire on fields in different parts of the country. The agricultural systems primarily relying on irrigation systems, predominantly the mechanized rice-wheat belt of northwest India is more prone to this problem (IARI 2012). However, there is a paradox; setting the residual crops to fire and prevailing insufficiency of fodder co-occur in the country, which thereby leads to a noteworthy and perpetual intensification in costs of fodder. But, the ease of removal and lack of awareness is sufficient enough to persuade the farming community to set the residual crops to fire. As per the reports of the Ministry of New and Renewable Energy, India burns around 92 MT of crop residues on an annual basis (Bhuvaneshwari et al. 2019).

The burning of these residual crops directs the generation of smoke as well as soot elements which results in severe animal and human health-related complications. Additionally, this act is also blamed for the release of several gases responsible for the greenhouse effect, such as, nitrous oxide and carbon dioxide which direct the happening of the phenomenon of global warming coupled with the harm to important plant nutrients. The act of burning also leads to the depletion of several valued possessions which have the potential of being utilized as a valuable basis of organic carbon, bio-active complexes, forage, and energy for rural households and small industries. The heat energy produced by the burning of crop leftovers is also responsible for elevating the temperature of soil which results in the mortality of diverse advantageous microbes. The burning of the crop remains leads to an immediate upsurge in the exchangeable $NH4^+$-N and bicarbonate- extractable P content, but there is no buildup of nutrients in the profile. Long-term burning reduces total N and C, and potentially mineralizable N in the upper soil layer. A diverse array of pollutants that originate in enormous amounts from biomass smoke are alleged to be potent carcinogens and thereby might be a chief source of concern directing numerous air-borne diseases (IARI 2012). Figure 13.1 illustrates the diverse consequences of agro-residue burning faced by different biotic and abiotic elements of the ecosystem which are associated with mankind in either a direct or an indirect manner.

The capability of the resources derived from biomass is getting ever-increased attention, and thus, has become a focus for ever-increasing research and debate as

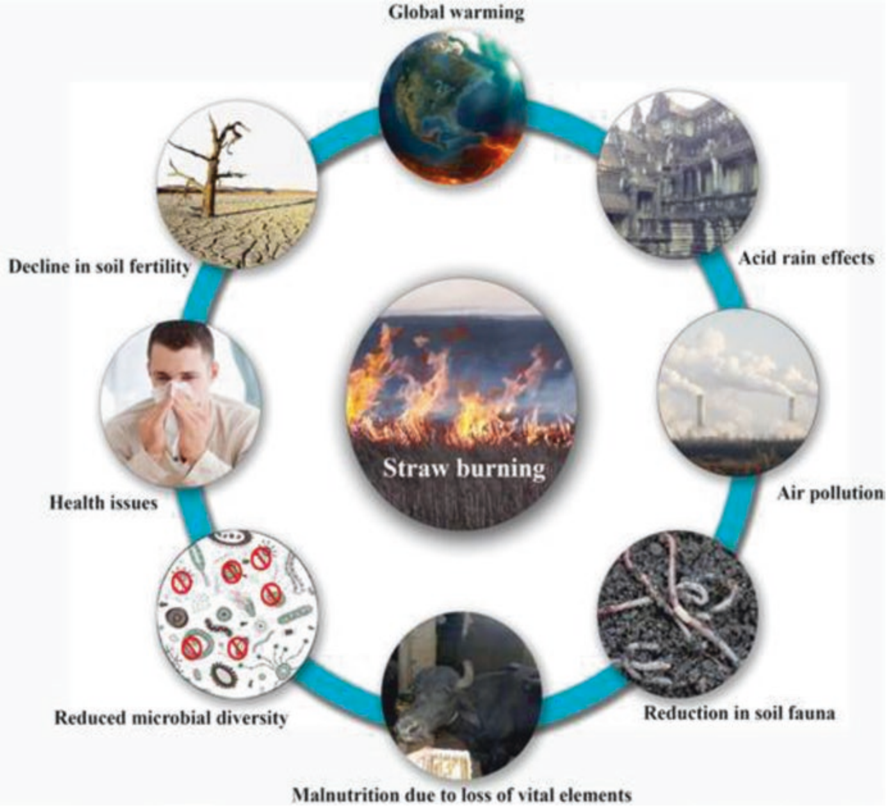

Fig. 13.1 Adverse effects of burning crops residues

well. There are numerous agreements happened across the globe, for instance, the Kyoto Protocol, EU Directives along with several policies such as the European 20:20:20 Plan and the US Recovery and Reinvestment Act which have collectively laid enormous pressure on the political proportion which in turn has directed the focus of mankind as well as the scientific community towards use of agricultural residues as a potential alternate candidate as effective energy carrier (Bentsen et al. 2014). Moreover, the perpetual and unexpected increase of the prices of crude oil in the year 2008 also carved commercial consideration for alternate energy assets. The United Nations have speculated that the global population will upsurge to 9.1 billion by the year 2050 (United Nations 2011), which as a consequence will lead to the increased demand for food, materials, and energy. The International Energy Agency estimates that the energy consumption will increase with an expected 1.6% annual rate from 2005 to 2030 (Hiloidhari et al. 2014).

Thereby, the numerous ill-effects of burning crop leftovers and present management practices coupled with their energy potential have directed the concerns of the global scientific community to find a potent and easily approachable alternate for managing the crop residues in such a way that would advocate sustainability, be economically viable and easy to execute (Table 13.1). Therefore, microbes having tremendous potential for remediation of agricultural residues seem to be an effective and viable means for managing crop residues. Microbes are potent enough to biologically transform the agro-residues into valuable feed for promoting animal health and into compost for up upgrading soil health thus indirectly promoting human health and alleviating stress from the petrochemical industries. The microbial systems bring out the biotransformation process through secretion of various primary and secondary metabolites (Kapoor et al. 2020; Sharma et al. 2019). Thereby, in this chapter, an attempt has been made to club the information available on microbial management of diverse agricultural residues.

13.2 Residue Potential in India

The non-eatable parts of plants that are left in the field after harvest is said to be crop residues. The wastes produced during the processing of crops and from crop-packing plants are also deliberated to crop residues (Sadh et al. 2018). The residues generated by diverse crops fluctuate extensively in terms of their approximate quantity. There is no direct measurement of these crop leftovers rather the estimates are made based on data on the area and manufacture of diverse crops, and research facts on the straw/grain ratio. The wastes engendered during the harvesting as well as the processing of agrarian vegetative crops are extensively classified into two types: (1) Field residues: these are the materials that are left in the cultivated land or plantation areas after reaping the crop. These are usually comprised of stalks, seed pods, stems, and leaves. Such residues can be nurtured unswervingly into the ground or burned first. The appropriate supervision of such leftovers can lead to an effective accomplishment of the irrigation proficiency along with an operative check on the soil erosion. (2) The other type of residues is called process residues: which results from the processing of a crop into a utilizable resource (Fig. 13.2). Such residues are often represented by seeds, bagasse, roots, husks, and molasses. Such residues have the capability of being utilized as fodder for animals and fertilizers for soil health enhancement (Ali et al. 2019).

It has been assessed that around 686 million tons of total residue is generated in India per year as a result of cultivation of 26 different crops which results in 39 types of the crop remains. A major proportion of around, 545 million tons is collectively added through the production of pulses, cereals, sugarcane, and oilseeds. The horticultural crops, primarily, banana, coconut, and areca nut contribute to around 61 million tons of residues whereas 80 million tons is contributed by other crops such as jute and cotton. If the classes of crops are concerned than the highest proportion of 368 million tons is contributed by the cereals which are equivalent to around 54% of the total residue generated. The residues generated by sugarcane are although much less as compared to those of cereals but they represent a significant proportion of around 16% equaling 111 million tons of residues. If the individual

Table 13.1 Environmental issues of agricultural residues

Agricultural residues	Problem associated with residue burning	Area of study	Environmental and health hazards	References
General crop residues	Enhancement in $PM_{2.5}$ and PM_{10} during crop residue burning period	Agra, India	The smoke plume originated from burning of agricultural crop-residue release particulate matter, carbon monoxide, carbon dioxide, nitric oxide, and volatile organic carbons.	Kumari et al. (2020)
Burning of wheat and paddy straw of about 20.3 and 9.6 million tons in Punjab and Haryana	Emission of 137.2 and 56.9 gigagrams of $PM_{2.5}$ and 163.7 and 72.1 gigagrams of PM_{10} for Punjab and Haryana, respectively	North India	The emissions of elemental carbon, organic carbon, and polycyclic aromatic hydrocarbons were 8.6, 45.7, and 0.08 gigagrams in Punjab, whereas in Haryana emissions were 3.7 Gg, 17.7 Gg, and 0.03 gigagrams, respectively. These were produced as a result of wheat and paddy straw burning in around 30,000 and 8500 active fires in Punjab and Haryana, respectively.	Singh et al. (2020)
Crops harvested in autumn	Increase in the levels of $PM_{2.5}$	North China plain	The levels of $PM_{2.5}$ during the harvesting and post-harvesting periods increase by a factor of 1.20 and 1.73, respectively.	Li et al. (2020)
General crop residues	Increased concentration of pollutants	North India	The average concentration of PM_{10}, $PM_{2.5}$, and PM_1 were 196.7 ± 30.6, 148.2 ± 20, and 51.2 ± 8.9 μgm^{-3} and daily average concentration were found several times higher than national ambient air quality standards for 24 h.	Ravindra et al. (2019a)
General crop residues	Air pollution	Nepal	More than 80% of air pollutants were generated during the months of February to May from the open burning of crop residue leading to health impact and regional warming.	Das et al. (2020)

(continued)

Table 13.1 (continued)

Agricultural residues	Problem associated with residue burning	Area of study	Environmental and health hazards	References
Rice straw	Sub-acute effect on pulmonary functions of healthy subjects	India	Crop residue burning events are highly dangerous for the health of the citizens.	Agarwal et al. (2012)
Rice and wheat residue burning	High PM levels	North India	Significant reduction in the Forced Vital Capacity and Peak Expiratory Flow and the lung capacity of children recovers only up to 80% after the crop residue burning events.	Gupta et al. (2016)
488 MT of total annual crop residue	Emissions of 824, 812, 58 and 239 gigagrams of $PM_{2.5}$, PM_{10}, elemental Carbon and organic Carbon respectively and 211 teragrams of CO_2 equivalent greenhouse gases	India	Residue burning emissions will increase by 45% in 2050. The crop residue has the potential to meet 10% of the current energy demands of India.	Ravindra et al. (2019b)
Paddy straw	Increased levels of benzenoids, acetonitrile, and isocyanic acid	India	Benzene exposure increase risks of cancer, cardiovascular diseases, and cataracts by 25 per million children and 10 per million adults.	Chandra and Sinha (2016)
General and regional crop residue	Increased levels of polycyclic aromatic hydrocarbons	Indo-Gangetic Plains of India	Increased levels of Anthracene, fluoranthene, pyrene, benzo[a] anthracene, and chrysene which be carcinogenic.	Singh et al. (2013)
Paddy straw	Soil health deterioration	Indo-Gangetic Plains, India	Burning of paddy straw cause rapid deterioration of soil microbial population and enzyme activity which compromise agricultural productivity.	Kumar et al. (2019)
Wheat straw	Reduced microbial dynamics of soil	Pakistan	Burning wheat residue significantly declines the soil microflora and also interfere with soil chemical and physical attributes like reduced soil carbon and nitrogen content along with the degradation and deterioration of soil.	Raheem et al. (2019)

(continued)

Table 13.1 (continued)

Agricultural residues	Problem associated with residue burning	Area of study	Environmental and health hazards	References
Wheat straw	Increase in atmospheric concentration of low-molecular weight monocarboxylic acids	China	High abundances of low molecular weight organic acids in the atmosphere can adversely affect the quality of air, human health and also increase the acidity of rainwater. Burning of agricultural residues also contributes to the formation of organic aerosols.	Mochizuki et al. (2017)
General and regional crop residue	Increase in aerosols over the South China Sea	China	Atmospheric aerosol particles can significantly affect the Earth's climate directly by absorbing and scattering solar irradiation, and indirectly by acting as cloud condensation nuclei.	Song et al. (2018)
Pinus sylvestris, P. abies	Reduced abundances and species richness of soil meso- and macrofauna	Sweden	Residues burning reduce the diversity of soil fauna and disturb the food chain which reduces ecosystem productivity due to the decreased number of predators and fungivores.	Malmström et al. (2009)

crops are considered for residue generation than rice is found to be dominating the league with a residue generation of around 154 million tons followed by wheat (131 million tons). However, if only the availability of surplus residue is considered than the national potential is found to be 234 million tons on an annual basis which represents around 34% of the gross residue generated in India.

The highest amount of surplus residue is also contributed by the cereals which are equivalent to 89 million tons of annual residue. It is mainly followed by the sugarcane with an annual residue generation of 56 MT, others (47 million tons), horticultural crops (23 million tons), oilseeds crops (14 million tons), and pulses (five million tons). If an individual crop is considered for the generation of surplus residue than sugarcane is found to be dominating the field with an annual production of around 56 million tons, followed by cotton with a residue potential of 47 million tons and rice (43 million tons). However, rice was found to be dominating in the gross residue production but it is lagging behind sugarcane when the generation of surplus residue is considered. This phenomenon is attributable to the fact that the residues generated by rice crops in the form of husk and straw often find more contending usages such as in cattle and animal feed, in packaging materials, and as fuel for heating and cooking purposes as compared to the residues generated by the

Fig. 13.2 Origin of diverse agricultural residues

sugarcane. The surplus residue generated by horticultural crops primarily coconut and banana also contribute to a significant proportion equivalent to 10 and 12 million tons, respectively.

The residue potential also varies state-wise, for instance, Uttar Pradesh generates a maximum crop residue of 121 million tons whereas Mizoram generates only 0.21 million tons of crop residues on an annual basis. Uttar Pradesh, being an agriculturally important state usually dominates in the crop production of sugarcane, wheat, and rice thereby a major proportion of around 90% of the crop residues generated is contributed by these three prime crops. Punjab follows Uttar Pradesh by generating an annual residue of 83 million tons. However, if only the generation of surplus residue is considered, still then, Uttar Pradesh dominates by an annual production of 40 million tons which is closely followed by Maharashtra by generating 31 million tons surplus residue, and by Punjab with a surplus residue generation of around 28 million tons (IARI 2012; Hiloidhari et al. 2014).

According to the Ministry of New and Renewable Energy India, a major proportion of the crop residues is set to fire at the field conditions. On a collective basis around 92.81 million tons of crop residues are burned on an annual basis. Uttar Pradesh is leading here as well with an annual burning of around 21.92 million tons of residues. Uttar Pradesh is followed by Punjab where 19.65 million tons residue is burned each year. Haryana and Maharashtra are also significant contributors in this race by annually burning of 9.08 and 7.42 million tons of crop residues, respectively (NPMCR 2014).

13.3 Current Management Practices

13.3.1 Bedding and Feed for Animals

The Indian farming community has traditionally been utilizing the crop remains as animal feed in their native form or by accompanying some additives. Conversely, the crop remains, are largely unpalatable and often show low digestibility, thereby, cannot be utilized solely as the feedstock. They are also low-density fibrous materials, having low nitrogen content, soluble carbohydrates, minerals, and vitamins. They may also have varying degrees of lignin content which acts as the physical constraint and obstructs the microbial breakdown of feed. Therefore, the residue needs to be preprocessed to meet the nutritional requirements of animals. It is also used in combination with other green fodders and legume (sun hemp, horse gram, cowpea, and gram) straws. Other low-quality residues are also often being used as bedding material for animals.

13.3.2 No-Tillage and Recycling of Crop Residues

It is a farming practice wherein the soil is not disturbed through the process of tillage. The crop residues are allowed to prevail in the field and are subject to natural decay. This practice is acclaimed for prevention of soil erosion since the crop residues hold the soil tightly and protect the soil from wind or water erosion (Triplett and Dick 2008; Telles et al. 2018); but there is a considerable drop in the yield of the crops.

The crop remains can also be recycled directly, by their amalgamation into the soil using several means. The crop residues can also be used as mulches and are often returned to the field in combination with animal manures. However, this is an indirect but traditional practice of agriculture that has made significant and irreplaceable contributions for promoting agricultural yield along with the advocation of environmental sustainability. The soil receiving such treatments are found to be rich in soil organic matter, facing very little soil erosion, enhanced water storage ability, and are collectively healthier as compared to others (Smil 1999; van der Wiel et al. 2019).

13.3.3 Biochar Production

Biochar is a high carbon material that is produced by slowly heating the biomass in the absence of oxygen. It is a fine-grained type of charcoal and is largely capable of storing carbon in the soil for a longer period. Conversely, the elevated production costs make its production process a highly costly affair, therefore, the practice of using biochar is not much prevalent in the farming community. However, the utilization of all the valuable goods and co-products, for instance, heat energy, hydrogen gas, and bio-oil that are generated during the process of biochar formation could make it an economically viable process. So, the development of a low-cost production process for generating biochar can also popularize its use.

13.4 Microbes for Residue Management

The wastes of agricultural origin are of significant importance and their proper management can prove to be highly economical due to the possession of numerous hidden capabilities. The microorganisms can be unbelievable agents for managing agricultural residues. Since the act of burning the residues at the field, conditions result in several ill effects. It leads to the loss of soil nutrients and therefore strongly affects the soil properties. Moreover, the emission of greenhouse gases deteriorates environmental health. The burning of agro residues also disturbs the microbial population as well as diversity at the field conditions which are considered to be very important elements for maintaining soil fertility. The ease of disposal often compels the farming community to burn the residues but it brings a gamut of challenges with it. Therefore, alternate ways for the management of agro residues are one of the most favorite agricultural technique being sought for.

The potential of microbes is often utilized to return the agricultural residues in the form of compost for elevating the nutrient status of the agricultural farms. The lignocellulolytic microbes are endowed with the capability of recycling and reusing agricultural wastes by transforming them into other forms. Therefore, the unique potential of microbes is being quested for managing the residues sustainably and more easily that could also be economically viable. The potential of microbes has already been explored for the transformation of biomass into biofuels and other useful products. Figure 13.3 depicts a diagrammatic representation of different products that can be produced by treating the agricultural residues with definite microbes.

13.5 Residue Management by Compost Preparation

Agriculture and food industries are among the ancient practices of mankind, but they too lead to the generation of a gamut of wastes thereby are strongly correlated with other industrial sectors in this particular aspect. The administration, as well as

Fig. 13.3 Products from microbially-treated agro-residues

control of wastes generated by the food and agronomic sector, is going to play an imperative role in the near future as per the preservation of diverse natural possessions is concerned. The process of composting agricultural residues is a much efficacious strategy utilizing the principles of microbiology for managing the residual products of agro-ecosystems sustainably. The word “composting” means the process of controlled and organized biological development where the complex forms of organic matter found their origin from either animal or plant resources is disintegrated into materials having shorter molecular chains, enhanced stability, clean, humus-rich, and are advantageous for the cultivated crops and recycling of soil organic matter (Sequi 1996; Sánchez et al. 2017). A diverse array of microbes is known for mediating the process of composting: bacteria, fungi, actinomycetes, algae, and protozoa, which contribute naturally to the organic biomass or are added artificially (Tuomela et al. 2000; Sánchez et al. 2017).

The process of composting agro-residues is largely governed by the activity of lignocellulolytic microbes which seem to be proficient agents for managing as well as recycling the lignocellulosic wastes having a great pecuniary competence. The recycled matter on application to the soil systems enhances the fertility as well as the health of the soil. The process of composting allows the biological degradation and steadying of the organic matter under a set of conditions that promote the thermophilic microflora to proliferate as an outcome of the biologically originated heat (Gaur 1999; Onwosi et al. 2017).

Initially, there is a succession of mesophilic microbes which consume the nutrients and are responsible for raising the pile temperature. The next phase allows the progression of thermophilic microbes which further results in a stable final product that is devoid of any kind of pathogen and suitable for application to the fields. A diverse array of agricultural wastes can be utilized for composting like paddy straw, sugarcane trash, and other agro-residues. The process of composting experiences the natural succession of microbes. Several fungi are known to play a significant role in degrading lignocellulosic wastes during composting, for instance, *Phanerochaete chrysosporium*, *Trichoderma harzianum*, *Polyporus ostriformis*, and *Pleurotus ostreatus* (Singh et al. 2012).

The higher lignin content of the crop residues is often responsible for restricting the enzymatic attack by microflora; which is largely responsible for the long periods required for composting. Numerous members belonging to the group fungi are known for their possession of lignocellulolytic activity. They are broadly classified into three major groups: soft rot fungi, brown rot fungi, and white-rot fungi (Kirk 1983; Singh et al. 2012). The soft rot fungi, such as *Chaetomium globosum*, *Phialophora malorum*, *P. mutabilis*, *Aspergillus niger*, *Penicillium chrysogenum*, and *Chaetomium globosum* are eminently capable of degrading cellulose but they decompose lignin slowly and almost incompletely. The brown rot fungi, for instance, *Oligoporus placenta*, *Coniophora puteana*, *Fomitopsis palustris*, *Coniophora puteana*, and *Poria placenta* preferably degrade the carbohydrate constituents and are also responsible for the demethylation of lignin. White rot fungi, such as *Schizophyllum commune*, *Pleurotus sajor caju*, *Trametes versicolor*, and *Phanerochaete chrysosporium* are endowed with the incredible capability of decomposing cellulose as well as lignin. There are numerous other bacteria and actinomycetes which convert the complex matter into simpler ones that are suitable for soil application. The application of compost to the soil systems is highly beneficial for enhancing soil as well as plant health.

The application of compost in the soil is an important way of improving the physical, chemical as well as biological properties of the soil. It also works well for restoring the organic carbon pool of the soil. It results in better mineral nutrition of the plant, and hence, is responsible for increasing the yield of agricultural produces. The composts enriched with a particular mineral are potent enough to compete with the costly chemical fertilizers. The application of composts is also acknowledged for the suppression of soil-borne pathogens (Singh et al. 2012). The action of composts also restricts the bioavailability of toxic heavy metals owing to the occurrence of different humic substances and iron oxide in composts. The steadied organic matter is deliberated to form multiplexes with metals which results in the constrained movement of heavy metals and thus reduction in their availability for plant systems (Paré et al. 1999; Piccolo et al. 2019). The dynamic activity of microbial systems throughout the progression of composting has the potential to hasten the disintegration of xenobiotic compounds in the soil (Büyüksönmez et al. 2000). The soil dehydrogenase activity also increases considerably by the addition of compost to the soil. Therefore, the act of composting of agro-residues not only helps in getting rid of the complex agricultural residues but can also be utilized effectively for uplifting the health status of agro-ecosystems.

13.6 Transforming Residues into Biofuel

The atmospheric level of carbon dioxide along with other greenhouse gases is increasing ever since the commencement of domestication of plants for agricultural systems 10,000 years ago (Ruddiman 2003). The onset of the Industrial Revolution since about 1850 has directed the international attention in ascertaining newer approaches for a reduction in the levels of gaseous emissions (IPCC 2000). The agro-ecosystems can be a basis as well as a basin for the atmospheric carbon dioxide as per the land use patterns and its management options are concerned. The transformation of biomass into biofuel received major consideration in the course of the 1970s on account of the insistence of accomplishing energy autonomy. The quest for mitigating global climatic changes developed an improved concentration in biomass energy since the mid-1990s. The approach of utilizing crop residues as a potential substrate for biofuel production has significant implications for comprehending these goals.

The crop residues have the capability of becoming a chief source of energy attributable to their influence on compensating emissions resulting from the use of fossil fuels. The crop leftovers are supposed to have a heating worth of around 3×10^6 kilocalories/megagram, which is approximately 50% of that of coal and 33% of that of diesel. The fuel value of 1 megagram of crop residue is appraised at 18.6×10^9 Joule, 2 barrels of diesel, 3×10^6 kilocalories, or 16×10^6 British Thermal Units (Lal 2005). Therefore, it can be said that agriculture is deliberated to be a rich source of energy because it fabricates biomass, which has the potential of being utilized as biofuel and is a renewable resource (Table 13.2). However, the energy content of different crop residues varies among crop species.

13.6.1 Bioethanol Production

The ethanol originated from the biomass resources is highly potent to be used as a sustainable fuel for transport, along with a fuel oxygenate that has the capability of replacing gasoline. The energy content of ethanol is further found to be higher than the energy required to produce it (Wang 2000; Kim and Dale 2004). Brazil and the US are deliberated to be major producers of ethanol and they account for 62% of global ethanol production. The foremost substrate, however, used in Brazil is sugar cane, while corn grain is utilized for ethanol production in the US. The increasing debate over the food/feed vs fuel issue and the ever-increasing global attention for managing the residual crops have directed the focus of mankind towards the

Table 13.2 Agricultural residues as substrates for microbial production of biofuels

Biofuels	Residue type	Pretreatments	Mechanisms/ processes	Microorganisms involved	Significant findings	References
Bioethanol	Coffee husk, cassava stem, and coconut coir	Popping pretreatment	Saccharification and fermentation	*Saccharomyces cerevisiae* KCTC 7906	Production of bioethanol from mixed biomass is a more promising approach	Nguyen et al. (2017)
	Rice residue	Microwave-assisted alkali and acid pretreatment	Saccharification and fermentation	*Trichoderma reesei* NCIM 1052 for Saccharification and *Pichia stipitis* NCIM 3499 for fermentation	*P. stipitis* NCIM 3499 gave a yield of 25.3 g/L of ethanol which exhibits its commercial potential.	Prasad et al. (2020)
	Paddy straw	Pretreatment with white-rot fungus, *Trametes hirsuta*	Saccharification and fermentation	*Saccharomyces cerevisiae*	Biological pretreatment proved to be a feasible method generating higher sugar yields.	Arora et al. (2016)
	Wheat straw	Pretreatment with H_3PO_4 plus H_2O_2	Simultaneous saccharification and fermentation	*Saccharomyces cerevisiae*	15.5 g ethanol was harvested from 100 g wheat straw which indicates its commercialization potential.	Qiu et al. (2018)
Biohydrogen	Wheat straw	Ozonation	Simultaneous enzyme hydrolysis and dark fermentation	Native microbiota of a slurry mixture of cow manure and a sediment	The ozone pretreatment efficiently degraded wheat straw lignin, and the delignification increased with an increase in the applied ozone dose	Wu et al. (2013)
	Fruits and vegetables waste and corn Stover	Acid pretreatment	Dark Fermentation	Hydrogenogenic inoculum was obtained from an anaerobic digester fed	Hydrogen production presented economic benefits such as net revenues of 0.009 USD per kg of co-substrates	Rodríguez-Valderrama et al. (2020)

	Fruit wastes	Moist heat treatment	Fermentation	*Clostridium strain* BOH3	*Clostridium strain* BOH3 has the unique capability of excreting saccharolytic and pectinolytic enzymes and producing a high level of hydrogen	Mahato et al. (2020)
	Waste corn cobs	Alkaline pre-treatment	Dark fermentation	Native microbiota of mixed wastewater sludge from the municipal sewage treatment plant	The alkaline pretreatment method proved to be effective	Kucharska et al. (2020)
Biodiesel	Corncob	Acid hydrolysis	Yeast based fermentation	*Rhodotorula taiwanensis* AM2352	Yeast based conversion of corncob hydrolysate into microbial lipid followed by the lipid transmethylation for biodiesel production	Miao et al. (2020)
	Barley Hull	Acid hydrolysis	Conversion of barley hull hydrolysate into lipid by yeast	*Trichosporon cutaneum*	*Trichosporon cutaneum* is a promising yeast for biodiesel production	Guerfali et al. (2018)
Biobutanol	Rice straw	Acid and Alkali pretreatment	Fermentation	Co-culture of *Saccharomyces cerevisiae* and *Pichia*	Addition of *S. cerevisiae* promoted butanol synthesis pathway which led to the higher butanol concentration	Mohapatra et al. (2020)
	Potato peel	Acid pretreatment	Acetone-butanol-ethanol fermentation	*Clostridium acetobutylicum*	Acetone-butanol-ethanol concentration of 24.8 g/L indicates that potato peel is an appropriate substrate for butanol production	Abedini et al. (2020)
	Banana peel	Acid pretreatment	Separate enzymatic hydrolysis and co-fermentation	Co-culture of *Saccharomyces cerevisiae* and *Pichia*	Co-culture of *Saccharomyces cerevisiae* and *Pichia* proved better for butanol production as compared to monocultures	Mishra et al. (2020)

(continued)

Table 13.2 (continued)

Biofuels	Residue type	Pretreatments	Mechanisms/ processes	Microorganisms involved	Significant findings	References
Biogas	Paddy straw	NaOH-microwave pretreatment	Anaerobic digestion	Native microbiota of digested biogas slurry	Supplementation of microwave irradiations enhanced the effectiveness of NaOH	Kaur and Phutela (2016)
	Wheat and pearl millet straw	Alkaline pretreatment	Anaerobic digestion	Biogas slurry of another biogas plant was used as inoculum	Mild alkaline pretreatment was effective in enhancing biogas production from both straws	Kumar et al. (2019)

agricultural residues as the potential substrate for ethanol production (Wyman 2018). The abundant biomass resources are largely comprised of agricultural and forestry residues and various other woody and herbaceous crops that are often cultivated on underutilized lands.

The net release of carbon dioxide gas that can contribute to global climate change can be practically zero by employing biomass as the substrate. The behaviors of ethanol as a cleaner fuel with low emissions of carbon monoxide and its capability to improve combustion in addition to gasoline strongly advocate its production (Lynd et al. 1991; Tyson 1993; Gupta and Verma 2015). In the US ethanol is blended with gasoline at the rate of 10% whereas in Brazil it is blended at the level of 22%. Surprisingly, India, being an agrarian country is lagging in executing such environmental policies up to this level. The vast volume of agro-residues generated in India offers a low-cost substrate for ethanol production which would surely decrease the reliance on petroleum resources along with the transformation of the problematic crop leftovers into cleaner fuel.

The crop residues are usually called as lignocellulosic substrates. The worldwide generation of plant biomass is approximately 200×10^9 tons/year; however, nearly 8×10^9–20×10^9 tons is potent enough to be employed as a substrate for biofuel production (Zabed et al. 2017) which is either available at no cost or at a low cost thereby attracting attention as a potential substrate for bioethanol production. A major proportion of lignocellulosic biomass of about 35–50% is comprised of cellulose and 20–35% is made up of hemicellulose. The bulky portion of the residual material is made up of lignin.

Cellulose and hemicellulose together represent around 65–75% of the total lignocellulosic biomass composition; these materials can be broken down into their component sugars for fermentation into bioethanol, as much as for starch conversion to sugars. However, producing sugars from cellulose and hemicellulose at high yields is far more difficult than deriving sugars from corn or sugar cane. Therefore, even though the cost of lignocellulosic biomass is far less than that of sugar and starch crops, the cost of obtaining sugars from such materials for fermentation into bioethanol has historically been far too high to attract industrial interest. However, with the emergence of new technology, economics have improved considerably (Wyman 2018).

Globally rice straw can produce 205 gigalitres of bioethanol, which is the largest amount from a single biomass feedstock. The next highest potential feedstock is wheat straw, which can produce 104 gigalitres of bioethanol (Kim and Dale 2004). The microbes that could be employed for bringing out such fermentation processes should be resistant to the presence of inhibitory compounds, should be tolerant to higher ethanol levels along with the ability for production of higher ethanol yields. The yeast *Saccharomyces cerevisiae* is usually used for such fermentation processes.

However, several other yeast strains, for instance, *Pichia stipitis* (NRRL-Y-7124), and *Kluyveromyces fagilis* (Kf1) are described as potent ethanol producers from diverse substrates. The hemicellulose is largely comprised of a mixture of pentose and hexose sugars. Only a few yeasts belonging to the genera *Pichia*, *Schizosaccharomyces*, *Candida*, and *Pachysolen* are proficient enough to ferment pentose sugars to ethanol (Mussatto et al. 2012). The yeast *K. Marxianus* has got the unique capability of co-fermenting both hexose as well as pentose sugars (Yanase et al. 2010).

The major hindrance in bioethanol production is the problem in pentose fermentation which can be resolved by using hybrid, genetically modified, or co-culture of two yeast strains. The hybrid yeast cells have the unique ability to utilize pentose as well as hexose concurrently for ethanol production. The genetically engineered yeast strains contain genes from other microbes which makes them capable of utilizing a previously non-utilizable substrate. The approach of using co-culture employs two diverse yeasts simultaneously in the same reactor. It gives an elevated yield as compared to the employment of pure cultures.

The yeast which is capable of pentose fermentation, for instance, *Pichia fermentans* and *Pichia stipitis* can be employed with a hexose fermenting yeast such as with *S. cerevisiae* with the intention of effective co-consumption of hexose as well as pentose sugars (Azhar et al. 2017). The bacterium *Zymomonas mobilis* is also capable of bringing out such conversions. But they are often limited by their capability of utilizing only a single substrate for bioethanol production coupled with the complexity of the biomass substrates. However, several attempts have been made to genetically modify other microbes like *Escherichia coli* and *Klebsiella oxytoca* to upgrade their substrate utilization range as well as capability.

13.6.2 Biobutanol Production

Ethanol has been widely accepted as a biofuel and has also been found much suitable than methanol owing to its renewability; therefore, it has been widely employed as an additive or alternate fuel in several nations like the US, China, Brazil, etc. Conversely, the employment of ethanol invites numerous grave concerns which further need to be addressed for use of ethanol as a fuel. The use of ethanol is found to corrode the prevailing pipelines by common corrosion, wet corrosion as well as dry corrosion. The general corrosion is, however, a result of different ionic contaminations whereas the dry corrosion is accredited to the ethanol molecule as well as its polarity (Jin et al. 2011).

There are various metals, for instance, lead, aluminium, and magnesium which are vulnerable to be attacked chemically by dry ethanol. Ethanol, by absorbing moisture from the air is also responsible for wet corrosion which results in oxidation of most of the metals. It is also known to affect various nonmetallic parts in different ways (Hansen et al. 2005). Therefore, butanol seems to be a much competent biofuel attributable to its diverse advantages. It is also a biomass-derived biofuel that is renewable in nature and can be produced by fermentation processes using biomass feedstocks as substrates. Although, being a 4-carbon entity, it is much complex as compared to simpler alcohols; however, it is equally competent to be blended with gasoline. Furthermore, it has the incredible ability to get blended with diesel oil also. Since it contains more oxygen than methanol, ethanol can effectively reduce soot generation when used with diesel oil. It requires a lower temperature for combustion, therefore, owes a greater heat of evaporation thus can also help in the reduction of NOx discharges (Rakopoulos et al. 2010). Consequently, the employment of butanol as biofuel seems to be more appealing as equated to the extensively used ethanol as well as biodiesel.

Butanol is produced by the process of fermentation by several rod-shaped, spore-forming, anaerobic, and Gram-positive bacteria called clostridia. The industrial production of butanol is restricted by several factors and one among them is the elevated substrate cost coupled with lower yields. The economics behind the production process is largely governed by the fermentation substrate. Therefore, various renewable, as well as economically realistic substrates are always a matter of concern (Lépiz-Aguilar et al. 2011). Therefore, the easily available and low-cost lignocellulose materials seem to be offering several potential benefits over prevailing, energy-demanding bioethanol manufacturing methods. An acetone-butanol-ethanol fermentation plant in Russia is supposed to be the only fermentation plant that works at an industrial scale by utilizing lignocellulosic waste materials as a substrate for butanol fermentation (Jin et al. 2011). The bacteria *Clostridium acetobutylicum*/*Clostridium beijerinckii* are most often employed for the production of acetone–butanol–ethanol. Furthermore, it is also found that pentose sugars accompanied by hexose sugars are competently utilized by the same microbial culture.

The usage of both the sugars as substrate at similar times may make acetone–butanol–ethanol fermentation much striking than ethanol or any other solvent production process (D'Aquino 2007). The additional benefit of employing these bacteria as compared to others is their capability of utilizing both these lignocellulosic hydrolysate sugars as opposed to conventional ethanol-fabricating yeast species which are unable to use them. It has been reported by several researchers that the agricultural residues receiving proper pretreatment are fermented by the microorganism especially by *Clostridium beijerinckii* without any inhibition. Furthermore, it has also been found that the bacteria are capable of fermenting the agro-residues at a rate quicker than the control fermentations utilizing glucose as the substrate (Qureshi et al. 2008). There are numerous microbial strains that can also be utilized for butanol production, for example, *Clostridium acetobutylicum* P262 (renamed as *C. saccharobutylicum*), *C. beijerinckii* P260, *C. acetobutylicum* NRRL B643, *C. acetobutylicum* ATCC 824, *C. beijerinckii* LMD 27.6, *C. acetobutylicum* B18, *C. beijerinckii* BA101, *C. saccharobutylicum* P262, *C. aurantibutyricum*, *C. butylicum*, and *C. tetanomorphum*.

All these producer strains have earlier been used for industrial production processes. However, the culture of *Escherichia coli* has also been manipulated genetically for enhanced production of butanol (Qureshi and Ezeji 2008). Several other microorganisms have also been manipulated genetically in the quest to produce more robust and butanol tolerant species comprising *Corynebacterium glutamicum*, *Lactobacillus brevis*, *Pseudomonas putida*, *Bacillus subtilis*, and *Lactobacillus* *buchneri* (Qureshi et al. 2013a). Several agricultural residues have been studied for the production of butanol, for instance, wheat straw, corn stover, switchgrass, barley straw (Qureshi et al. 2013b). Therefore, the correct pretreatment coupled with optimal downstream processing could be an effective measure for exploiting agro-residues at an industrial scale for enhanced butanol production.

13.6.3 Biohydrogen Production

Nations across the globe are in continuous quest to find novel, pollution-free, and renewable sources of energy. The previous decades, however, have largely been dedicated to the production of bioethanol as well as biodiesel. The extra pressure levied by first-generation biofuels on the global food costs has largely added to the contemporary universal food crunch. Therefore, the utilization of agro-residues for energy production via biofuel synthesis seems to be a viable and renewable source of energy (Ni et al. 2006; Angelidaki et al. 2007). Hydrogen gas is deliberated to be one among the encouraging applicants for substituting fossil fuels. The progressions of biological origin are always reflected as the supreme eco-friendly substitutes for sustaining upcoming demands for hydrogen.

Like other biofuels, the production of biohydrogen by utilizing agro-wastes seems to be of many advantages owing to their abundance, low cost, renewability, and extreme biodegradability (Guo et al. 2010). Biohydrogen has the potential to be utilized unswervingly in combustion engines for conveyance, and after decontamination, it can also be used for generating electric power. It has a very high energy content per unit weight (142 kJ/g). The generation of water as the sole by-product by oxidative combustion, makes it a perfect and greatest eco-friendly substitute to fossil fuels (Piera et al. 2006). The elevated costs of hydrogen production, complex storage necessities, and distribution systems are the major factors that have largely restricted the employment of hydrogen gas as fuel (Dunn 2002). Presently, a major proportion of hydrogen is derived from fossil fuels (Nath and Das 2003) and the technique of water electrolysis has comprehensively advanced in current years. Nevertheless, all these methods are energy-demanding and are unsustainable progressions.

The hydrogen finding its origin from biological sources needs much less energy for its synthesis as compared to other methods. Furthermore, the utilization of agro-wastes which are further made up of multifaceted components by complex microbial systems by dark fermentation can prove to be the key technology for the production of biohydrogen gas by utilizing several crops remains (livestock waste and food waste) (Guo et al. 2010). The yield of hydrogen gas from different crop residues varies greatly. The yield of hydrogen is further found to be higher at thermophilic conditions as compared to mesophilic conditions (Karlsson et al. 2008). The varied contents of cellulose, hemicellulose and lignin are largely responsible for the variable yields of hydrogen gas.

The yield of hydrogen is considered to be inversely proportional to the lignin content of the crop residue (Guo et al. 2010). The hydrolytic activity of the producer microbes is often responsible for limiting biohydrogen production. The residue sample receiving suitable pretreatment can give elevated yields of biohydrogen (MTui 2009). A diverse array of microbial cultures is known to be eminent producers of biohydrogen. The pure cultures of hydrogen-producing bacteria mainly belong to *Enterobacter aerogenes*, *Bacillus coagulans*, *Clostridium butyricum*, and *Thermoanaerobacterium* spp. whereas the other bacteria that have been isolated from mixed cultures belong to *Clostridium saccharobutylicum*, *Clostridium pasteurianum*, *C. butyricum*, *Enterobacter aerogenes*, *Thermoanaerobacterium thermosaccharolyticum*, *Caldicellulosiruptor saccharolyticus*, *C. thermocellum*, and *Bacillus thermozeamaize* (Guo et al. 2010).

13.6.4 Biogas Production

Another important way of utilizing the dormant energy of crop residues is through anaerobic digestion. It is also a significant and sustainable measure of guaranteeing the energy supply and would surely play an imperative role in sustaining the future of energy supply from renewable and low-cost substrates. Biogas represents a multifaceted and renewable source of energy that has the potential to bring out the replacement of traditional fuels for producing power as well as heat. Furthermore, it can also be utilized as a gaseous fuel in motorized applications. The advanced form of biogas, biomethane, has the potential to replace natural gas in chemicals production.

Biogas is a renewable source of energy that is produced by the process of anaerobic digestion utilizing several organic and biodegradable substrates, such as, municipal wastes, animal and agricultural remains. It has a methane content of around 40–70% that can further be progressed (Mittal et al. 2018). It has also been well established that the biogas generated utilizing anaerobic digestion is significantly advantageous over other forms of bioenergy attributable to the energy-competent and eco-friendly aptitude of the production technology (Nishio and Nakashimada 2007; van Foreest 2012). In addition, the side product of this process, called digestate, is a high-value fertilizer utilized for crop farming and has the potential to replace common mineral fertilizers.

The biogas production technology is a well-established technology in European countries where the annual production has reached a level of 1.35×10^7 tons in the year 2014 (EurObserv'ER 2014). Germany is the pioneer nation in the worldwide production of biogas, with around 25% installed capability owing to the robust expansion of agricultural biogas plants on farms. It was also found that over and above 8000 agricultural biogas production units were functional in Germany in the year 2014 (Wagner 2015). A large number of countries are in a quest to develop novel pathways for biogas production utilizing biomass as well as residues and wastes as substrates (Edita 2015). The nations like US, China, and India are also spending a lot on developing substitute technologies for biogas production from cellulosic resources, and are deliberated to lead in future biogas production (Soetaert and Vandamme 2009).

The energy potential of agro-residues has also attracted major attention for being utilized as a substrate for biogas production, for instance, one hectare of cereal straw is endowed with an energy potential of 73 GJ which is approximately comparable to 200 liters of oil. However, straw and other products in this category have different combustion characteristics from those of woody fuels. Point transformation in ash and emission behavior of biomass-type straw means that different technical approaches are needed (Ionel and Cioabla 2010). Although it is highly suspicious that biogas derived from waste would meet the global energy consumption; however, the requirement for global sustainable waste management practices has led to research interest in alternate substrates based on agro-wastes (Weiland et al. 2009).

The crop residues generated as agricultural wastes are usually lignocellulosic in nature and appear to be an appealing substrate for biogas production. Their multifaceted structure proves to be a financial as well as a technical obstruction for operating bio-refineries (Yang and Wyman 2004). However, the efficiency can be fairly improved by the choice of proper pretreatment which indirectly governs the performance. The main objective behind the pretreatment is to make the process of anaerobic digestion quicker to increase the biogas yield. Once the complex substrate is converted into simpler units and is subjected to anaerobic digestion, it receives a succession of microbes which ultimately yields a mixture of gases with a major proportion of methane (Achinas et al. 2017).

In the Indian scenario, small and family-type biogas systems primarily exist and prevail in rural communities with their capacities usually alternating from 1 to 10 m^3 biogas production per day. Such plants mainly use animal excreta coupled with agricultural wastes as potential substrates for domestic biogas digesters, yielding biogas as well as bio-slurry with the potential of being used as organic fertilizers. Such plants are mainly accomplished by discrete families for generating energy for self-usage. On the flip side, outsized and industrial-level biogas units having a capacity above 5000 m^3 biogas predominantly use municipal or industrial organic wastes as substrates (Mittal et al. 2018).

However, there are continuous efforts by the government that introduce newer policies and offer biogas plants at subsidized rates for the promotion as well as increasing awareness among the people for setting up newer biogas plants along with the utilization of agricultural residues as substrates rather than burning them. Furthermore, the maintenance of soil organic matter is also advocated by utilizing agro-residues as substrates because the slurry is returned to the field as fertilizer. The slurry also contains a large part of chief nutrients such as phosphorus, nitrogen, potassium, etc. therefore the requirement of chemical fertilizers is reduced considerably since a considerable portion of the harvest from the field is being returned in another form. Additionally, the usage of agro-residues in combination with other substrates can also prove to be much efficacious.

13.7 Residues for Feed and Food Production

Rapid developments in science and technology have increased the living standard of human beings and have resulted in increased demands of food and animal feed. However, the introduction of high-yielding varieties along with chemical fertilizers has contributed a lot towards the enhancement in food crop production which has also led to the generation of huge biomass of agricultural wastes. The exploitation of such wastes is getting increased attention as the recycling of agro-wastes considerably advocates environmental sustainability by significantly reducing the environmental pollution. Moreover, the prevailing scarcity of feed for animals can also be addressed by fashioning new produce from crop residues through involvement of active microbes.

In addition, transforming crop residues into animal feed would be of dual benefit to mankind as it would also address the problem of waste disposal along with livestock feeding. The involvement of microbial systems can fairly improve the available nutrient content along with the enhanced digestibility. Besides, the crop leftovers can also act as a substrate for various kinds of solid-state fermentation for producing food like mushroom cultivation. Therefore, the extended applications of utilizing agro-residues for food and feed production can largely address the problem of food and feed scarcity.

13.7.1 Feed Production

The enhancement in animal feeding is among the significant and basic requirements for the proper management of livestock. The pitiable eminence of food is largely responsible for poor animal health as well as performance. The increased costs of feed have also contributed a lot to the deprivation of livestock from a good quality feed. Thus, acceptable and good-quality feed materials are among the most vital elements in farm administration. A major proportion of around 70% of the expenditure in livestock rearing is mostly for animal feed (Ajila et al. 2012). The fabrication of feed for animals is one of the most reasonable ways that pertain to utilize an extensive share of the agro-residues. However, this approach has been a part of conventional farming practices since the dawn of civilization. The manufacture of feed from agro-residues also signifies one of the chief cash returns to the farming community attributable to the fact that the claim for animal feed is always steady and enormous. The marketing is also relatively easy and the technologies involved are not too complicated.

The agro-residues for instance husk, pods, leaves, and tender stems are feeds with high nutritional value and contain a considerable amount of easily digestible protein. Therefore, they can be utilized for nourishing livestock in consort with the concentrate mixture (Ranjhan 1993; Bhatti and Khan 1996; Godoy et al. 2018). Furthermore, it has also been reported that the treatment of such residues with urea improves the digestibility as well as their nutritive value in a substantial way (Wanapat et al. 2009). The byproducts of one particular crop used in combination with the residues of other crops are also a useful measure of improving the nutritional status of the crop residues used as feed (Ranjhan 1993; Godoy et al. 2018). However, the higher concentration of a particular nutrient in a particular crop often limits its usage, for instance, the chief factor that limits the employment of legume byproducts as feed is the elevated concentrations of phosphorus in it, which results in inhibition of the feed consumption. Such residues are made suitable for consumption by the addition of various other substrates, such as the addition of a combination of molasses and diammonium phosphate or rather a balanced liquid supplement on chopped groundnut straw can help to overcome the feeding inhibition (Maglad et al. 1986; Ajila et al. 2012).

Different crop residues are often utilized directly for feeding animals, such as, wheat bran, which is also the main constituent for feed formulation, can be used to feed sick animals deprived of any side effect and is also known to produce laxative effects in the animal intestine. Bran has also a higher concentration of amino acids as compared to wheat and is also deliberated to be a rich source of water-soluble vitamins. The husk of rice is another important animal feed and is considered to be a good source of fibers. The use of rice bran alone as feed often results in colic pain because it leads to ball formation inside the intestine. Therefore, the use of rice bran in combination with some other residual crops can prove to be efficacious feed. The high oil content of rice bran makes it a suitable substrate for using it as a feed-in combination with other substrates. Maize gluten is also considered to be a very good feed attributable to its higher protein content. The wastes generated by the processing of agricultural products are also utilized as animal feeds. Although, there are certain advantages of using these residues as feed; however, their employment is often restricted by the presence of naturally occurring anti-nutritional elements, variability in the nutritional value of the feeds, and presence of pathogenic microbes and their toxins (Cheeke 1991; Ranjhan 1993; Ajila et al. 2012).

The agricultural residues are found to be highly contaminated due to the presence of mycotoxins which are highly toxic. Such mycotoxins can be reduced by using potent organisms endowed with the capability of bio-transforming mycotoxins into other non-toxic metabolites (Schatzmayr et al. 2006). Several bacterial and fungal species, for instance, *Flavobacterium aurantiacum*, *Aspergillus niger*, *Armillariella tabescens*, *Candida lipolitica*, *Corynebacterium rubrum*, *Trichoderma viride*, *Mucor*, *Neurospora*, *Rhizopu*s have the potential to detoxify numerous kinds of mycotoxins (Bata and Lásztity 1999). Several lactic acid bacteria are also found to be capable of degrading mycotoxins (Peltonen et al. 2001).

The crop residues are also found to be holding a higher proportion of lignin which is difficult to be digested by the ruminants. The treatment with appropriate microorganisms can considerably reduce the lignin content of the feed and thus can upgrade the nutrient status and improvement in the taste of the feed. Moreover, the chemical treatments for delignification are inapplicable for feed preparation due to the generation of toxic byproducts. Therefore, the crop residues receiving appropriate microbial treatment can prove to be easily digestible feed for animals. Treatment of processing residues with the co-culture of *A. niger* and *Candida utilis* has the potential to enhance the protein content of the residue (Bhalla and Joshi 1994). Several other microbes like *Kloeckera apiculafa*, *Saccharomyces cerevisiae*, *Pleurotus ostreatus*, *Rhizopus oligosporus*, *Gongronella butleri*, *Trichoderma longiobrachiatum*, *Pleurotus sajor-caju*, *Enterococcus faecium*, *Phaffia rhodozyma*, and *Sporobolomyces roseus* have the potential to upgrade the nutritional value of animal feed (Ajila et al. 2012).

Preparation of silage is another kind of transformation of agricultural residues into animal feed which results in a low pH feed for ruminant animals. It is a multistep fermentative process that decreases the pH of feed below 4 and thus makes it resistant to microbial spoilage. During this process, microbes break down the cellulose and hemicellulose constituents of the substrate into their corresponding sugars which are further metabolized to low molecular weight acids, generally lactic acid. This process is typically governed by the utilization of appropriate enzymes and microbial silage inoculants for silage making (Colombatto et al. 2004; Okine et al. 2005). The effective and competent fermentation process yields a pleasant and digestible feed. There is a strict requirement of anaerobic conditions in a quick manner to enable the lactic acid bacteria to grow and dominate so that the pH of a substrate can be brought down quickly (Arvidsson et al. 2008). This depresses the decay of the silage by putrefactive aerobic microbiota and also guarantees the maintenance of a major proportion of the nutrients in the final product.

13.7.2 Food Production

The process of photosynthesis can fix about 200 billion tons of organic matter on this beautiful planet on an annual basis (Zhang 2008). Conversely, a major proportion of this organic matter is not available for direct consumption of human beings as well as other animals and at several times such an organic matter also becomes a nuisance for humankind when it starts raising environmental concerns. The present world is, however, suffering from a continuous escalation in the prices and declining nutritional standards along with a perpetual decline in the accessibility of raw materials (Laufenberg et al. 2003). The generation of around four billion tons of crop remains on an annual basis where a major proportion finds its origin from cereals (Lal 2008) demands its up-gradation to other products of higher values by exploiting numerous chemical or biological progressions. The numerous possessions of lignocellulosic agro-residues mark their employment as a substrate with huge importance and biotechnological value. Such residual matter is endowed with the numerous potentials for being used as a substrate for solid-state fermentation to produce various food materials.

Mushroom cultivation is an economically effective process of great ecological significance that can be effectively utilized for bio-transforming agro-residues. The cultivated mushroom species is a wonderful food source and is endowed with numerous pharmacological possessions, for instance, antiparasitic, antiviral, antibacterial, antiatherosclerosis, antitumor, antidiabetic, antihypertension, hepatoprotective, anti-inflammatory, and immuno-modulatory effects. Industrial mushroom production is a biological process of small duration which marks the proteinaceous food production from the agricultural-residues attributable to the degrading aptitude of mushroom (Martınez-Carrera et al. 2000; Chiu and Moore 2001). The mushroom belonging to the class *Lentinula edodes* and *Pleurotus* species are endowed with exceptionally higher degradative potential with a capability of utilizing a vast number of lignocellulosic residues.

The mycelium of these organisms produces substantial magnitudes of numerous enzymes that are capable of degrading the complex lignocellulosic residues and exploit them as a source of nutrients for their growth as well as proliferation (Bushwell et al. 1996; Elisashvili et al. 2008). Conversely, the type and nature residue used for mushroom cultivation strongly affects the quality of the mushroom. The varieties of mushrooms that are cultivated globally are largely represented by *Agaricus bisporus*, *Pleurotus ostreatus*, and *L. edodes*, followed by *Auricularia auricula*, *Flammulina velutipes*, and *Volvariella volvacea*. Other mushroom species produced successfully on various substrates include *Agrocybe aegerita*, *Ganoderma* spp., *Grifola frondosa*, *Hericium erinaceus*, *Hypsizygus marmoreus*, *Lepista nuda*, *Coprinus comatus*, *Pholiota nameko*, and *Stropharia* spp. (Stamets 2000; Royse 2004).

The international mushroom harvest exceeds ten million metric tons, where China dominates the market followed by Europe and the US (Desrumaux 2007; Huang 2007). The marketable mushroom fabrication is also a solid-state fermentation process utilizing lignocellulosic materials at a much larger scale. The industry of mushroom cultivation is the biggest biotechnological industry which thrives on solid-state fermentation by utilizing lignocellulosic biomass as the feedstock (Moore and Chiu 2001).

The act of mushroom cultivation seems to be a much economic viable phenomenon attributable to the use of low-value remains of agro-ecosystems. Furthermore, these wastes are handled utilizing moderately cheaper microbial technologies to yield human foodstuff, which is further deliberated to be a functional food or as a source of numerous drugs and pharmaceuticals. Additionally, the operational utilization of resources finding their origin from agricultural leftovers is a comprehensive environmental protection approach (Zervakis and Philippoussis 2000). The process of mushroom cultivation is also a holistic approach to production. This approach attempts to join diverse goals, for instance, enhancement in the product quality, maximum production efficacy, and amalgamation of ecological characteristics into product formulation and food manufacturing. It is also an exceptional practice of crop management that manages the remaining growth medium after cropping as feed for animals as the mycelial tissue of mushroom improves the protein proportion, as fertilizer for soil attributable to its richness in nutrients and other diverse constituents that upgrade the soil structure, as a basis of enzymes, for the bio-control of plant pathogens and even utilized for the bioremediation drives as it encompasses a diverse community of microbes that is capable of digesting natural phenolic constituents of lignin (Philippoussis 2009).

13.8 Conclusion

Microbes are ubiquitous in nature and seem to be the last ray of hope when the prevailing practices seem to be challenging for mankind. The improper management of such a large volume of agricultural residues not only deteriorates soil health but is also responsible for the declining status of human health. The common practice of burning the agro residues generates a lot of particulate matter that is responsible for causing a large number of respiratory diseases along with several other metabolic disorders. Microbial systems can be carefully employed for altering the nature of resistant agro residues. The microbial treatment shapes them into new products which on application to soil significantly enhance the physical, chemical, and biological attributes of the soil. The transformation of the waste products into food and feed is of extreme importance as it can be used to combat the malnutrition and prevailing scarcity of food and feed.

In this era of technology, the comforts of human beings are largely guided by exploiting petroleum resources directly or indirectly which ultimately deteriorates the environmental status. The incredible capability of microorganisms of transforming waste products into different kinds of biofuels can prove to be a miracle for future generations under the limitations of petroleum resources. Furthermore, more robust technologies need to be developed to increase the yield of microbial fermentations regarding biofuel production. The major constraint experienced by the farming community in the composting of agro residues is the extended period taken during the compost preparation. This problem can be addressed by exploring the numerous hidden potentials of microbes or by isolating microbial members with enhanced capabilities of degrading the agro wastes. The development of such a microbial consortium that can degrade the agro residues on the field conditions in a quick manner can largely contribute to the prevailing concerns associated with the generation of such a vast amount of agro residues. In addition to it, microbes also generate several compounds that are synthesized at a level that is beyond the detection limits. Therefore, on a long way to the future, the sensitivity of currently operating detections systems will improve and multiple novel bioactive compounds of microbial origin will be identified. In addition, the bio-synthesis of these microbial origin compounds at the industrial level using waste products will see the future researcher's interest.

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Chapter 14
The Biotechnological Story of Microbial Genes from Soil to Transgenic Plants

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Abstract Microbial genes from fungi, virus, actinomycete, and bacteria have been widely explored for the improvement of crop plants. Here we review microbial genes-based transgenic plants for tolerance against abiotic stresses, improved nutrients, and disease resistance. Genetic engineering involves the transfer of desirable genes of foreign origin to plants. The expression of foreign genes induce changes at the biochemical, physio-chemical, anatomical, morphological, and physiological levels, which ultimately improve stresses tolerance and crop production. Foreign genes of microbial origin are delivered to the plant species by methods including *Agrobacterium* transformation, floral dip transformation, polyethylene glycol/ $CaCl_2$-mediated transformation, viral vectors, and the biolistic method. The efficiency of these methods depend on plant type, variety/cultivar, organ, cloning method, and gene size.

Keywords Abiotic stress · *Bacillus subtilis* · *cry* gene · Glycine Betain · Herbicides · Phytase · *Trichoderma virens*

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Abbreviations

2,4-D	2,4-dichloro-phenoxyacetic acid
dsRNA	Double-stranded RNA
EPSPS	5-enolpyruvylshikimate-3-phosphate
sgRNA	Single-guide RNA
siRNAs	Small interfering RNA
TYLCV	Tomato yellow leaf curl virus
VIGS	Virus-Induced gene silencing

14.1 Introduction

Nearly 3.5 billion years ago, prokaryotic cells were reported to be present on the earth (Bosch and Miller 2016; Bertrand et al. 2015, 2018; Javaux 2019). Since then, microbes have undergone a series of changes to be established as an important as well as a fundamental part of every plant ecosystem (Graham et al. 2016; Laforest-Lapointe et al. 2017; Lipson and Xu 2019). These microbes include bacteria, fungi, archaea, actinomycetes, yeasts ranging from single- to multi-cellular and present ubiquitously on earth (Delgado-Baquerizo et al. 2016; Nannipieri et al. 2003). Besides, microbes are playing an important role in agriculture since the beginning (Alori et al. 2017; Andreote and E Silva 2017; Besset-Manzoni et al. 2018; Delgado-Baquerizo et al. 2016; Qiu et al. 2019; Richardson et al. 2011).

Traditionally, microbes provide fundamental support to the cultivated plants, for example in acquiring nutrients, maintaining soil fertility, disease-resistance, and abiotic stresses tolerance (Alori et al. 2017; Enebe and Babalola 2019; Geisen et al. 2018; Ma et al. 2019; Turner et al. 2013). They also provide a myriad of options for biological management for pests as well as diseases (Dukare et al. 2019; Hammam et al. 2019; Jaber and Ownley 2018; Mishra et al. 2018; Nazir et al. 2019; Nguyen et al. 2016; Tjamos et al. 2010). On another hand, these microbes also afflict multiple diseases in plants cultivated on global scale such as, rice, barley, and sugarcane (Mehta et al. 2019; Rahman et al. 2019; Singh et al. 2019a), and significantly decrease the global crop yield even up to 40% (Mehta et al. 2019; Rahman et al. 2019; Singh et al. 2019a). The above description pointed out that the interaction of plants and microbes is indispensable in agriculture.

Furthermore, there is one noted fact that microbes were only used in the form of inoculants/formulations topically in agricultural practices till the 1990s (Bashan 1998; Boby and Bagyaraj 2003; Nakkeeran et al. 2002; Parr et al. 1994; Singh et al. 2016). Later in the 1990s, the microbial-origin genetic elements were first transferred to the plants like petunia, tobacco, sunflower through genetic engineering methodology (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983; Murai et al. 1983). This opened a way for the emergence of “transgenic breeding” in the scientific community (Milner et al. 2014; Rahman et al. 2019; Singh et al.

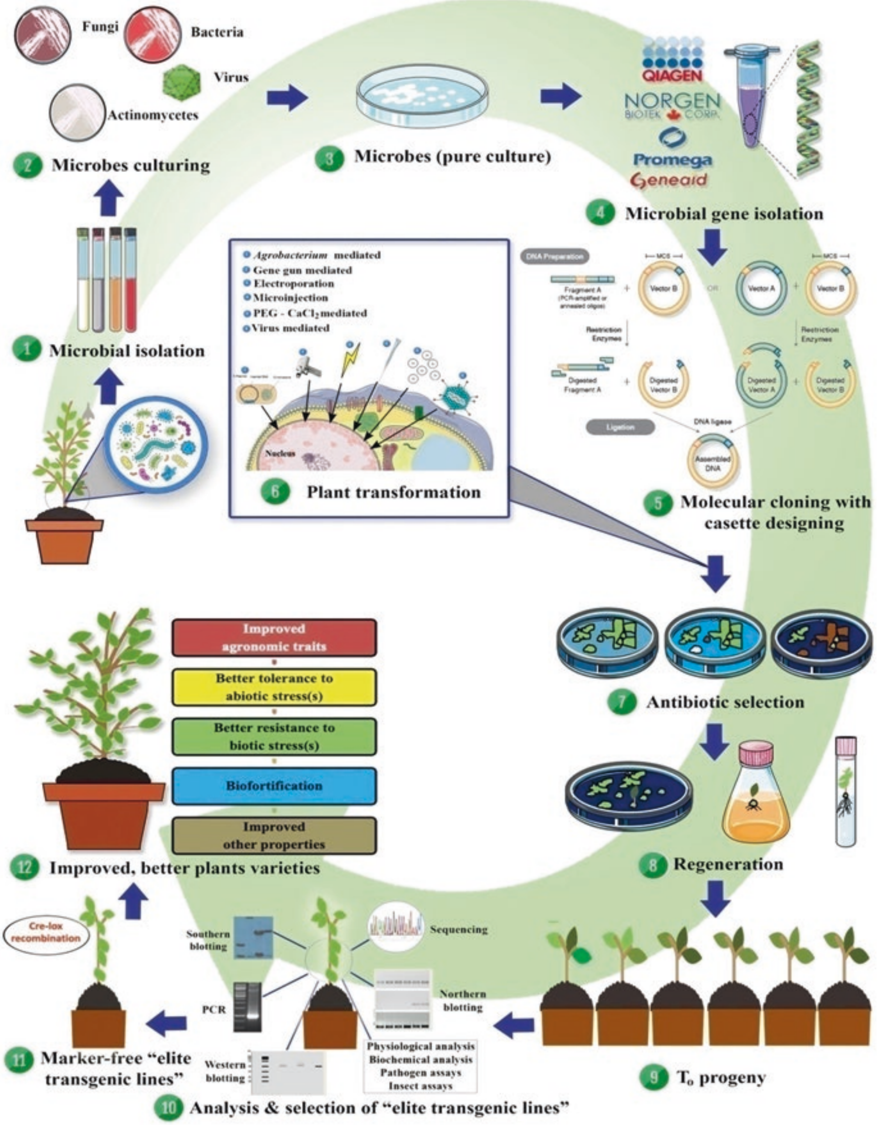

Fig. 14.1 Transgenic development using microbial genes

2019b; Syed and Tollamadugu 2019; Visarada et al. 2009; Zhong 2001). It provided a direct way to isolate the genes responsible for specific microbial properties precisely followed by transfer of the genes into the plant genomes (Kumar et al. 2018; Baloglu et al. 2018; Singh et al. 2019b; Syed and Tollamadugu 2019). A generalised scheme for development of transgenics using microbial genes has been described in Fig. 14.1.

After nearly three decades, transgenic breeding has been established as the most prominent method to modify the plant genomic architecture (Baloglu et al. 2018; Singh et al. 2019b; Syed and Tollamadugu 2019; Visarada et al. 2009); as well as an alternative to "conventional plant breeding" which is used by the humans since domestication. Table 14.1 elaborates on the differences in transgenic breeding with conventional breeding. As a result, multiple plants containing transgenic genes (microbial origin) and regulatory genetic elements have been cultivated by farmers worldwide (Baloglu et al. 2018; James et al. 2015; Kumar et al. 2018; Singh et al. 2019b).

Table 14.1 Comparison of conventional and transgenic breeding for crop improvement

Property	Conventional breeding (Hybridization method)	Transgenic breeding/genetically modified organisms
Method-description	Selectively mating crops of the same or similar species to allow the passage of desirable traits to the next generation	Transfer of a well-studied gene (from plants/animals/microbes) to a different species for the introduction of a specifically desired trait
Breeding method type	General method	Special method
Purpose	To combine desirable genes from two or more different sources	To introduce new traits for various desirable characters with the help of genetic engineering
Started	8000 BC	1996 onwards
Source of created variation	Recombination and segregation	Gene insertion
Background of crops improved	Both self-pollinating and cross-pollinating crops	Any type of crop with regenerating protocols available
Popularity currently	Very less	Highly popular
Minimum genotype required	Minimum two genetically different genotypes (parents) are required	One variety or genotype is sufficient along with gene(s) to be transferred
Sexual process involved	Yes	Bypassed
Crossing involved	Yes	No
Mutagenic treatment involved	No	No
Level of handling skills required	Low	Very high
Monetary cost involved	Low	Very high
Frequency of desirable plants	Adequate	Very low
Equipment and lab facility required	Simple, mainly field facilities	Well sophisticated lab
Effectiveness	Equally effective with oligogenic and polygenic traits	More effective with monogenic and oligogenic traits

(continued)

Table 14.1 (continued)

Property	Conventional breeding (Hybridization method)	Transgenic breeding/genetically modified organisms
Selection practiced	F_2 or later generations	Up to T_3 generation
Feature of the end product	Different from both of the parent's genotypes except backcrossing	Different from the parent genotype variety for the desirable trait involved
Number of genes affected	10,000–300,000	1–5
Knowledge of genes altered	No	Yes
Regulatory tests for human safety	No	Yes
Test for environmental impact	No	Yes
Calls for labeling	No	Yes
Time required for research and product development	5–30 years	3–5 years
Time required to release the variety	6–10 years	Depend solely on government policies
Seeds patentable	Yes	Yes
Use in organic farming	Yes	No
Activist opposition	No	Yes

Furthermore, a significant number of crop cultivars with microbial genes are in-pipeline for clearance to be released; however, their releases are still debatable with the Genetic Engineering Appraisal Committee and local public (Datta et al. 2019; Herring 2015; Kanaujia and Bhattacharya 2018; Panda 2016; Shelton et al. 2019; Shukla et al. 2018; Sudha Rani et al. 2018). As a result, this book chapter is an effort to provide insight into the microbial genes-based transgenic plants for tolerance against several abiotic stresses, improved nutrients (P and K) as well as disease resistance.

14.2 Enhanced Abiotic Stress Tolerance

Catastrophic environmental conditions hamper the growth of plants morphologically, physiologically, and genetically and additionally poses adverse penalties on productivity. Globally, these major stresses affecting the global land area are drought (64%), salinity (6%), and cold (15%) (Meena et al. 2017). With advanced techniques like genetic engineering, multiple microbial genes have been transferred to plants which ultimately increased the stress tolerance in plants (Table 14.2). There have been few earlier reports regarding use of microbial-origin genes for mitigating

Table 14.2 Transgenic crops enhanced tolerance against abiotic stress

Bacterial source	Gene	Transgenic plant	Stress tolerance	Effect	References
E. coli	*mtlD*	*Nicotiana tobaccum*	Salt	Mannitol concentrations exceeded 6 jumol/g (fresh weight) in the leaves and roots	Tarczynski et al. (1992)
Arthrobacter globiformis	*codA*	Cyanobacterium *Synechococcus*	Salt	Higher accumulation of glycine betaine, increased photosynthetic activity	Deshnium et al. (1995)
E. coli	*betA*	Tobacco	Salt	Higher content of choline, fresh weight was more in transgenic lines	Lilius et al. (1996)
Arthrobacter globiformis	*codA*	*Arabidopsis thaliana*	Salt and cold stress	Increase activity of Photosystem II, well-developed growth of root and leaves	Hayashi et al. (1997)
E. coli	*proA*, *proBosm*	*Nicotiana tabacum*	Salt	Tolerance to salt stress	Sokhansandzh et al. (1997)
Arthrobacter globiformis	*codA*	*Brassica juncea*	Salt	Significantly higher growth	Prasad et al. (2000)
Arthrobacter globiformis	*codA*	Pusa Basmati-1	Salt stress	50% of the transgenic plants could survive, wild type failed to recover	Mohanty et al. (2002)
Arthrobacter globiformis	*codA*	*Lycopersicon esculentum* Mill.	Chilling	Increased chilling tolerance at seed germination and fruit formation stage	Park et al. (2004)
Grifola frondosa Fr.	*TSase*	Tobacco	Drought and salt	Accumulate higher levels of trehalose, thick and deep-colored leaves	Zhang et al. (2005)
Pleurotus sajor-caju	*PsTP*	Tobacco	Drought	Showed good capacity to retain water	Han et al. (2005)
Arthrobacter pascens	Choline oxidase	Rice	Salt	Showed higher growth rate	Su et al. (2006)

(continued)

Table 14.2 (continued)

Bacterial source	Gene	Transgenic plant	Stress tolerance	Effect	References
Bacillus subtilis	*ProBA*	*Arabidopsis*	Salt	Increasing proline production, higher biomass accumulation	Chen et al. (2007)
Arthrobacter globiformis	*codA*	*Solanum tubersosum*	Salt and drought	Higher water contents and higher vegetative biomass	Ahmad et al. (2008)
Trichoderma harzianum	*Hsp70*	*Arabidopsis thaliana*	Heat, salt, and oxidative	Seed germination is higher in transgenic plants	Montero-Barrientos et al. (2010)
Trichoderma virens	Glutathione S-transferase	*Nicotiana tabacum*	Increase tolerance against cadmium	Increase activity of antioxidant enzymes	Dixit et al. (2011)
Arthrobacter globiformis	*codA*	*Lycopersicon esculentum* Mill.	Salt and drought	Higher levels of relative water content, chlorophyll content, and proline content	Goel et al. 2011
Trichoderma harzianum	*Thkel1*	*Arabidopsis thaliana*	Salt and osmotic stresses	80% of all transgenic plants were able to germinate and develop green cotyledons,	Hermosa et al. (2011)
Arthrobacter globiformis	*codA*	*Solanum tubersosum*	Drought	Higher chlorophyll content, lower malondialdehyde content	Cheng et al. (2013)
Nostoc commune	*wspa1*	*Arabidopsis thaliana*	Drought	More roots, high proline, low malondialdehyde content	Ai et al. (2014)
Candida tropicalis	*CtHSR1*	*Arabidopsis thaliana*	Drought	Increased osmotic potential	Martínez et al. (2015)
Arthrobacter globiformis	*codA*	*Ipomoea batatas*	Drought	Tolerance to methyl viologen, low malondialdehyde content	Park et al. (2015)
Trichoderma harzianum	*ThAQGP*	*Nicotiana tabacum*	Drought	Increased photosynthesis efficiency	Vieira et al. (2017)
Arthrobacter globiformis	*codA*	*Eucalyptus camaldulensis*	Salt	Increased level of glycine betaine content	Tran et al. (2018)

stress (Deshnium et al. 1995; Lilius et al. 1996; Tarczynski et al. 1992). An *E.coli* gene, *mtlD* encoding mannitol-l-phosphate dehydrogenase, transferred in tobacco under the *35S* constitutive promoter reported a higher accumulation of mannitol (Tarczynski et al. 1992).

Two *E. coli* proline pathway genes namely *proA* and *proBosm* encodes enzymes glutamyl-γ-semialdehyde dehydrogenase and γ-glutamyl kinase respectively were transformed in tobacco plants under the control of constitutive promoter *CaMV35S*. The transfer of genes mediated the increased level of L-azetidine-2-carboxylic acid, an analog of proline that provides tolerance to salt stress (Sokhansandzh et al. 1997). Following this, a wild-type and fusion gene *ProBA* gene from *Bacillus subtilis* was introduced in *Arabidopsis* under the control of a strong constitutive promoter (Chen et al. 2007). In their work, they noticed that after 20 days of NaCl treatment, transgenic plants started to have seeds whereas wild type died eventually. At the biochemistry level, the proline content was found to be two-fold higher than the wild-type plants (Chen et al. 2007).

Experiments show that glycine betaine transgenic plants confer resistance to cold, salt, drought, heat, and cadmium (heavy metals). The *Arthrobacter globiformis* bacterium contains a choline oxidase (cod) gene, which further converts it into glycine betaine (Ikuta et al. 1977). In another report, the induced-glycine betaine accumulation maintains the osmoticum, stabilizes membrane, and protects macromolecules, photosynthetic machinery such as Rubisco, photosystem-II complex from salt stress (Papageorgiou and Murata 1995). The *coda* (Choline oxidase) gene of choline oxidase from *Arthrobacter globiformis*, transformed *Arabidopsis* showed increased accumulation of glycine betaine in transgenic as compared to the wild type. Moreover, it prevented photosystem machinery from salt-induced damage (Hayashi et al. 1997). The leaves and roots of transformed *Arabidopsis* showed better development in 100 mM and 400 mM concentrations of NaCl as compared to wild type. The activity of photosystem-II was also observed increment in transgenic plants whereas decrement in mock-plants.

The accumulation of glycine betaine also provides tolerance to heat stress. Results showed that at 4 h, the reduction of photosystem-II activity was about 80% in wild-type plants whereas it was found to show nearly 45% decline in the transgenic plants (Hayashi et al. 1997). Along with this, at low temperature wild type showed symptoms like wilting and chlorosis as compared to transgenic. The above finding indicates that the *codA* gene improves salt and heat stress in *Arabidopsis*. The *CodA* gene of *Arthrobacter globiformis* in tomatoes showed early fruit set as well as improved chilling tolerance associated with enhanced catalase activity compared with water-treated controls (Goel et al. 2011; Park et al. 2004). Consequently, the *CodA* gene enhances photosynthetic system activity, increases antioxidant enzymes, high chlorophyll content, and better recovery to transgenic plants.

Cadmium increases the production of reactive oxygen species such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals result in the formation of cytotoxic 4-hydroxyalkenals which further inhibit protein and DNA function and synthesis (Dixit et al. 2011). Under stress-inducible promoter ABA-Inducible Promoter Complex, the choline oxidase gene from *Arthrobacter pascens* showed higher accumulation of glycine betaine in transgenic rice plants as compared to other transgenic lines under constitutive promoter (Su et al. 2006). The *codA* gene derived from *Arthrobacter globiformis* when transformed in *Eucalyptus camaldulensis* under the control of constitutive promoter *35S* showed 24 to 64 higher glycine betaine content in transgenic lines as compared to non-transgenic plants (Tran et al. 2018). Additionally, 20% of wild tomatoes died when subjected at 3 °C for 7 days whereas all transgenic tomatoes survived. *Arthrobacter globiformis* encoding *codA* gene in *Solanum tubersoum*, the cyanobacterium *Synechococcus*, tobacco and *Arabidopsis* confers tolerance to salt, light, heat, and drought tolerance (Table 14.2).

Expression of *Trichoderma* species glutathione S-transferase gene in transgenic tobacco imparts enhanced tolerance against cadmium stress. Transgenic plants showed a lower level of lipid peroxidation, and a higher level of antioxidant enzymes such as catalase, guaiacol peroxidase, ascorbate peroxidase, and superoxide dismutase as compared to non-transgenic plants (Dixit et al. 2011). A trehalose biosynthetic gene *Grifola frondosa* Fr was expressed in tobacco and showed high tolerance to drought, salt stress as well as higher accumulation of trehalose content in transgenic tobacco plants as compared to wild-type plants (Zhang et al. 2005).

The *Hsp70* gene was found to provide thermo-tolerance, oxidative, salt, and osmotic stress. The transcription level of *Hsp70* in *Trichoderma* showed 3.6 fold increase compared to the strain T34 at 28 °C (Montero-Barrientos et al. 2008). The t-hsp2 strain showed 13.6 fold-increase in *Hsp70* transcripts in the presence of 10% NaCl as compared to the wild-type *Trichoderma* strain (Montero-Barrientos et al. 2008). The scientists have cloned the *Hsp70* gene under the control of constitutive promoter 35S of *Trichoderma* in *Arabidopsis* and found tolerance against heat. Seed germination percentages of the *H1613* and *H1651* lines were found higher than in Col-0 seeds in the presence of NaCl, mannitol, and H_2O_2 (Montero-Barrientos et al. 2010).

Similarly, Lee et al. (2014) isolated the *Arthrobacter* cold-shock protein gene (*ArCrpA*) and over-expressed in the tobacco plant which enhanced the tobacco plant's tolerance against cold, drought, and salt. Tran and group (2018) generated a novel transgenic *Eucalyptus camaldulensis* having *codA* gene expressed under the *CaMV35S* promoter control and nopaline synthase terminator with enhanced salt stress tolerance and hiked glycine betaine concentration. More recently, Shim and co-workers also reported the heterologous expression of bacterial trehalose pathway genes in potato plants which enhanced the trehalose accumulation without any incurred fitness cost (Shim et al. 2019).

14.3 Enhanced Resistance Against Biotic Factors

Fungi constitute a wide range of omnipresent, eukaryotic organisms. Most of the fungi are saprophytes which are known to feed on plants and dead animals and ultimately absorb nutrients from them. Fungi cause disease in plants which have a great impact on yield reduction (Infantino et al. 2007; Knogge 1996; Matsumoto and Hoshino 2008; Mehta et al. 2019; Singh et al. 2019a; Rahman et al. 2019). The fungus also has properties of producing anti-fungal, glucanase, and chitinase enzyme which results in resistance against other several deadly fungi such as *Venturia*, *Rhizoctonia*, *Alternaria*, *Colletotrichum*, and *Fusarium* (Asif et al. 2019). Using this basic fact, multiple transgenic plants have been produced with the incorporation of microbial-origin genes that impart tolerance against various pathogenic fungi (Table 14.3). Similarly, many bacterial genes like *Anthrobactor* species, *E. coli* and *Grifola frondosa* are listed in (Table 14.3) which showed enhanced resistance against other biotic stresses.

Table 14.3 Transgenic crops enhanced resistance against fungal pathogens

Sources	Genes	Transgenic plants	Effects	References
Trichoderma harzianum	*Ech42*	Apple	Resistance against *Venturia inaequalis*	Bolar et al. (2000)
Trichoderma virens	*ech1*	Cotton	Resistance against *Rhizoctonia solani* and *Alternaria alternata*	Emani et al. (2003)
Trichoderma species	Chitinase-*42*	Strawberry	Tolerance to *Colletotrichum acutatum*	Pascual et al. (2005)
Pseudomonas fluorescence	Microbial factor3	Carrot	*Alternaria dauci*, *Alternaria radicina*, *Botrytis cinerea*	Baranski et al. (2007)
Trichodermaharzianum	*CHIT36*	Carrot	Tolerance to *Alternaria radicina* and *Botrytis cinerea*	Baranski et al. (2008)
Metarhizium anisopliae	*CHIT42*	Tobacco	Resistance against *Rhizoctonia solani*	Kern et al. (2010)
Trichoderma virens	*ech42*	*Nicotiana tabacum*, *Lycopersicon esculentum*	Resistance against *Fusarium oxysporum* sp. *Alternaria solani*	Shah et al. (2010)
Trichoderma harzianum	Chitinase (HarChit), Chitosanase (HarCho)	*Sorghum bicolor* L. Moench	Tolerance to anthracnose	Akosambo-Ayoo et al. (2011)
Bacillus thuringiensis	*Cry2Aa*	Chickpea	Resistance to *Lepidoptera*	Mehrotra et al. (2011)
Trichoderma harzianum	*ThEn-42*	Cavendish banana	Resistance against *Mycosphaerella fijiensis*	Vishnevetsky et al. (2011)

(continued)

Table 14.3 (continued)

Sources	Genes	Transgenic plants	Effects	References
Trichoderma harzianum	HarChit and HarCho	*Triticum aestivum* L.	Tolerance to *Erysiphe graminis* f. sp. *tritici*	Rana et al. (2012)
Trichoderma virens	*cht42*	Rice	Resistance against sheath blight disease	Nicolas et al. (2014)
Streptococcus pyogenes	*SpCas9*	*Nicotiana tabacum*	Eradicate 'Tomato yellow leaf curl virus'	Ali et al. (2015)
Bacillus thuringiensis	*Cry3A*	Potato	Resistance to *Leptinotarsa decemlineata*	Mi et al. (2015)
Trichoderma virens	*ech42*	*Brassica juncea*	Resistance against *A. brassicae* and *A. brassicicola*	Kamble et al. (2016)
Streptococcus pyogenes	*SpCas9*	*Solanum lycopersicum*	Low accumulation of 'Tomato yellow leaf curl virus' genome	Tashkandi et al. (2018)

Trichoderma species are mycoparasitic fungus that produces an endo-symbiotic relationship with the host plants, through penetration to plant epidermis and release of several metabolites and influence plant growth, yield and protects against a wide range of fungi (Emani et al. 2003). Many studies suggest that chitinase genes from fungi provide resistance against many biotic stresses (Kannojia et al. 2017). Chitinases are evolutionary-conserved in archaea, bacteria, plants, and fungi and found to have a diverse role including defense mechanism to the pathogen, abiotic stress, growing hyphae, and increase nutrition (Guevara-González and Torres-Pacheco 2006). It is used to digest the structural polysaccharide chitin in the cell wall of fungi thereby inhibiting fungal growth (Hamid et al. 2013). They are well characterized in several groups of fungi like *Trichoderma virens*, *T. harzianum*, *T. atorviridae*, and *Metarhizium anisopliae.*

Trichoderma has three sub-groups of chitinases; of which groups A and C are considered as exochitinases and B is endochitinases which have a carbohydrate-binding module (CBM)-1 domain at their C-terminus. In addition to this, subgroup C chitinases contain, at the N-terminus of their GH18 module, CBM18 (chitin-binding), and CBM50 (LysM) domains, which contribute to the antifungal activity through their binding to chitin in the cell wall of fungi (Hamid et al. 2013). One such famous report in which expression of chitinase gene provided tolerance to fungus is by Pascual et al. (2005). They enhanced the tolerance against *Colletotrichum acutatum* in transgenic strawberries by using the same strategy. Endochitinase gene *chit1* from another fungus *Metarhizium anisopliae* under the control of 35S promoter showed resistance to *Rhizoctonia solani* (Kern et al. 2010).

Black leaf spot disease and grey leaf spot disease are one of a deadly disease that causes crop losses even up to 47% (Meena et al. 2002). Transfer of *Ech42* gene in *Brassica* confers tolerance to *Alternaria* blight disease. Over-expression of *Ech42* in transgenic apple under *35S* promoter provides tolerance to apple scab disease. These plants showed a high level of chitinase activity as well as the presence of healthy green leaves (Bolar et al. 2000). The *Ech1* gene from *T. virens* transformed in cotton was found to have higher endochitinase activity as compared to non-transgenic lines. All lines were fertile, and their leaves showed significant protection from disease (Emani et al. 2003). PB-1 rice variety transformed with *cht42* gene under the control of *35S* promoter was found to have 4.6 fold higher chitinase activity, up to 62% reduction in sheath blight disease as well as tolerance against sheath blight (Shah et al. 2009). Over-expression of *ech42* gene in T1 transgenic lines of tomato and tobacco resulted in higher endochitinase activity as compared to control wild-type plants measured by fluorimetric method (Shah et al. 2010).

Transgenic carrot plants assayed for three phytopathogens showed 40% enhanced resistance against *A. dauci*, *A. radicina*, *and Botrytis cinerea* as compared to non-transformed carrots (Baranski et al. 2008). In the detached leaf bioassay, *Brassica* transgenic line showed tolerance to two deadly fungi and delayed onset of fungal infection (Kamble et al. 2016; Table 14.3). Tsai and group from the University of Toronto and Joint Bio-Energy Institute jointly constructed *Arabidopsis thaliana* transgenic lines which were constitutively expressing the *PcGCE* and *AnAF54* hemicellulases genes (Tsai et al. 2017). Of the two, the *PcGCE* lines demonstrated improved xylan extractability as well as biotic stress tolerance against *Aspergillus*.

Insects also cause a significant loss of crops and reduce the overall productivity (Huang et al. 2015; Wei and Li 2016; Stanton et al. 2017; Liu et al. 2019; Roland et al. 2019). In this aspect, gathered reports indicate that bacterial genes have also been applied to increase the resistance in plants against insects and pests (Baloglu et al. 2018). One of the world-known bacteria used is *Bacillus thuringiensis*. Genetically modified crops with *Bt* genes were globally planted over 35 million hectares in 13 different countries in 2014, and they constitute 15% of all genetically modified crops (James et al. 2015). This bacterium produces crystalline (Cry) proteins during sporulation which have been established to be highly toxic to insects (mainly against lepidopteran, coleopteran, dipteran, and nematodes), but non-toxic to humans and animals (Palma et al. 2014).

Initially, insects ingest cry protein (protoxin form), which is further solubilized by the gut (high pH) to solubilized protoxins which in turn get activated by digestive enzymes into smaller fragments. These toxic fragments bind to receptors on the membrane of the insect's midgut epithelial cells, aggregate, and form ion-permeable pores that lead to osmotic shock, gut dysfunction, lysis of gut epithelial cells, and eventual death of the insect. The specific activity of *Bacillus thuringiensis* on a particular insect species is determined by the forms of the cry genes carried by the bacterium. As per the reports, bacterial cry genes have been transferred in tobacco, tomato, potato, cotton, rice, and maize for developing resistance against several lepidopteran insect pests (Abbas 2018).

More than 40 different cry genes have been incorporated in plants to provide resistance against insects (Mi et al. 2015; Tabashnik et al. 2008). The synthetic *cry1Ac* gene was cloned and expressed in *Brassica juncea* that resulted in enhanced resistance to *Plutella xylostella* larva as compared to the control plants (Kamble et al. 2013). In insect bioassay, the pyramided *cry1Ab* + *cry1Ac* transgenic plants showed a high level of resistance and protection to pod borer insect *Helicoverpa armigera* then transgenic lines expressing a single *cry* gene (Mehrotra et al. 2011). It has been shown that the transgenic line of alfalfa showed greater resistance against *Alfalfa weevil* in comparison to control plants (Tohidfar et al. 2013). Many *Bt* transgenic plants have been produced via *Agrobacterium*-mediated transformation and showed greater tolerance against insects (Table 14.4). More recently, Liu and group (2019) from Nanjing Agricultural University (China) reported the successful creation of Cotton (cv. Sumian16) lines-highly resistant to bollworm larvae using the cassette p7RPSBK-mGNA-NPTII via the pollen-tube pathway method (Baloglu et al. 2018; Kumar et al. 2018; Kumar and Selvaraj 2019).

Viruses can be best described as the most significant non-living threat to food security worldwide (Gilbertson et al. 1998; Mehta et al. 2019; Rahman et al. 2019). To tackle the ill-effects of virus infection, multiple virus-resistant cultivars have been generated through traditional breeding; however, the results are highly discouraging (Brown and Corsini 2001; Solomon-Blackburn and Barker 2001). One of the approaches which have given satisfactory results up to a limit is genetic modification (Bucher et al. 2006; Liu et al. 2011; Panter et al. 2012; Qu et al. 2007; Thakur et al. 2014). This approach has enabled the insertion, replacement, and addition of desired genes to provide better virus-resistant varieties (Ahmad et al. 2011; Anjanappa et al. 2016; Scorza et al. 2016; Shetty et al. 2018; Taylor et al. 2012; van Esse et al. 2020).

Additionally, VIGS (Virus-Induced Gene Silencing) has emerged as a successful technique that employs the viral genome to provide resistance against several viruses in plants (Baulcombe 2015; Ding et al. 2018; Fujita et al. 2019; Mehta et al. 2018). It is best described as a post-transcriptional gene silencing technique in which the viral vector is designed by inserting a fragment of the host plant's target

Table 14.4 Transgenic crops enhanced resistance against insects

Source	Gene	Crop	Effects	References
Bacillus thuringiensis	*Cry2Aa*	Chickpea	Resistance to *Lepidoptera*	Mehrotra et al. (2011)
Synthetic gene	*Cry1Ac*	*Brassica juncea L. Czern and Coss*	Resistance to *Plutella xylostella*	Kamble et al. (2013)
Bacillus thuringiensis	*Cry3a*	Alfalfa	Resistance to *Coleoptera*	Tohidfar et al. (2013)
Bacillus thuringiensis	*Cry1Ab* and *vip3H*	Rice	Resistance to *Sogatella furcifera*	Lu et al. (2014)
Bacillus thuringiensis	*Cry3A*	Potato	Resistance to *Leptinotarsa decemlineata*	Mi et al. (2015)

gene (Becker and Lange 2010; Mehta et al. 2018; Senthil-Kumar and Mysore 2011). Following the generation, recombinant vectors are then transferred into plants via *Agrobacterium* Ti-plasmid transformation (Mehta et al. 2018).

In plants, the gene of interest transcribed by the RNA-dependent RNA polymerase enzyme leads to the production of double-stranded RNA (dsRNA). The dsRNA recognized by Dicer (like enzymes cleave dsRNA into 21–25 nucleotide small interfering RNA (siRNA) (Becker and Lange 2010; Senthil-Kumar and Mysore 2011). The siRNAs are recognized by the RNA-Induced Silencing Complex and unwound into single-stranded RNA which further finds the complementary region (target region) in the plant genome and induces cleavage. Researchers have been using the VIGS strategy to improve virus resistance in several crops like tomato, *Nicotiana*, *Capsicum*, *Hordeum vulgare*, *Triticum aestivum*, *Oryza sativa*, and *Zea mays* (Baulcombe 2015; Ding et al. 2018; Fujita et al. 2019; Lange et al. 2013; Mehta et al. 2018; Ramegowda et al. 2014).

Since last decade, the most revolutionizing genome-editing approach *i.e.*, CRISPR/Cas9 system have also been employed for better resistance against several viruses (Ali et al. 2015; Ji et al. 2015; Mahas and Mahfouz 2018; Mehta et al. 2020; Zhang et al. 2018, 2019). In this process, the Cas enzyme bind to the RNA sequence then further attached to the target region by complementary and ultimately leads to DNA editing. Several Cas proteins with sequence-specific nuclease activity have been identified and introduced in crops (Arora and Narula 2017; Pearson et al. 2015). In the year 2015, CRISPR/Cas9 system consisting of *Streptococcus pyogenes Sp*Cas9 and a synthetic single-guide RNA (sgRNA) specific for coding and non-coding sequence of *Tomato yellow leaf curl virus* (*TYLCV*) was delivered into *Nicotiana benthamiana.* This transfer showed less *TYLCV* accumulation as well as annihilate the symptoms of infection (Ali et al. 2015). In *Solanum lycopersicum*, *TYLCV* titer as confirmed through semi-quantitative PCR and rolling circle amplification assay was found to be low in transgenic plants (T3 progeny plants) as compared to wild type (Tashkandi et al. 2018).

Herbicides are chemicals that kill or reduce the growth of unwanted, obnoxious, non-economic plants *i.e.*, weeds (Achary et al. 2017; Ashton and Crafts 1973; Chandrasekhar et al. 2014; Fartyal et al. 2018; Manna et al. 2016; Ossana et al. 2019). All these weeds compete with the desired vegetation for space, light, water, and nutrients so their eradication is a must for the farmers. Therefore, scientists have adopted genetic engineering compared to the in-efficient manual weeding which is highly expensive and manpower-consuming. This approach enables the transfer of bacterial genes which provide resistance against many herbicides likes 2,4-D (2,4-dichloro-phenoxyacetic acid), glyphosate, dicamba, glufosinate, basta, and atrazine (Green and Owen 2010; Hamid et al. 2011; Vats 2015).

One such famous herbicide is glyphosate, a broad-spectrum herbicide that interrupts the production of all three aromatic amino acids (phenylalanine, tyrosine, and tryptophan) by blocking the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme. In 1996, Monsanto developed a genetically engineered soybean known as "Roundup Ready soybean" by expressing bacterial *Cp4*gene from *Agrobacterium tumefaciens* (Harrison et al. 1996). This microbial gene translational aspect was so successful that currently the Roundup Ready Soybean has been commercialized in multiple countries including Canada, Argentina, Japan, Mexico, and United Nations. Similarly, few other microbial genes have been also used to make many glyphosate-resistant plants (Dill 2005; Dill et al. 2008; Duke and Powles 2009; Gianessi 2008; Green 2018).

Furthermore, 2,4-dichloro-phenoxyacetic acid was the first herbicide to be commercialized to kill only dicots (Peterson et al. 2016). It mimics auxin, a phytohormone, and causes uncontrollable plant growth and ultimately death. For the first time, Syngenta company developed 2,4-D resistant transgenic maize which detoxifies 2,4-D herbicide by side-chain degradation and degrades the R-enantiomers of aryloxyphenoxypropionate herbicides (Table 14.5). Likewise, many transgenic plants have been developed to improve the herbicides resistance cotton, maize, and soybean against dicamba (2-methoxy-3,6-dichlorobenzoic acid) by using gene *Dmo* gene from *Stenotrophomonas maltophilia* which encode dicamba mono-oxygenase enzyme and nitrilase is encoded by *bxn* gene from *Klebsiella pneumonia* which detoxify bromoxynil to nontoxic benzoic acid in Cotton, Argentina canola and Tobacco (ISAAA 2019).

Similarly, the *PqrA* gene isolated from *Ochrobactrum anthropi* bacteria when introduced in *Nicotiana benthamiana* under control of 35S promoter showed resistance against20μM paraquat concentration as compared to the wild-type plants. Additionally, the chlorophyll content in transgenic lines was a little less than 15% at 50 μM paraquat concentration. Moreover, the transgenic rice showed resistance against two other herbicides-Basta and Bensulfuron Methyl (Table 14.5). T3 transgenic line of rice when further analyzed for basta and BM it was observed that wild-type plants have necrotic symptoms, turned yellow, and died after 20 days inoculation with Bensulfuron Methyl while transgenic line showed no necrotic symptoms with green leaves. Likewise, transgenic rice plants were treated with a 2% basta solution and noticed that wild-type plants showed necrotic symptoms and died after 15 days

Table 14.5 Transgenic crops with microbial genes showing herbicide resistance

Source	Gene	Transgenic plants	Effect	References
Agrobacterium tumefaciens	*CP4 EPSPS*	Maize, cotton, wheat, and potato	Confer increased tolerance to glyphosate herbicide	Barry et al. (1992)
Sphingobium herbicidovorans	*aad-1*	Maize	2,4 D herbicide tolerance	www.syngenta-us.com/herbicides/resistance-fighter
Agrobacterium tumefaciens	*CP4 EPSPS*	*Soybean*	Glyphosate tolerance	Padgette et al. (1995)
Ochrobactrum anthropic	*pqrA*	*Nicotiana tabacum*	Paraquat resistance	Jo et al. (2004)
Streptomyces	*Bar*	Indica rice cultivar Swarna	Resistant against Bensulfuron Methyl and basta herbicides	Fartyal et al. (2018)

(Fartyal et al. 2018). Many crops like cotton, soybean, corn, and canola have been made resistant to bialaphos herbicides by using bar genes from *Streptomyces* and commercialized worldwide (Fartyal et al. 2018).

14.4 Nutrient Availability

14.4.1 Nitrogen

Nitrogen is a major nutrient for a plant's growth, development, and productivity as it is primarily involved in protein structure, nucleic acid structure, cell signaling, and nitrogen fixation (McAllister et al. 2012). It is present in abundant quantity as a gaseous N_2 molecule on earth; however, plants uptake N in the form of nitrate and then convert it to ammonium via the nitrite pathway (Seiffert et al. 2004). Earlier plants were given synthetic nitrogen results in good yield despite this it is absorbed by the aquatic animals, caused algal blooms and increased greenhouse emission which leads to concern towards the ecosystem. Researchers have used genetic engineering techniques to improve N uptake efficiency by using the microbial genes which incur no fitness cost to the environment. A gene *Asparagine synthetase* showed higher total amino acids glutamine and asparagine in *Brassica napus* (Seiffert et al. 2004). Transgenic tomato over-expressing *glutamate dehydrogenase A* (*gdhA*) gene from *Aspergillus nidulans* under the control of constitutive promoter showed higher amino acids, as well as twofold higher glutamate (Kisaka and Kida 2003). Transgenic *Lactuca* plants made using the *Asparagine synthetase* gene A from *E. coli* under the control of *pMAC* promoter showed better growth as compared to wild-type plants (Giannino et al. 2008; Table 14.6).

14.4.2 Phosphorous

Phosphorous is an essential nutrient important for plant growth, development, photosynthesis, macromolecules formation, and energy metabolism and affects ultimately agriculture worldwide. Phosphate is mainly present in phytate form which accounts for 60–80% of P in soils with low solubility in plants (Reddy et al. 2017). Phytase is a phosphatase enzyme that breakdown phytic acid and organic phosphorous into the inorganic form of phosphate which is readily absorbed by the plant's roots. It is ubiquitously found in bacteria, plants, fungi, and animals (Dersjant-Li et al. 2015; Jain and Singh 2016; Madsen and Brinch-Pedersen 2019; Dailin et al. 2019). Moreover, scientists have isolated and characterized various bacteria (*E. coli*) and fungi (*Aspergillus niger*, *A. ficuum*, *A. awamori*, and *A. terreus*) and cloned them in the many crops via *Agrobacterium* mediated-transformation to mitigate the unavailability of phosphorous (Table 14.6). The *E.coli* phytase gene *appA* cloned in

Table 14.6 Transgenic crops with microbial genes showing enhanced agronomic traits

Sources	Genes	Transgenic plants	Effects	References
Aspergillus niger	*phyA*	*Triticum aestivum*	4% increase in phytase activity	Brinch-Pedersen et al. (2000)
E. coli	*GDH*	*N. tabacum*	Increased biomass and leaf number and ammonium assimilation	Ameziane et al. (2000) and Mungur et al. (2005)
Aspergillus nidulans	*gdhA*	*Lycopersicon esculentum*	A higher level of amino acids (Glutamine)	Kisaka and Kida (2003)
E. coli	*AsnA*	*Brassica napus*	Higher seed N content	Seiffert et al. (2004)
E. coli	*asnA*	*Lactuca sativa*	Increased leaf number and more leaf surface	Giannino et al. (2008)
A. niger	phyA	Maize	Increased phytase activity, higher seed germination	Chen et al. (2008)
E. coli	*appA*	*Solanum tuberosum*	Higher phosphate acquisition, phytase activity, and increased chlorophyll content	Hong et al. (2008)
A. niger and *Peniophora lycii*	*phyA*	*Nicotiana tabacum*	Higher phosphorous accumulation	George et al. (2009)
A. ficuum	*AfPhy*A	*Glycine max*	Improved phytase activity and inorganic phosphate levels	Li et al. (2009)
A. niger, *E. coli*	*phyA*, *appA*	*Brassica napus*	Larger rosette diameter, high shoot dry weight, seed yield, improved P uptake	Wang et al. (2013)
Pantoea agglomerans, *Bacillus subtilis*	*paPhC*, *168phyA*	*Arabidopsis thaliana*	Phytase activity	Valeeva et al. (2018)

Solanum tuberosum showed appreciated higher phytase activity in the range of 8000–20,000 U/kg in leaves, 6000–14,000 U/kg in stems, and 40,000–50,000 U/kg in tubers while no phytase activity was detected in non-transgenic lines (Hong et al. 2008).

Transgenic tobacco with the over-expressed phytase gene from *Aspergillus niger* and *Peniophora lycii* under the control of *CaMV35S* promoter showed higher phytase accumulation as well as significantly higher shoots biomass as compared to the control plants (George et al. 2009). Over-expressed *phyA2* gene isolated from fungus *A. niger* in Maize resulted in a significant increment in phytase enzyme activity as compared to the control plants as well as increased Pi level in the transgenic seeds. Additionally, the results were supported by the enhanced T4 and T5 seed germination which was 75–88% and 80–92.5% in the field and greenhouse, respectively. Two phytase encoding bacterial genes *paPhyC* from *Pantoea agglomerans* and *168phyA* from *Bacillus subtilis* were transferred to the *Arabidopsis thaliana* and the transgenic lines demonstrated impressive plant growth under phosphorous deficient conditions. Phytase activity in roots of transgenic lines was 3.07 and 5.75-fold higher and rosette diameter also increased in the transgenic lines (Valeeva et al. 2018; Table 14.6).

14.5 Conclusion

In their living environment, every member of various plant family is exposed to a wide range of living and non-living factors including beneficial microorganisms as well as phytopathogens/pests which concomitantly affect every aspect of the host life-cycle. Since 1990s, transgenic breeding/genetic engineering has been established as a technique that enables cloning as well as the efficient transfer of genes (both homologous and heterologous) for traits manipulation in the various (crops, model, and non-model plant species). This approach has enabled the researchers to introduce, remove or edit one or more genes with no/minimal undesired changes in the rest of the plant genome. Eventually, due to the ill-effects of post-1840 human activities, the global food demand is not in accordance with food production which leaves genetic engineering only the sustainable option to look for. The reason lies in the nature to utilize a variety of gene sources including a plethora of microbes in contrast to conventional plant breeding. Besides, the era of genetic engineering has also decreased the dependency on agrochemicals inputs.

Moreover, in the last three decades, extensive work has been carried out to transfer multiple fungal, viral, actinomycete, and bacterial genes to several agriculturally important crop plants including rice, mustard, brinjal, tomato, sugarcane, wheat, cotton, etc. This introduction in plants ultimately translates into better plant traits like abiotic stress tolerance, improved biological yield, better aroma, hiked disease resistance, altered herbicide tolerance, nutrient availability, and fortification. This is also supported by the fact that the planting areas of genetically modified crops have increased more than 150-fold within the last 2.5 decades. Despite the expansive published scientific reports on microbial genes transfer to transgenic plants, a major pool of the generated transgenic plants is unavailable to the farmers for use. The reason for lab-to-field lagging is that the genetically modified plants get stuck in a pre-release pipeline due to the risk and perception of the general public, policymakers, and international traders. This can also be better understood by the mass protests of the Indian public against *Bt* Brinjal and/or genetically modified Mustard in the past 5 years. Nevertheless, the major reasons for genetically modified crop oppose lies in the possibility of horizontal gene transfer of microbial genes leading to the emergence of super-virulent strains.

Interestingly, the plants and microbes have co-evolved, and every plant contains various types of microbiomes (phyllospheric, endospheric, spermospheric, carpospheric, and caulospheric microbiome) in their parts/organs. Additionally, people are consuming a significant quantity of uncooked/cooked food (which contains microbes and microbial DNA) as well as using many plant-derived materials since the inception of agriculture. Hence in a pronounced way, non-engineered microbial DNA/genes along with food particles and other plant products are in a treaty of harmony with the global human community. In this discussed context, crops even carrying the microbial genes do not pose any serious threat to food safety and security.

In the near way to future, the continuous researcher's efforts will result in overcoming the present-day difficulties like low transformation frequencies, inappropriate transgene expression levels, durable resistance incurred cost, restricted range of pathogenic species in consideration, fewer in-field trials/assessment, and commercialization. Taking all these points into consideration, improved plants stacked with microbial genes will be cultivated by farmers in their fields for sustainable agriculture in the future.

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Chapter 15
Microbial Biosurfactants for Green Agricultural Technology

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Abstract Surfactants are used in the formulation of pesticides and fertilizers to improve distribution by micelle formation for reducing surface and interfacial tension. Many actual synthetic surfactants are non-biodegradable and exhibit toxic effects, calling for safer compounds such as biosurfactants. Only about 25–30% of surfactants used in agricultural fields are of biological origin owing to a higher production cost. Here we review biosurfactants and their applications in agriculture. Biosurfactants increase the solubility of plant nutrients and xenobiotics, which favors nutrient intake and xenobiotic biodegradation. Low molecular weight biosurfactants such as glycolipids, phospholipids, lipopeptides and lipoproteins lower surface and interfacial tension, whereas high molecular weight biosurfactants such as polysaccharides, proteins, lipopolysaccharides, and lipoproteins, stabilize oil in water emulsions. Efficient biosurfactants have lower critical micelle concentration for effectively reducing surface tension. We discuss mechanisms by which biosurfactants enhance plant growth.

Keywords Biosurfactants · Surface tension · Critical micelle concentration · Bioremediation · Biopesticide · Plant growth stimulators · Bioavailability

Abbreviations

CMC	Critical Micelle Concentration
HLB	Hydrophobic Lipophilic Balance
PGPR	Plant Growth Promoting Rhizobacteria
SDS	Sodium Dodecyl Sulfate

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15.1 Introduction

Agricultural intensification has resulted in extensive use of hazardous non-biodegradable chemicals in agriculture and allied activities. Accumulation of such toxic chemicals over prolonged period has led to soil pollution. Chemical surfactants are one of such chemicals which are increasingly used for better distribution of pesticides and fertilizers and are used as part of their basic formulations. According to some reports millions of tons of chemically derived surfactants are used in variety of agro-formulations and even used for crop protection (Silva et al. 2014a; Morais et al. 2017). These chemical surfactants are of petrochemical origin. The by-product obtained during their manufacturing process as well as the production processes itself are very hazardous (Morais et al. 2017). Although such synthetic surfactants are crucial to meet increasing vegetation demand, they tend to accumulate in soil. The persistence of these non-biodegradable compounds in soil for prolong periods imparts harmful effects on environment.

However, to maintain soil health, any agent applied in soil should not cause any harm to environment, have high turnover rate and be eco-friendly in nature. With stricter policies of environmental laws and regulations, some alternatives are required for substitution of chemical surfactants (Banat et al. 2000). Alternative to such chemical surfactant is natural compounds having similar property and of biological origin termed as biosurfactants. This review summarizes the prevalence of biosurfactants in agro-ecosystems and proposes its application for sustainable agriculture practice. The aim is to focus on multifaceted approach of biosurfactant mediated green farming.

15.2 Biosurfactants

Biosurfactant unlike chemical or synthetic surfactant is of biological origin. Its amphibolic nature due to hydrophilic as well as hydrophobic domain makes it amenable to reduce surface and/or interfacial tension between two immiscible surfaces or interfaces (Banat et al. 2000; Fakruddin 2012; Singh et al. 2007). Thus, it increases the solubility of poorly soluble or insoluble molecules. Structurally, biosurfactant is composed of two domains, one is hydrophobic, and another is hydrophilic in nature (Fig. 15.1a). The hydrophobic domain consists of a fatty acid which is either long chain fatty acid, hydroxyl fatty acid, alpha alkyl or beta hydroxyl fatty acid whereas the hydrophilic domain is composed of non-ionic, anionic, amphoteric or cationic molecules (Campos et al. 2013). The molecule on which biosurfactant is acting has been referred to as ‘substrate’ in this article.

Microorganisms tend to produce structurally diverse low molecular weight surface active agents extracellularly or on their cell surface (Sachdev and Cameotra 2013). Biosurfactants are structurally more complex than synthetic surfactants (Morikawa et al. 1993). Biodegradability, no or low toxicity, structural diversity,

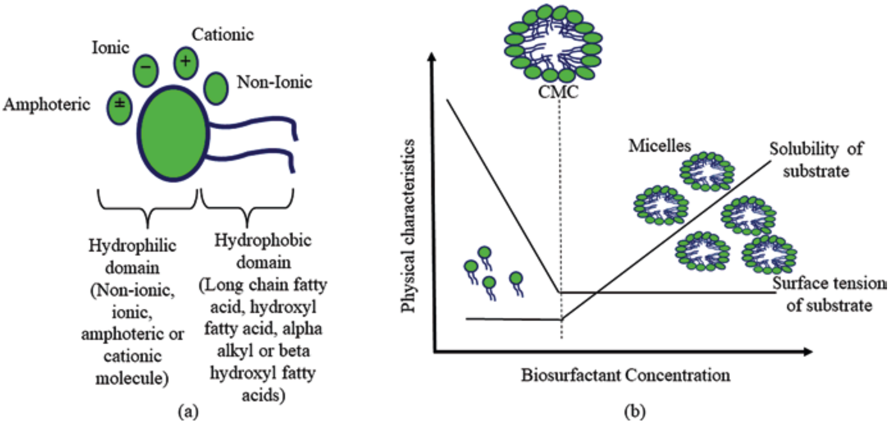

Fig. 15.1 (**a**) General structure of biosurfactants. Hydrophilic and hydrophobic domains of biosurfactant make it amphibolic molecule. Hydrophilic domain can be non-ionic, anionic, amphoteric or cationic whereas hydrophobic domain can be long chain fatty acid, hydroxyl fatty acid, alpha alkyl or beta hydroxyl fatty acid. (**b**) Correlation between biosurfactant concentration and physical characteristics of substrate. Inverse relation is observed between solubility and surface tension of substrate. CMC-Critical Micelle Concentration

low critical micelle concentration, efficacy with wide range of substrates, effectiveness at wide range of pH, salinity as well as temperature, anti-microbial property, anti-adhesive property and production from variety of substrates are the unique characteristics of biosurfactants, making them advantageous over chemically derived surfactants (Santos et al. 2018). Biosurfactants are also derived from natural resources like plants such as saponins, lecithin and soy proteins. They have better emulsification capability but are less soluble, less hydrophobic and their large-scale production is also costly. Hence, microbe based biosurfactant have advantages over plant based biosurfactants (Randhawa and Rahman 2014).

15.3 Biosurfactants and Critical Micelle Concentration

Critical Micelle Concentration (CMC) is the lowest possible concentration of biosurfactant required for micelle formation. Good biosurfactant possesses lower critical micelle concentration for effectively reducing surface tension. Micelle can be defined as the aggregates of biosurfactant molecules with hydrophilic and hydrophobic domains facing in or out depending on the type of surrounding environment. Figure 15.1b represents the relation between concentration of biosurfactant and physical properties of water insoluble compounds or substrates. Hydrophobic Lipophilic Balance (HLB) is another way of measuring efficacy of biosurfactant. HLB indicates ability of biosurfactant to form water in oil emulsion because of low

HLB or oil in water emulsion because of high HLB. HLB value varies from 0 to 20 and is determined by counting hydrophilic and hydrophobic regions of molecule (Campos et al. 2013; Pacwa-Płociniczak et al. 2011).

15.4 Classification of Biosurfactants

Biosurfactants can be classified as low molecular weight compounds, which lowers surface and interfacial tension like glycolipids, phospholipids, lipopeptides or lipoprotein and as high molecular weight compounds, which stabilize oil in water emulsion like polysaccharide, protein, lipopolysaccharide, lipoprotein and complex mixture of all these polymers (Ron and Rosenberg 2001; Pacwa-Płociniczak et al. 2011). Table 15.1 presents the type of microorganisms known to produce specific type of biosurfactants. From this table it becomes clear that not only bacteria even yeast is noted as having ability to produce biosurfactants.

15.5 Economics of Biosurfactants Production

Despite of varieties of benefits of biosurfactant over petro-chemically derived surfactant, economically large-scale production of biosurfactant is not feasible. Mostly 70–75% of surfactants used in industrialized nation are of petrochemical origin. In 2012, out of 12 million tons of surfactants, only 3.5 million tons were biosurfactants (Silva et al. 2014a). Companies such as Tee Gene Biotech (UK), AGAE Technologies LLC (USA), Jeneil Biosurfactant Co. LLC (USA), Paradigm Biomedical Inc. (USA), Rhamnolipid Companies Inc. (USA), Fraunhofer ICO (Germany), Cognis Care Chemicals (China, Gaermany, USA), SARAYA Co. Ltd. (Japan), Ecover Belgium (Belgium), Groupe Soliance (France), MG Intobio Co. Ltd. (South Korea), Synthezyme LLC (USA), Allied carbon solutions (ACS) Ltd. (Germany), Henkel (Germany), Lions Chemicals (USA) and Kanek Co. (Japan) are few biosurfactant producing companies located worldwide (Randhawa and Rahman 2014). The main factor affecting the manufacturing of biosurfactant at large industrial scale is usage of pure growth substrate for its microbial production. Moreover, the product obtained is in impure form and yield of product is also low.

The cost of purifying the impure product again raises the manufacturing cost. Also, downstream processing involves utilization of antifoaming agent which is a kind of contaminant. All these ultimately add onto the expenditure for biosurfactant production. Few solutions regarding this will be finding the appropriate microbial isolates capable of utilizing cheaper and crude or waste grade growth substrates for biosurfactant production as shown in Table 15.2. Along with this, developing an efficient bioprocess which allows recovery of pure product with high yield and concentration will be advantageous. Construction of recombinant strains having capabilities of over production of biosurfactant can also be the strategy for economic production of biosurfactants (Makkar et al. 2011; Banat et al. 2014).

Table 15.1 Biosurfactants produced by microorganisms

Biosurfactants	Microbes	Surface tension (mN/m)	CMC	Inter-facial tension (mN/m)	Emulsification index (%)	Applications	References
Low molecular weight							
Glycolipids							
Glycolipid	*Rhodococcus erythropolis*	36	NA	NA	44	Washing of crude oil Contaminated soil	Pacheco et al. (2010)
	Alcanivorax borkumensis	NA	NA	NA	NA	NA	Abraham et al. (1998)
	Serratia rubidaea	26	NA	NA	NA	NA	Matsuyama et al. (1990)
	Rhodococcus erythropolis 3C-9	33.4	50 mg/l	NA	NA	Oil spill remediation	Peng et al. (2007)
Rhamnolipid	*Pseudomonas aeruginosa*	NA	NA	NA	NA	Induces production of reducing sugars when added for rice straw decomposing process	Qiuzhuo et al. (2008)
Mono Rhamnolipid	*Pseudomonas aeruginosa* 9027	28	0.1 mg/l	2	NA	Increase solubility and enhance rate of phenanthrene biodegradation	Zhang et al. (1997)
Di Rhamnolipid	*Pseudomonas aeruginosa* 9027	36	0.1 mg/l	5	NA	Enhance alkane biodegradation	Zhang and miller (1995)

(continued)

Table 15.1 (continued)

Biosurfactants	Microbes	Surface tension (mN/m)	CMC	Inter-facial tension (mN/m)	Emulsification index (%)	Applications	References
Sophorolipids	*Rhodotorula babjevae* YS3	35.8	130 mg/l	NA	100% against crude oil, 25% against diesel, 62.26% against motor oil, 33.33% against sunflower oil, 25.19% against n-hexadecane	Possesses antifungal activity	Sen et al. (2017)
Mannosyl erythritol lipids	*Pseudomonas siamensis* CB5 9960	30.7	4.5×10^{-6} M	NA	NA	NA	Morita et al. (2008)
Phospholipids							
Phosphatidyl ethanolamine	*Pseudomonas fluorescens*	NA	NA	NA	NA	Heavy metal remediation	Appanna et al. (1995)
Lipopeptides							
Lipopeptide	*Bacillus cereus* NK1	38	45 mg/l	NA	62% against hexadecane, 55.5% against diesel oil, 80.36% against motor oil, 70% against crude oil, 50.47% against vegetable oil, 44% against petrol	Reduces biofilm formation by pathogens and have antimicrobial activity against gram positive and gram-negative bacteria and fungi	Sriram et al. (2011)
Lipopeptide	*Bacillus licheniformis*	28	12 mg/l	NA	NA	Antimicrobial	Yakimov et al. (1995)
Lipopeptide	*Bacillus licheniformis* JF-2	NA	10 mg/l	0.006	NA	NA	Lin et al. (1994)

Arthrofactin	*Arthrobacter* sp. strain MIS38	24	1×10^{-5} M	NA	NA	NA	Morikawa et al. (1993)
Lipopeptide	*Bacillus subtilis*	31	5.7 mg/l	NA	60% against olive oil, 57% against fish oil, 52% against palm oil, 36% against coconut oil, 32% against diesel	Remove Ca^{2+} and Cr^{2+} from aqueous solution	Saranya et al. (2014)
High molecular weight							
Lipopolysaccharide							
Emulsan	*Acinetobacter calcoaceticus* RAG-1 ATCC3102	NA	NA	NA	1.5 U/mg	NA	Kim et al. (2000)
Polymeric							
Mixture of rhamnolipid rrha-rha c10-c10 and phosphatidyl ethanolamines	*Pseudomonas putida* BD2	31	130 mg/l	NA	70% against vegetable oil and 51–60% against n-hexane, xylene, hexadecane and petroleum ether	Inhibit adhesion of pathogenic bacteria and fungus	Janek et al. (2013)
Biodisperson (anionic polysaccharide)	*Acinetobacter calcoaceticus*	NA	NA	NA	NA	NA	Rosenberg et al. (1988)
Alasan (complex of polysaccharide and protein)	*Acinetobacter radioresistens*	NA	NA	NA	100%	Bioemulsifier	Toren et al. (2001)
Mannoprotein	*Saccharomyces cerevisiae*	NA	NA	NA	Varies from 95 to 98%	Bioemulsifier	Cameron et al. (1988)

NA not available, *CMC* critical micelle concentration

Table 15.2 Microorganisms producing different types of biosurfactants from cheap nutrient sources

Biosurfactants	Waste-grade substrates	Microorganisms	References
Lipopeptide	Orange peel	*Bacillus licheniformis* KC710973	Kumar et al. (2016)
	Molasses, whey, extracts of potato, orange and banana peels and bagasse	*Bacillus subtilis* ANR88	Rane et al. (2017)
	Potato peels	*Bacillus subtilis* DM-03 and *Bacillus subtilis* DM-04	Das and Mukherjee (2007a)
Rhamnolipid	Non-edible vegetable de-oiled seed cakes	*Pseudomonas aeruginosa* AB4	Hazra et al. (2011)
	Molasses and corn steep liquor	*Pseudomonas aeruginosa* GS3	Patel and Desai (1997)
	Soap stock	*Pseudomonas aeruginosa* LB1	Benincasa et al. (2004)
	Corn steep liquor + molasses	*Pseudomonas aeruginosa* #12	Gudina et al. (2015)
	Mahua (*Madhuca indica*) oil cake	*Serratia rubidaea* SNAU02	Nalini and Parthasarathi (2014)
	Waste vegetable oils	*Pseudomonas aeruginosa* WJ-1	Xia et al. (2012)
	Non-edible oil like Mesua ferrea seed oil	*Pseudomonas aeruginosa* MTCC 7815	Singh et al. (2013)
Surfactin	Potato process effluents	*Bacillus subtilis*	Thompson et al. (2000)
Trehalose lipid	Sunflower oil	*Tsukamurella spumae* and *Tsukamurella pseudospumae*	Kugler et al. (2014)
Mannosyl erythritol lipid	Soyabean oil	*Candida spp.* strain SY16	Kim et al. (2006)
	Castor oil	*Pseudozyma tsukubaensis*	Yamamoto et al. (2013)
	Cellulosic material	*Pseudozyma Antarctica* PYCC 5048 and *Pseudozyma aphidis* PYCC 5535	Faria et al. (2014)
Lunasan	Soybean oil + corn steep liquor	*Candida sphaerica* UCP 0995	Luna et al. (2011)

(continued)

Table 15.2 (continued)

Biosurfactants	Waste-grade substrates	Microorganisms	References
Glycolipid	Molasses	*Pseudomonas* spp. 2B	Aparna et al. (2012)
	Sunflower oil	*Tsukamurella* spp.	Vollbrecht et al. (1998)
	Waste motor lubricant oil and peanut oil cake	*Cornybacterium kutscheri*	Thavasi et al. (2007)
	Jackfruit seed powder	*Deinococcus caeni* P05	Chooklin et al. (2014)
	Coconut oil cake	*Pseudomonas* spp.	Murugan and Rengaswamy (2011)
	Low cost agro-based raw material	*Pseudomonas aeruginosa* AB4	Hazra et al. (2011)
	Animal fat and corn steep liquor	*Candida lipolytica* UPC 0988	Santos et al. (2013)
	Wheat bran, oil seed cake, tannery pretreated sludge, pretreated molasses, distillery waste	*Nocardiopsis lucentensis* MSA04	Kiran et al. (2010)
	Tannery waste	*Brachybacterium paraconglomeratum* MSA21	Kiran et al. (2014)
	Groundnut oil + corn steep liquor	*Candida sphaerica* UCP0995	Luna et al. (2013)
Sophorolipid	Delignified corncob residue	*Wickerhamiella domercqiae* Var. *sophorolipid* CGMCC 1576 and *Cryptococcus curvatus* ATCC 96219	Ma et al. (2014)
	Mango kernel fat	*Starmerella bombicola* NRRLY 17069	Parekh et al. (2012)
	Jatropha oil, Karanja oil and neem oil	*Starmerella bombicola* (ATCC 22214)	Bhangale et al. (2014)
	Fish oil	*Wickerhamieella domercqiae*	Li et al. (2013)
	Jatropha oil	*Starmerella bombicola* NBRC 10243	Imura et al. (2013)
	Sugarcane molasses	*Starmerella bombicola* NBRC 10243	Takahashi et al. (2011)
	Virgin and waste frying oil	*Starmerella bombicola* (ATCC 22214)	Wadekar et al. (2012)
	Sugarcane molasses	*Candida albicans* O-13-1	Yang et al. (2012)
	Soybean dark oil	*Candida bombicola* ATCC 22214	Kim et al. (2005)
Polymeric substances (lipid, carbohydrates, proteins)	Soya bean oil waste and corn steep liquor	*Cunninghamella echinulata*	Silva et al. (2014b)

15.6 Biosurfactants Favouring Bioremediation of Xenobiotic Compounds

In this modern era, advances in industrial and agricultural process leads to generation of tons of xenobiotic compounds which affect the quality of soil. Out of total organic pollutants, only 10% is disposed off safely and major part of the remaining organic pollutants accumulates in environment. These hazardous pollutants have carcinogenic or mutagenic effects (Ellouze and Sayadi 2016). Moreover, oil spill at petroleum dwelling site adds diverse range of petroleum hydrocarbons and contaminates the soil. Worldwide, approximately 400,000 metric tons of oil sludge leakage has been observed in environment (Cheng et al. 2017). Industrialization also contributes to the pool of organic pollutants. Persistence of such pollutant in soil for longer duration is known to impart adverse effects on flora and fauna. Contamination of subsurface soil consequently may have impact on groundwater (Mohanty et al. 2013). Bioremediation is the eco-friendly way for recovering such polluted soil. The role of biosurfactant in bioremediation process is to make the pollutant bioavailable for biodegradation.

15.6.1 Hydrocarbon Remediation

Among varieties of xenobiotic pollutants, hydrocarbons are the principal organic pollutants which accumulate in soil matrix. Bioremediation is an effective policy for improving the quality of contaminated soil. The main factor affecting the bioremediation is the bioavailability of pollutant. Hydrocarbons due to sorption with soil matrix get accumulated in the soil and become inaccessible for the microorganisms. Biosurfactant increases the solubility of pollutant by reducing surface tension and makes them bioavailable for hydrocarbon eating microbes. This enhances the bioremediation process. Figure 15.2 shows the role of biosurfactant in the process of bioremediation. Another strategy of biosurfactant to improve bioremediation is its interaction with cell surface, as it increases the hydrophobicity of surface allowing hydrophobic substrate to associate more easily with such surface (Sachdev and Cameotra 2013).

To stimulate bioremediation, biosurfactant can be straightaway applied to soil by spraying where *in situ* microbes can actively degrade the pollutants which are solubilized or else bioaugmentation approach can be implemented. Bioaugmentation involves addition of biosurfactant producing hydrocarbon degrading microbes (Lawniczak et al. 2013). *Pseudomonas* spp. P-1 strain, an efficient rhamnolipid biosurfactant producing organism, had been reported to degrade hexadecane as well as crude oil and its fraction A5 (light boiling fraction) and P3 (high boiling fraction). It can utilize low-cost raw material molasses as nutrient source for growth as well as biosurfactant production. Hence it can be a cost-effective strategy for obtaining biosurfactant. Rhamnolipid production was 30.23 ± 2.97 g/l and the surface tension of

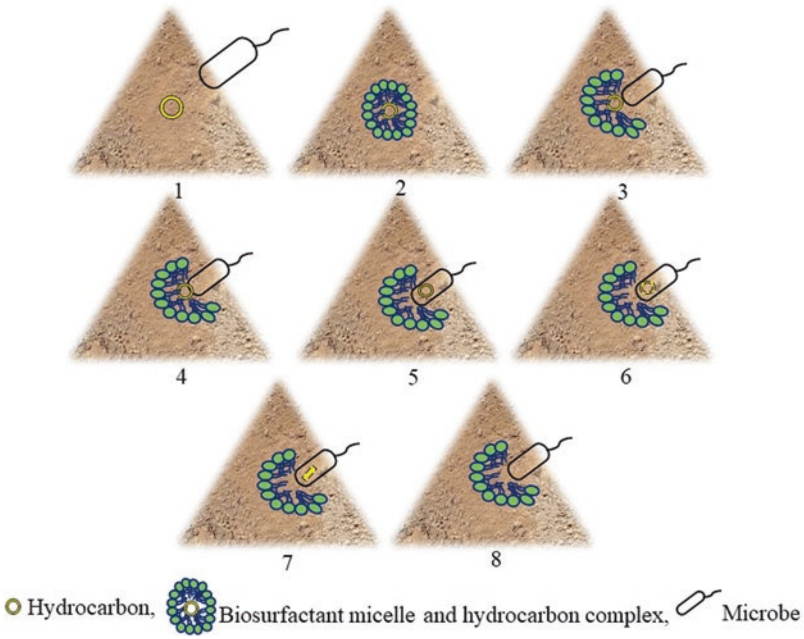

Fig. 15.2 Events depicting biosurfactant aided bioremediation. (1) Hydrocarbon obscured within soil matrix and inaccessible to hydrocarbon eating microbe. (2) Entrapment of hydrocarbon by biosurfactant (3), (4) and (5) Hydrocarbon being accessible to microbe capable of degradation. (6) Commencement of hydrocarbon degradation. (7) Metabolism of hydrocarbon and (8) Elimination of hydrocarbon

cell free supernatant was reduced to 26.67 mN/m. Flask study indicated 39%, 27% and 13% reduction in A5, P3 and hexadecane content (Pacwa-Płociniczak et al. 2014).

Comparative study between synthetic surfactant and biosurfactant has also been done. *Lactobacillus pentosus* produced glycopeptides or glycoprotein type biosurfactant from hemi-cellulosic waste like vineyard pruning waste. The efficiency of this biosurfactant producing strain to degrade octane was compared with a synthetic surfactant, SDS (Sodium Dodecyl Sulfate). Soil contaminated with 7000 mg/kg of octane showed 591 mg/kg reduction in octane concentration after 30 days when inoculated with *Lactobacillus pentosus,* while 430 mg/kg when treated with SDS. This indicates the superiority of biosurfactant over chemical surfactant. Also, burning of vineyard pruning waste generates green house gases as well as carcinogenic polycyclic aromatic hydrocarbons and hence utilizing such waste for biosurfactant production can confer dual advantages (Moldes et al. 2013).

Nowadays researchers are exploring for finding better consortium giving good results. Biosurfactant producing consortium comprising of *Bacillus pumilus* KS2 and *Bacillus cereus* R2 was capable of degrading 84.15% of Total Petroleum Hydrocarbon within 15 weeks at flask level (Patowary et al. 2016). Similarly, *Pseudomonas aeruginosa* ZS1 isolated from petroleum oil sludge produced rhamnolipid and helped in uptake and degradation of crude oil (Cheng et al. 2017). USEPA had listed pyrene as one of the priority pollutants indicating its removal from environment. Two strains *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and MN secreted biosurfactant when grown on pyrene as sole carbon source. Biosurfactant had capacity to increase solubility of pyrene by factor of 5–7 and influenced the hydrophobicity of cell surface and hence helped in bioremediation (Das and Mukherjee 2007b). *Corynebacterium aurimucosum* MKS1, *Acinetobacter baumannii* MKS2 and *Microbacterium hydrocarbonoxydans* MKS3 isolated from crude oil and crude oil contaminated soil sample produced glycolipid based biosurfactant and showed respectively 52%, 43% and 74% reduction in Total Petroleum Hydrocarbon (Muthukamalam et al. 2017).

15.6.2 Heavy Metal Remediation

Heavy metals are non-degradable, thus their removal from soil becomes difficult. Some microorganisms are known to use metal ions as co-factor for enzyme or as electron donor or acceptor for energy generation. Thus, microorganisms which can tolerate metal ions can be used for removing heavy metals from soil (Franzetti et al. 2014). Removal of heavy metal using biosurfactants is advantageous as they chelate heavy metal with their functional group and accelerate their mobility (Yang et al. 2018). Ion exchange, precipitation-dissolution and counter ion binding are the possible mechanisms for removal of metal ions by biosurfactants (Rufino et al. 2012). Anionic biosurfactants follow electrostatic attraction principle for removal of metal. They create nonionic biosurfactant micelle-metal complex by forming ionic bond with metal ion and allow desorption of metal from soil. The ionic bond so formed is stronger than metal-soil bond. Cationic biosurfactants can replace metal ions following ion exchange principle. They replace metal ions from soil surface by competing for some but not all negatively charged surface on soil (Franzetti et al. 2014; Pacwa-Płociniczak et al. 2011). Hence, biosurfactants can make heavy metal bioavailable to microorganisms. As shown in Fig. 15.3 biosurfactant forms complex by binding with metal ion which is sorbed into soil matrix and release the metal ion outside of complex making it bioavailable to microbes (Pacwa-Płociniczak et al. 2011; Mulligan et al. 2001).

Biosurfactants impart two mechanisms for heavy metal removal. Firstly, biosurfactants can form complex with free metal ion present in solution and reduce their availability; this, in turn, will reduce the toxicity of metal ion according to Le Chatelier's principle which states that change in concentration adjusts the disturbed equilibrium in such a way that the effect of change gets reduced. Secondly, formation of biosurfactant and metal ion complex at solid-liquid interface reduces the interfacial tension and allows complex to accumulate at solid-liquid interface (Miller 1995). The second strategy of biosurfactants can be employed for desorption of metal ion. But co-existence of some heavy metal with hydrocarbon

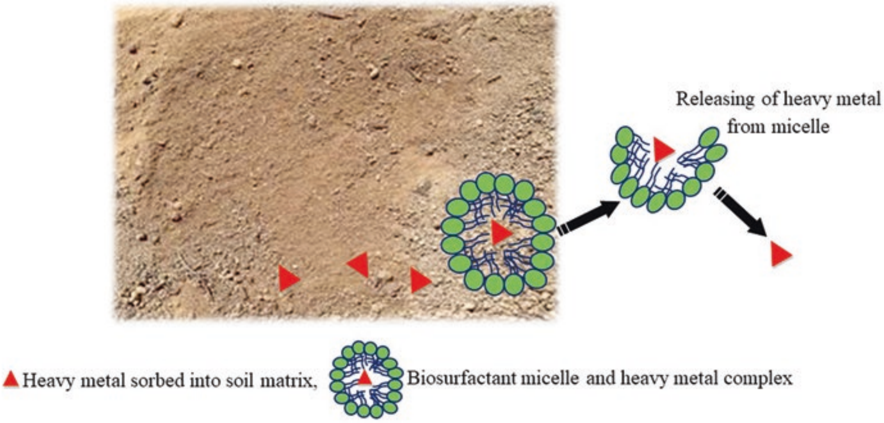

Fig. 15.3 Biosurfactant mediated bioleaching of heavy metals. Biosurfactants have the ability to increase the bioavailability of sorbed heavy metal. It forms complex with the heavy metal sorbed into the soil matrix and forms biosurfactant micelle. Upon contact with microorganisms, it releases the heavy metal making it bioavailable

pollutants in soil imparts inhibitory effect on bioremediation mechanism since few heavy metals have been reported to exert lethal effects on microorganisms or interact with hydrocarbon catabolic enzymes (Russel et al. 2018). Desorption of heavy metal from environment adjacent to biodegrading microorganisms can overcome this lethal effect (Yang et al. 2018). Stimulating biosurfactant production of indigenous microorganisms of contaminated site or using biosurfactant produced by microorganisms capable of bioremediation will be advantageous (Olaniran et al. 2013).

Bioleaching of heavy metal has been reported with mixture of rhamnolipid biosurfactant R1 (RLL) and R2 (RRLL). It was obtained from *Pseudomonas aeruginosa* with trademark JBR425 from JENEIL Biosurfactant Co. (USA) and was applied in the form of foam to column containing sandy soil contaminated with 1710 ppm of Cd and 2010 ppm of Ni. Result showed 73.2% and 68.1% reduction in Cd and Ni respectively whereas liquid solution of this biosurfactant showed 61.7% and 51.0% reduction in Cd and Ni respectively (Mulligan and Wang 2006). Efficiency of rhamnolipid biosurfactant obtained from *Pseudomonas aeruginosa* strain BS2 was investigated in column study of heavy metal spiked soil sample. It showed 92% Cd and 88% Pb removal as compared to 2.7% Cd and 9.8% Pb removal by tap water as control (Juwarkar et al. 2007). Biosurfactant produced by heavy metal resistant *Rahnella sp.* RM isolated from subsurface soil contaminated with chromium showed 74.3%, 72.5% and 70.1% reduction in Cu, Cr and Pb concentration respectively within 48 h in flask spiked with 100 mg/l of individual metal (Govarthanan et al. 2017). *Starmerella bombicola* CGMCC 1576 has potential to synthesize sophorolipid biosurfactant which removed about 83.6% Cd and 44.8% Pb from artificially contaminated subsurface clay soil. The removal efficiency based on sophorolipids was found to be higher compared to that of distilled water, SDS or Tween-80 (Qi et al. 2018). Monorhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* 9027 reduces Cd toxicity and enhances biodegradation of naphthalene by *Burkholderia* sp. (Sandrin et al. 2000).

15.6.3 Pesticide Remediation

Pesticide application in agriculture field has increased tremendously in favour of farmers for gaining better crop yield. Pesticide allows pest control but it's over usage creates additional contamination problem. Many of them have been known to accumulate in soil over time even after its intended/targeted use is over because of their persistent nature. One of the eco-friendly solutions is bioremediation. But problem associated with bioavailability of pesticide affects bioremediation potential of soil microorganisms. Biosurfactants can be used in way similar to hydrocarbon remediation for bio-removal of pesticide (Odukkathil and Vasudevan 2013). *Pseudomonas sp*. (ChID) produced rhamnolipid biosurfactant with 0.2 g/l of CMC which in flask experiment indicated 98% degradation of chlorpyrifos pesticide in 120 h on addition to 0.1 g/l of biosurfactant. The same organism showed 84% removal of pesticide in absence of biosurfactant (Singh et al. 2009). Further, since the biosurfactants are biodegradable in nature, they themselves are turned over soon after application in soil for bioremediation purpose. Further, application of biosurfactant as pesticide is described ahead.

These above-mentioned examples indicate about the potential of biosurfactant in green cleaning of polluted soil ecosystem. Further employing such techniques at huge scale may turn out to be globally beneficial. With the advancements in computational techniques, the biological databases have become rich in information. BiosurfDB is a database comprising information related to mechanism and role of biosurfactant production for bioremediation (Oliveira et al. 2015). Such databases can be used to derive molecules with greater efficiency for interacting with variety of hydrocarbon compounds.

15.7 Biosurfactants as Virulence Factor Against Plant Pathogens

Existence of plant pathogens in soil imparts perilous effects on vegetation. Pesticides were developed as a remedy for getting rid of plant pathogens, but their intensive application give rise to environmental issues like bioaccumulation of Endocrine Disrupting Compounds. Cost of synthetic pesticide is higher and at the same time, pathogens are rapidly becoming resistant to such expensively produced pesticides (Aboutorabi 2018). Some biosurfactant have antimicrobial characteristics and some also stimulate antagonistic mechanism of plant growth promoting microorganism

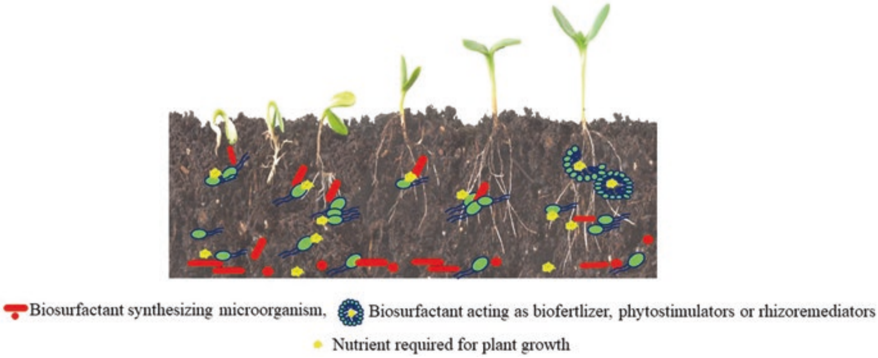

Fig. 15.4 Biosurfactant acting as biopesticides. Biosurfactants can stimulate virulence activities of plant growth promoting microorganisms like enhancing parasitism, antibiosis or competition with native flora. Such biosurfactants can efficiently kill plant pathogens and act as biopesticide

such as parasitism, antibiosis, and competition (Sachdev and Cameotra 2013). Biosurfactants like sophorolipids, cellobiose lipids and mannosyl-erythritol-lipids had been proven to be effective against hazardous phyto-fungi. Glycolipid is a prominent agent against mosquitoes. Rhamnolipids have shown to induce systemic plant immune system (Mnif and Ghribi 2016). Therefore, biosurfactant can also be used as an effective agent for controlling plant pests. Figure 15.4 represents the role of bioremediation in eliminating plant pathogens.

Pseudomonas aeruginosa JS29 produce antifungal rhamnolipid biosurfactant. Activity of this biosurfactant in plant assay and *in vitro* experiment was effective against spore as well as mycelia of *Colletotrichum capsici* which is the causative agent of anthracnose disease in chilli. Biosurfactant also showed inhibitory action against pathogen in storage condition. Therefore, it proves to be valuable biopesticide in storage and field condition (Lahkar et al. 2018). Synergism between a fungi and nematode *Fusarium oxysporum* and *Meloidogyne incognita* respectively results in loss of flower from *Gerbera* which is commonly known as Barberton daisy or African daisy. Iturin and Surfactin produced by *Bacillus subtilis* Bbv57 were the lipopeptide antibiotic biosurfactants having biocidal and systemic resistance induction activity. Both surfactants showed inhibitory action against *F. oxysporum* and *M. incognita* complex (wilt root-knot nematode complex) affecting *Gerbera* (Ramyabharathi et al. 2018).

Lipopeptide biosurfactant of *Bacillus sp.* exerted antifungal activity against *Fusarium* and *Trichoderma* species. *Bacillus amyloliquefaciens* FZB42, *Bacillus sp*. NH 217, *Bacillus sp.* NH-100, *Bacillus atrophaeus* 1765, *Paenibacillus polymyxa* CC125 and *Bacillus subtilis* 168 were proven to have inhibitory action against *Fusarium moniliforme* (rice bakanae disease), *Fusarium oxysporum* (root rot disease), *Fusarium solani* (root rot disease) and *Trichoderma atroviride* (ear rot and root rot disease) (Sarwar et al. 2018). *Bacillus licheniformis* AG-4 produced lipopeptide antifungal biosurfactant and was found to be effective against *Rhizoctonia* *solani*, causative agent of root rot in faba beans (*Vicia faba* L.) plants (Akladious et al. 2018).

Mixture of lipopeptide biosurfactant from *Bacillus amyloliquefaciens* 32a affected biofilm formation by *Agrobacterium tumefaciens* C58 and B6 which is causative agent of crown gall disease (Abdallah et al. 2018), this indicates that biosurfactant can also have biofilm inhibiting characteristics. Pot study regarding rhamnolipid biosurfactant from *Serratia rubidaea* SNAU02 revealed its antagonistic effects against *Fusarium oxysporum f. sp. melongenae* which is the causative agent of *Fusarium* wilt in eggplant (Brinjal) (Nalini and Parthasarathi 2017). *Pseudomonas fluorescens* MFS03 produces rhamnolipid biosurfactant which acts as biopesticide and removes cypermethrin residues in Spinach (Govindammal and Parthasarathi 2013).

These studies give an indication of utilizing virulence activity of biosurfactant in a positive way. Demand of pesticide free food is growing universally and hence using biosurfactant as biopesticide instead of synthetic pesticides will definitely be advantageous (Compant et al. 2005). Field level study of such biosurfactant will be favourable for attaining sustainable agriculture practice. Liquid formulation of such biosurfactant may allow feasible application of biosurfactant for controlling pests.

15.8 Plant Growth Promoting Activity of Biosurfactants

The importance of plant growth promoting rhizobacteria (PGPR) is well known for the plant growth. Microorganisms can be applied as biofertilizer, phytostimulators, or rhizoremediators in agro-ecosystems (Lugtenberg et al. 2002). Motility, capability of synthesizing and releasing biosensing molecules and performing quorum sensing is beneficial for microorganism in order to interact with plant for stimulating plant growth. Biosurfactants have been reported to have a vital role with respect to mechanisms mentioned above. Biosurfactant produced by rhizobacteria increases bioavailability of compounds and these compounds may act as nutrient for plant as shown in Fig. 15.5. Biosurfactant also enhances the effectiveness of chemical fertilizer in soil (Sachdev and Cameotra 2013). Some biosurfactants regulate the attachment and detachment of microorganism to and from surfaces which may be crucial for microbial activity towards plant growth promotion (Ron and Rosenberg 2001). All these indicate the use of biosurfactant for enhancement of plant growth.

Bacteria like *Pseudomonas* and *Bacillus* are considered efficient biosurfactant synthesizing agents. Study by Adnan et al. (2018) gives account on endophytic fungi *Xylaria regalis* obtained from cones of *Thuja plicata* as biosurfactant producing agent which promoted plant growth. Plant leaves contains hydrophobic waxy cuticle coating on their surface which is impermeable to water and dissolved solids. Numerous epiphytic microorganisms inhabiting leaf surface produce biosurfactant which increases the availability of water by acting as mediator between non-polar cuticle coating and polar water, thus increases the water content on leaf. The water

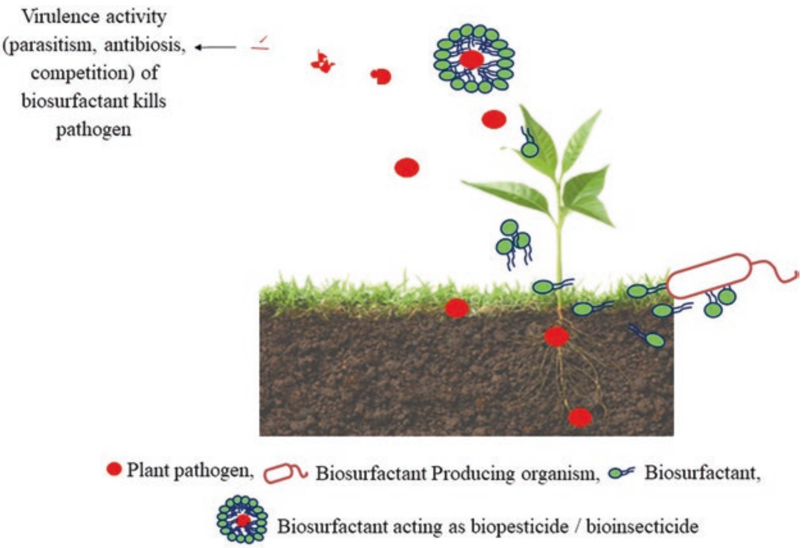

Fig. 15.5 Biosurfactant enhancing plant growth. Biosurfactant produced by plant growth promoting rhizobacteria can increase the availability of nutrients essential for plant growth. Thus, such microorganisms can act as biofertilizer, making the nutrients available, phytostimulators, enhancing the production of plant growth hormone such as Indole Acetic Acid by *Azospirillum* or rhizoremediator, which can degrade pollutants present in soil while they grow using root exudates as nutrients (Hassen et al. 2018; Lugtenberg et al. 2002)

allows leaching of nutrients making them available to epiphytic microorganisms (Zeisler-Diehl et al. 2018). Limited water availability is a problematic issue in dessert area which might affect plant growth in such habitats. Biosurfactant can play a key role in promoting growth of plant in such habitats. Biosurfactant produced by *Bacillus sp.* L45, *Bacillus sp.* R43 and *Rhizobium sp.* V3E1 improved water holding capacity and humidity retention ability of sandy soil compared to when treated with triton–X or tap water. Such biosurfactant producing isolates directly or biosurfactant itself from such isolates can be applied as additives in irrigation water used for reducing stress of plants during drought (Raddadi et al. 2018).

15.9 Biosurfactant with Multiple Characteristics

As evident from above discussions, any component with multiple efficacies will be beneficial to find economical applications as far as agriculture practices are concerned. *Pseudomonas rhizophila* S211 isolated from pesticide contaminated agricultural soil produced rhamnolipid biosurfactant which promoted PGPR activity and solubilized pentachlorophenol (PCP) pesticide as well. The pesticide solubilization efficiency of this biosurfactant was better than tween-80 and was similar to SDS (Hassen et al. 2018). This example highlights the potential of biosurfactant in two aspects, for plant growth enhancement and for pesticide remediation. Commercialization and field level application of such biosurfactant possibly will prove to be beneficial.

Heavy metal imparts detrimental effects on vegetation due to toxicity factor at high concentrations. Existence of cadmium in water employed for germination purpose of onion root cell was shown to affect 40% of actively divided root tip cells in mitotic dividing state. *Anopheles culicifacies* is the major mosquito vector causing malaria in India. *Bacillus tequilensis* strain CH produced cyclic lipopeptide biosurfactant which has shown lethal action against larvae of *Anopheles culicifacies* present in contaminated water and was also able to reduce Cd toxicity (Pradhan et al. 2018). Using this biosurfactant as bioinsecticide and bioremediating agent for regaining the quality of water used for agricultural purpose offers dual benefit.

15.10 Future Challenges

Regardless of broad relevance of biosurfactant in agriculture, major drawback associated with them is their large-scale production (Makkar et al. 2011; Banat et al. 2014). Currently pure substrates have been used for microbial production of biosurfactants which adds to the production cost apart from complex downstream processing (Kumar et al. 2016; Banat et al. 2014). If raw or crude substrates can be used, the process would be more economical, and we can witness the commercial application of biosurfactants (Kiran et al. 2014). Further, investigating multi-functional biosurfactants will offer a great help (Hassen et al. 2018).

15.11 Conclusion

Biosurfactants bearing hydrophobic as well as hydrophilic group, increase the solubility of substrate by lowering the surface tension and hence can become part of various agro-formulations ensuring their rapid dispersion and turnover. Biosurfactants imparting low critical micelle concentration are considered as superior over others. Utilization of economically cheap substrate such as agricultural and industrial organic waste for manufacturing of biosurfactant may ensure its commercial application possible. Since biosurfactants increase the solubility of organic pollutants present in soil, increased bioavailability can aid in bioremediation activity. They also support the activity of plant growth promoting microorganisms enhancing crop production. Apart from this, dual targets of bioremediation and plant growth promotion can be achieved by employing biosurfactants having multiple characteristics. Therefore, biosurfactants are efficient candidates for sustainable agriculture practice.

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Chapter 16
Role of Microbes in the Synthesis of Industrial Products from Lignocellulosic Materials

Balwinder Singh Sooch and Yogita Lugani

Abstract Agricultural residues are a major renewable source available on earth. In developing countries, food, agriculture, and forestry and industries produce high amounts of lignocellulosic wastes that cause disposal problems because these wastes are not easily degraded. Here we review the microbial synthesis of industrial products from lignocellulosic wastes. Lignocellulose is a complex compound of plant cell wall, composed of cellulose, hemicellulose, lignin, pectin and inorganics. As a consequence, feedstock pretreatments are required before the microbial production of industrial products through microbial intervention. Pretreatment techniques include physical, chemical, and biological methods. We discuss the microbial synthesis of industrial products such as enzymes, bioethanol, xylitol, and vinegar.

Keywords Lignocellulosic biomass · Cellulose · Hemicellulose · Lignin · Agricultural waste · Pretreatment · Hydrolysis · Industrial products · Xylitol · Bioethanol · Microorganisms

16.1 Introduction

Lignocellulose, mainly consists of cellulose, hemicellulose and lignin, is one of the key components of woody and non-woody plants. Lignocellulosic materials are obtained from three sources namely primary sources like short rotation energy plantations, sugar cane etc., secondary sources like corn cob, rice husk, straw, bagasse, and tertiary sources like sewage treatment sludge, municipal solid waste, and wood trimmings (Fischer and Schrattenholzer 2001). Primary sources have economic and social barriers for their utilization; hence, secondary and tertiary sources are

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commonly used as potential feed stocks for synthesis of industrial products. Lignocellulosic materials are produced in large amount annually by developing, and agricultural countries like India from food, agricultural, and forestry industries. These wastes cause serious disposal problems and cause harmful effects on environment because these wastes are often disposed of by burning in many developing countries (Demirbas 2008). Lignocellulose acts as major substrate for the production of enormous products of biotechnological value due to its chemical properties (Malherbe and Cloete 2003). The current focus of researchers is towards utilization of such renewable sources for microbial synthesis of industrial important products.

From the past few decades, considerable improvements have been made in green biotechnology by utilizing these lignocellulosic materials. These wastes are promising feedstocks for the production of numerous value-added products like animal feeds, amino acids, biofuels, chemicals, cheap energy sources, enzymes, organic acids, polyols, and human foods (Anwar et al. 2014; Sooch et al. 2019; Kauldhar et al. 2021; Lugani et al. 2019, 2021a, b). The global market of these bio-based products is enhancing at a very fast pace due to environmental concerns and continuous depletion of fossil fuels including petrol and diesel.

The major steps involved in production of industrial products using biomass as feedstocks are pretreatment, hydrolysis/saccharification, fermentation and purification. The schematic representation for biosynthesis of value-added industrial products from lignocellulosic biomass is shown in Fig. 16.1. The further steps vary with the type of product and form of its availability in the market. Pretreatment, hydrolysis, and purification are three major steps which regulate overall product market cost (Amiri and Karimi 2018). One of the major obstacles for using lignocellulosic materials is their recalcitrant nature due to their complex geometry, and hence, different pretreatment and hydrolysis methods are used to release fermentable sugars from these complex polysaccharides (Kumar and Sharma 2017). Different physical, chemical, biological, physico-chemical, thermo-chemical and thermo-physical methods are available for pretreatment of various feedstocks (Lugani et al. 2019, 2021b; Sooch et al. 2019; Mann and Sooch 2020; Kauldhar et al. 2021). The selection of pretreatment method relies on type of substrate, type of hydrolysis method, and type of microbial strain and fermentation mode. Hydrolysis can be achieved either by chemical or enzymatic methods but enzymatic methods appear to be more promising due to environmental and safety concerns.

Fermentation is an attractive approach for currently used green biotechnology in which microbial (bacteria, yeast, fungi and algae) strains are used for the synthesis of bio-products in the form of primary and secondary products (Box 16.1). Due to less productivity with the use of wild microbial strains, some improved approaches such as synthesis of genetically modified strains and use of computational models have been developed to produce industrial important strains in the past. Immobilization of biocatalyst is one of the alternative methods for development of continuous systems with reusability of enzymes.

Separation and purification of product is generally carried out on the basis of the type of compounds being separated using various chromatographic techniques such as affinity chromatography, dye-ligand chromatography, gel permeation

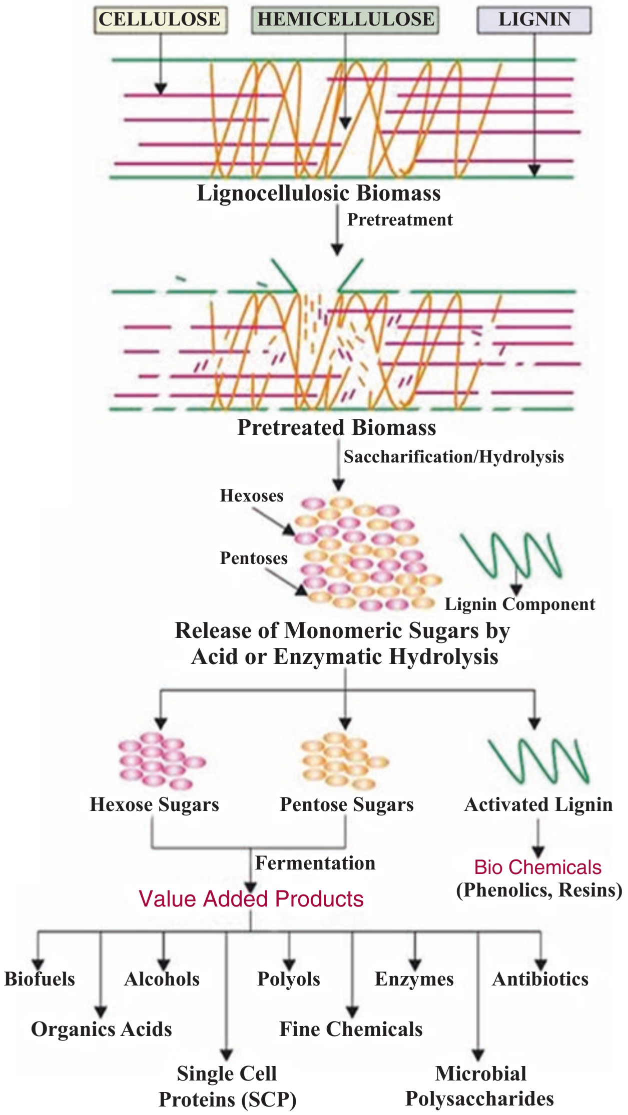

Fig. 16.1 Biosynthesis of value-added industrial products from lignocellulosic biomass

chromatography, high pressure liquid chromatography, hydrophobic interaction chromatography, ion exchange chromatography and gas chromatography (Coskun 2016). Deep eutectic solvents are used for the extraction of value-added components like flavones (apigein, luteolin), lignans (acetoxypinoresinol), phenolic acids, phenolic alcohols, secoiridoid derivative (aglycone, ligstroside), tocopherols and tocotrienols (Jablonsky et al. 2018). The current chapter enlightens the significance of lignocellulose biotechnology with emphasis on different pretreatment and

Box 16.1 Primary and Secondary Metabolites Produced by Microorganisms

Primary metabolites	**Examples**
Amino acids	L-alanine, L-arginine, L-glutamic acid, L-glutamine, L-histidine, L-proline, L-serine, L-valine, L-tyrosine, L-tryptophan, L-threonine, L-phenylalanine, L-leucine, L-isoleucine
Organic acids	Acetic acid, butyric acid, citric acid, lactic acid, succinic acid, fumaric acid, Itaconic acid, Gluconic acid, Xylonic acid
Alcohols	Ethanol, 1,3-Propanediol, 2,3-Butanediol
Enzymes	Cellulase, protease, pectinase, lipase, xylanase, xylose reductase, laccase, peroxidase, arabinase, mannase
Proteins	Single cell protein, Phycobiliproteins
Vitamins	Riboflavin, ascorbic acid
Trace elements	Ca, Fe, Mg, Zn, P, K, S, N, Mo, cl, Zn
Sugars	Xylitol, mannitol, sorbitol
Secondary metabolites	**Examples**
Antibiotics	Penicillin, cephalosporin, cyclosporine A, neomycin, Oxytetracycline, Rifamycin A, tetracycline
Phenolic compounds	Benzene, biphenyls, catechol, cyclohexane, toluene, xylene, lutein, Astaxanthin, zeaxanthin, β-carotene
Essential oils	Docosahexaneoic acid, Eicosapentaneoic acid, terpenes, lactones, aldehydes, ketones, paraffin, monoterpenoid alcohols
Chemicals	Furfural, ethylene, propylene, phenazines, quinolines, vanillin
Polymers	Poly-3-hydroxybutyrate

hydrolysis steps, production of various value-added products and their current market status. Some other aspects like improved strategies for production of industrial products including development of genetically modified microbial strains, immobilization and computation techniques have also been reviewed in this chapter.

16.2 Types and Composition of Lignocellulosic Biomass

Lignocellulosic biomass is typically nonedible plant materials mainly comprises of cellulose (40–50%), hemicellulose (25–30%), lignin (15–20%), and traces of inorganic and nitrogen compounds along with pectin (Mori et al. 2015). Cellulose is the plentiful compound on earth with special features such as hydrophobicity, biocompatibility, and stereoregularity, and the framework of cell wall is determined by its structure (Tayyab et al. 2018). It is a linear syndiotactic, rigid, crystalline homopolymer in which D-glucose residues are linked together by β-1,4-glycosidic linkage. The non-covalent interactions (hydrogen bond and van der Walls forces) present help in packing of cellulose polymers into microfibrils. The hydrolysis of cellulose into monomeric sugars is influenced by its crystallinity and polymerization, and it varies with plant species (Gray et al. 2006). Hemicellulose is an amorphous structure which provides structural strength through linkage of cellulose fibers into micrifibrils, and their crosslinkage with lignin. It comprises of short, linear, heterogenous, highly branched chains, and it is a mixture of polysaccharides including pentose sugars (D-xylose, D-arabinose) and hexose sugars (D-glucose, D-galactose, and D-mannose) and uronic acids (Saha and Cotta 2007; Saha et al. 2017). Hemicellulosic component require a great variety of enzymes for complete hydrolysis into fermentable sugars due to diversity of hemicellulosic sugars (Limayem and Ricke 2012). This polysaccharide does not possess crystalline regions, and it has low degree of polymerization, therefore, it can be easily hydrolysed into monomeric units (Karimi and Taherzadeh 2016).

Lignin is a non-sugar based complex polymer of phenyl propanic alcohol (p-coumaryl, coniferyl, and sinapyl alcohol), which provides impermeability and structural support to plants. It also confers resistance against oxidative stress and microbial attack due to its distinctive properties such as water insolubility, optical inactivity, and amorphous nature (Howard et al. 2003). A great amount of energy is produced by burning lignin; hence, it is used in biorefinery for combined heat and power production in environmental-friendly manner (Bonawitz and Chapple 2010). Along with cellulose, hemicellulose, and lignin, the lignocellulosic materials contain small amount of ash, extractives (chlorophyll, nitrogenous materials, and waxes), proteins, and pectin (Kumar et al. 2009). There is great variation in composition of biomass constituents among different plant sources based on species, tissue, maturity of plant cell wall, variety, soil fertility, climate, and species. The composition of some lignocellulosic materials is shown in Table 16.1.

The methods for determining chemical composition of biomass have been developed by some global organizations like Technical Association of the Pulp and Paper Industry, National Renewable Energy and Laboratory, and American Society for Testing and Materials. The purest sources of cellulose are cotton (80–95%), flax and chemical pulp (60–80%), however, approximately 45% cellulose is present in hardwoods, and softwoods (Demirbas 2005). Monterey pine is a preferred substrate for production of industrial products due to its rapid growth, high cellulose content (48%), and compositional uniformity (Dong et al. 2018). Hemicellulosic content is found maximum in crop residues like wheat straw, rice straw, and corn stover (Foody and Foody 1991). The main hemicelluloses in hardwood species are glucuronoxylans (O-acetyl-4-O-methyl-D-glucuronoxylan), and in softwood species are galactoglucomannans (O-acetyl-galactoglucomannans). Hardwood species have higher xylan and lower mannan content than softwood; therefore, softwood species are more recalcitrant to enzymatic action (Alvarez et al. 2016). Lignin content is highest in softwood barks (30–60%), followed by hardwood barks (30–55%), whereas, minimum lignin content is found in grasses (10–30%), and agricultural

Table 16.1 Composition of lignocellulosic materials

Lignocellulose material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Ash (%)	References
Eichhornia crassipes	18.2	48.7	3.50	NA	Nigam (2002)
Switchgrass	5–20	30–50	10–40	5–6	McKendry (2002)
Wheat straw	30	50	15	NA	Sun and Cheng (2002)
Sorted refuse	32.1	24	18	NA	Howard et al. (2003)
Waste paper from chemical pulp	60–70	10–20	5–10	NA	Howard et al. (2003)
Primary wastewater solid	8–15	NA	24–29	NA	Howard et al. (2003)
Swine waste	6	28	NA	NA	Howard et al. (2003)
Spruce	43.0	29.4	27.6	0.6	Demirbas (2005)
Banana waste	13.2	14.8	14	NA	John et al. (2006)
Rice straw	32.1	24.0	18.0	NA	Prassad et al. (2007)
Hardwood	45–47	25–40	20–25	0.80	Swart et al. (2008)
Softwood	40–45	25–29	30–60	0.50	Swart et al. (2008)
Sponge gourd fibers	66.59	17.44	15.46	NA	Guimaraes et al. (2009)
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7	NA	Singh et al. (2011)
Nut shells	25–30	25–30	30–40	NA	Singh et al. (2011)
Water hyacinth	18.4	49.2	3.55	NA	Singh et al. (2011)
Sweet sorghum	45	27	21	NA	Kim and Day (2011)
Miscanthus	38–40	18–24	24–25	5.5	Brosse et al. (2010) and Rabemanolontsoa and Saka (2013)
Corn leaves	26.93	13.27	15.18	10.95	Rabemanolontsoa and Saka (2013)
Corn cob	42–45	35–39	14–15	3.53	Rabemanolontsoa and Saka (2013)
Sugarcane bagasse	42–48	19–25	20–42	NA	Saini et al. (2015)
Rice straw	28–36	23–28	12–14	19.8	Saini et al. (2015)
Jatropha waste	56.3	17.5	23.9	NA	Nikolic et al. (2016)
Grasses	25–40	25–50	10–30	NA	Nikolic et al. (2016)
Switch grass	45	31.4	12	NA	Kumar and Sharma (2017)
Newspaper	12	40–45	25–40	NA	Kumar and Sharma (2017)
Cotton seed hairs	80–95	5–20	0	NA	Kumar and Sharma (2017)
Garlic and onion	41–50	16–26	26–39	NA	Reddy and Rhim (2018)
Rice straw	41.94	25.58	0.80	NA	Wang et al. (2021)

NA data not available

residues (3–15%) (Demirbas 2005). The complex chemical structure of lignocellulosic biomass is the major challenge for their utilization to develop and commercialize value-added industrial products (Tayyab et al. 2018). Hence, it is necessary to select suitable, cost-effective and economic pretreatment method for their utilization as raw materials with minimum formation of by-products.

16.3 Pretreatment and Hydrolysis of Lignocellulosic Biomass

Pretreatment and saccharification/ hydrolysis are two crucial steps used for the release of monomeric sugars from polysaccharides. Pretreatment causes alteration in the structure, and chemical composition of biomass resulting in improved substrate porosity by decreasing cellulose crystallinity, increasing surface area, size reduction, and lignin removal (Zhu et al. 2009). The fermentable sugars are released from pretreated biomass either by enzymatic hydrolysis or acid hydrolysis (Azhar et al. 2017).

16.3.1 Methods of Pretreatment

There are many physical, chemical, biological, electrical, physico-chemical, thermo-physical, and thermo-chemical methods available for pretreatment of lignocellulosic materials, and each method has some merits and demerits. Figure 16.2 shows different methods available for pretreatment of lignocellulosic biomass but none of these methods could be adapted successfully at industrial scale, and researchers are still focusing on development of some cost and energy efficient pretreatment method. The physical methods used for the disruption of recalcitrant biomass are size reduction (Maurya et al. 2015), microwave irradiation (Amin et al. 2017), and pyrolysis (Den et al. 2018). These methods do not use chemicals and disrupt the biomass by reducing its size by mechanical forces (milling, grinding, and chipping), generating heat from magnetic and electrical components, and rapid decomposition by producing gaseous products (Amin et al. 2017). However, physical pretreatment methods are not economical for commercial use due to high power consumption, and slow rate of reaction.

Different chemicals such as acids (Wyman et al. 2005), alkali (Kumar et al. 2009), ionic liquids (Capolupo and Faraco 2016), organic solvents (Monavari et al. 2009), surfactants (Qing et al. 2010), and lime (Sierra et al. 2009) are used in chemical pretreatment. The most conventional chemical pretreatment method is acid

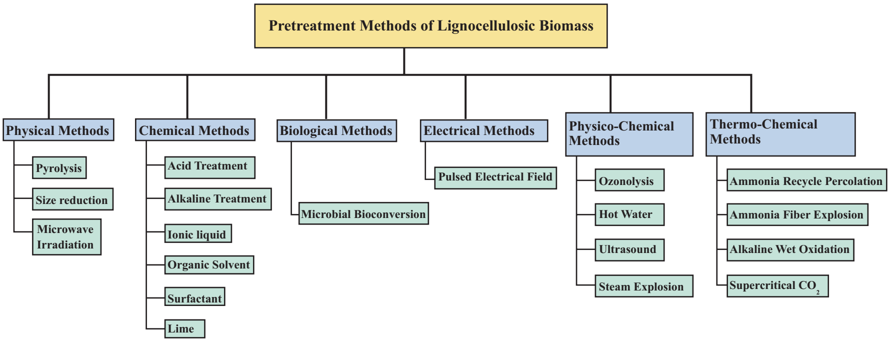

Fig. 16.2 Methods for the pretreatment of lignocellulosic biomass

hydrolysis in which acids like H_2SO_4, HNO_3, HCl, and H_3PO_4 are used for disrupting covalent and non-covalent interactions. Dilute acid treatment methods are simple with less generation of toxic products (Wyman et al. 2005). Alternatively, the pretreatment method using concentrated acids result in rapid extraction of reducing sugars from diverse feedstocks; however, reducing sugars are easily degraded by this method (Zhu et al. 2009). Hemicellulose fraction is rapidly hydrolysed by alkaline agents like KOH, NaOH, NH_4OH, and $Ca(OH)_2$ (Mosier et al. 2005). Simultaneous hydrolysis and delignification for hardwood and softwood can be achieved by using organic solvents (ethanol, methanol, tetrahydrofurfuryl alcohol, acetone, ethylene glycol) (Pan et al. 2005), however, this method is expensive, and leads to synthesis of toxic inhibitors (Eggeman and Elander 2005). Surfactants possess both hydrophilic and hydrophobic properties, and the most commonly used surfactants are polyethylene glycol 4000, polyethylene glycol 6000, Tween 20, and Tween 80 (Zhang et al. 2016a).

Biological pretreatment method is energy-saving and environment friendly. It is conducted at mild temperature and pressure conditions using microbial cells or enzymes as biocatalyst (Tayyab et al. 2018). The major drawbacks for using this method at industrial scale are requirement of chemical mediators, large space, and long residence time with optimum growth conditions. Pulsed electrical field is electrical pretreatment method which needs simple equipment, and ambient conditions (Ammar et al. 2011). Ozonolysis is a physico-chemical method which is conducted at mild temperature, and pressure, and effective for lignin removal, however, this method is expensive and leads to production of toxic inhibitors (Cubero et al. 2009). Hot water (Banerjee et al. 2009), ultrasound (Ivetic et al. 2017), and steam explosion (Pielhop et al. 2016) are thermo-physical methods in which physical and chemical forces cause loosening of lignocellulose materials which become highly susceptible to hydrolysis (Den et al. 2018). The methods used in thermo-chemical pretreatment are alkaline wet oxidation (Monavari et al. 2009), supercritical CO_2 (Kumar et al. 2009), ammonia recycle percolation (Chaturvedi and Verma 2013), and ammonia fiber explosion (Kim 2018), and these methods are reported to be more effective for biomass containing high lipid fraction (Kumar et al. 2009).

16.3.2 Factors Affecting the Pretreatment

There are various factors affecting the pretreatment of lignocellulosic materials and particle size is an important factor of size reduction pretreatment process because it affects the power requirement, and high-power consumption can make the process economically non-feasible (Hendriks and Zeeman 2009). High decomposition rate of biomass can be achieved using pyrolysis at low temperature in the presence of sodium carbonate or zinc chloride as catalyst (Singh et al. 2011). Dielectric properties of lignocellulosic material influence the performance of microwave irradiation (Amin et al. 2017). Dilute acid pretreatment is an economical method and used with abroad range of feedstocks like agricultural waste materials, hardwood, herbaceous crops, municipal solid waste, and softwood for complete removal of hemicelluloses. The temperature-time combination is very crucial during selection of method for pretreatment, this can be carried out at high temperature (180 °C) for short time or low temperature (120 °C) for long time (Myat and Ryu 2016). The efficiency of pulsed electric field pretreatment method relies on various parameters such as electric field strength, treatment time, and pulse parameters, and moisture distribution of plant tissue (Barba et al. 2015). The major factors like temperature, particle size, and residence time affect the efficacy of pretreatment by steam explosion method.

There is enhanced removal of hemicellulose from solid fraction and cellulose digestibility at higher temperature (Oliva et al. 2003). High frequency ultrasonic waves are used in ultrasound pretreatment, and the effectiveness of this method is governed by ultrasonic frequency, reactor geometry, and type of solvent used (Den et al. 2018). In organosolv process, different organic acids like salicylic, acetyl salicylic, and oxalic acid are used as catalyst, however, the use of catalyst is insignificant for satisfactory delignification at high temperature i.e., above 185 °C (Aziz and Sarkanen 1989). Steam explosion pretreatment method is economic and environment friendly which used high pressure saturated steam for explosive decompression of hemicelluloses.

Ammonia fiber expansion is an ammonia-based pretreatment method aimed to improve the susceptibility of lignocellulosic biomass for enzymatic action. In this process, ammonia load and residence time are the critical factors affecting the economics of the process. The conditions optimized for this method are like temperature of 90 °C, liquid ammonia dosage of 1–2 Kg ammonia/Kg dry biomass, and residence time of 30 min (Sun and Cheng 2002). The energy consumption by steam explosion method affects the energy efficiency ratio. The energy efficiency ratio is less (0.26 Kg sugar/ MJ) for steam explosion than organosolv (0.31–0.40 Kg sugar/ MJ) (Zhu et al. 2010), hence steam explosion pretreatment method is a method of choice for wide variety of feedstocks including industrial hump (Sipos et al. 2010), wheat straw (Erdei et al. 2012), wood chips (Pielhop et al. 2016), and corn stover (Walker et al. 2018; Sulzenbacher et al. 2021).

16.3.3 Methods for the Hydrolysis of Pretreated Feedstock

The fermentable sugars are released from pretreated biomass using saccharification/ hydrolysis, which is generally conducted by acid or enzymatic treatment (Azhar et al. 2017). Acid hydrolysis is carried out through dilute or concentrated acids (Kim et al. 2005). Organic acids, phosphoric acid, nitric acid, sulfuric acid and hydrochloric acid are commonly used for acid hydrolysis (Zhou et al. 2013). Enzyme hydrolysis, also known as biological hydrolysis, is another method for hydrolysis of pretreated materials in which biocatalysts (cellulases, and hemicellulases) are used, and biological method is observed to be more promising over acid hydrolysis for commercial applications due to less chemical and energy requirement, less generation of toxic by-products, and high product yield (Madadi et al. 2017). The factors affecting enzymatic hydrolysis involve enzyme mediated factors such as source of enzymes, combined action of two or more enzymes and adsorption of enzymes in addition to lignin content, and substrate related factors such as accessible surface area, cellulose crystallinity, hemicellulose content, particle size and specific surface area ratio and degree of polymerization (Lukajtis et al. 2018). Other physical and chemical parameters like temperature, pH, substrate concentration, enzyme dosage, and treatment time also affect the enzyme mediated hydrolysis (Kamzon et al. 2016).

Bacteria and fungi can be easily grown under laboratory conditions due to their rapid growth and less generation time, and species of *Aspergillus, Clostridium, Cellulomonas, Fusarium, Neurospora, Penicillium* and *Trichoderma* possess the ability for production of cellulases and hemicellulases (Chandel et al. 2007). Cellulase mediated hydrolysis has been conducted by synergistic action of three enzymes namely, endo-glucanase, exo-glucanase and β-glucosidase, and three major steps are involved in this process for production of monomeric sugars from polysaccharide cellulose (Madadi et al. 2017). A complex group of hemicellulases involving endo-β-1,4-xylanase (EC 3.2.1.8), α-D-galactosidase (EC 3.2.1.22), acetyl xylan esterase (EC 3.1.1.72), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), and ferulic acid esterase (EC 3.1.1.73) was employed for degradation of complex hemicellulose structure (Ivetic et al. 2017).

16.4 Production of Industrial Products from Lignocellulosic Biomass

Lignocellulosic biomass is found to be one of the primary raw materials for renewable fuels, chemicals, and different value-added products (Liu et al. 2014), and the major sources of lignocellulosic biomass are agricultural sources like crop residues, perennial grasses and woody crops, logging and wood processing mill waste, and biomass from forest waste (Wahlstrom and Suurnakki 2015). It has been reported that more than 200 value-added compounds have been obtained from lignocellulosic biomass by development of novel cost-effective technique (Kumar et al. 2018a). There are different microbial factories which have been involved in biotransformation of lignocellulosic biomass into value-added products. The major factors influencing rate of microbial growth and production of different primary and secondary metabolites are temperature, pH, inoculum size, inoculum age, aeration rate, type and concentration of carbohydrate, concentration of salt, osmolarity, and ethanol concentration (Sooch and Lugani 2017). The three major steps involved in transformation of lignocellulosic biomass into high value compounds are pretreatment, hydrolysis and fermentation (Kumar et al. 2018b). Different value-added industrial products produced by bioconversion of lignocellulosic biomass have been described in Table 16.2.

Table 16.2 Biosynthesis of value-added industrial products from lignocellulosic biomass

Type of products	Products	Ligno-cellulosic waste	Pretreatment and hydrolysis methods	Microorganisms	Operation conditions	Product yield/titre	References
Industrial enzymes	Xylose reductase	Sugarcane bagasse	Hydrolysis with 98% H_2SO_4 followed by detoxification with CaO and activated charcoal	*Candida guilliermondii* FTI 20037	Xylose: 30 g/L, pH of medium: 5.5, inoculum size: 1.0 g/L, operated at 30 °C under 1.0 vvm (aeration rate) for 12 h (batch bioreactor)	2.5 U/mg of protein	Milessi et al. (2011)
	α-amylase	Banana peel	Homogenization in blender with addition of water 10% (w/v)	*Aspergillus niger* NCIM 616	Fermentation media with NH_4NO_3 (10 g/L), $CaCl_2$ (5 g/L), water (30%), operated at 37 °C for 72 h (solid state fermentation)	1350 U/mg of protein/g peel	Krishna et al. (2012)
	Polygalactouronase	Wheat bran	Particle size reduction to 100–250 μm	*Aspergillus sojae* ATCC20235	Inoculum size (10^7 spores/g substrate) at 37 °C for 96 h (solid state fermentation)	535.4 U/g substrate	Demir and Tari (2014)
	Cellulase	Rice bran and wheat bran	NA	*Aspergillus niger*	Substrate (10 g/L), inoculum size of 10^8 spores at 30 °C under 180 rpm for 168 h (solid state fermentation)	29.81 U/g FPase activity, 25.2 U/g CMCase activity	Reddy et al. (2015)
	Lipase	Coconut dregs	Size reduction (1 mm), H_2SO_4 (1.5% v/v) treatment, pH adjustment, pretreatment with cellulase (30,000 U/g of cellulysin) at 40 °C for 96 h	*Bacillus stratosphericus* PW3	Basal media containing $MgSO_4.7H_2O$ (0.1 g/L), KH_2PO_4 (0.1 g/L), peptone (5 g/L), inoculum size (5%, v/v), pH:7, temperature: 37 °C at 200 rpm for 32 h (submerged fermentation)	140 U/mL	Zin et al. (2017)

(continued)

Table 16.2 (continued)

Type of products	Products	Ligno-cellulosic waste	Pretreatment and hydrolysis methods	Microorganisms	Operation conditions	Product yield/titre	References
	Protease	Organic municipal solid waste	NA	*Bacillus subtilis* AKAL7	Basal media containing solid waste (30 g/L), glucose (10 g/L)), yeast extract (5 g/L), peptone (5 g/L), pH of 9.0, temperature 30 °C under stirring conditions (120 rpm) for 24 h (batch bioreactor)	141.8 U/mL	Hakim et al. (2018)
	Xylose reductase	Rice straw hydrolysate	Size reduction (1 cm), H_2SO_4 treatment and enzymatic pretreatment	*Pseudomonas putida* BSX-46	Xylose (30 g/L) from pretreated rice straw, urea (10 g/L), malt extract (10 g/L), ZnSO4 (1 mg/100 mL), inoculum size (5.0%, v/v), inoculum age (6 h), agitation rate (200 rpm), aeration rate (0.4 vvm), pH (6.5), temperature (30 °C), fermentation time (24 h)	213.14 ± 0.47 IU/mg of cells	Lugani et al. (2021b)

Biofuels	Biobutanol	Soft wood (*Moringa oleifera*)	Chipping and milling, drying at 60 °C for 4 h, pretreatment with H_2SO_4 (2%, w/v)	*Clostridium* sp. MTCC 1349	Fermentation media having beef heart (196 g/L), peptone (40 g/L), dextrose (4 g/L); NaCl (10 g/L) under anaerobic conditions at 37 °C for 168 h	12.8 g/L	Bharathiraja et al. (2015)
	Bioethanol	Rice straw	Pretreatment with aqueous ammonia with solid: liquid ratio of 1:12, hydrolysis with cellulase (15 FPU/g substrate) and xylanase (100 XU/g substrate) at 50 °C for 16 h	*Saccharomyces cerevisiae*	Fermentation media containing yeast extract (10 g/L), peptone (20 g/L) at 37 °C under agitation conditions (200 rpm) for 48 h (semi-solid-state fermentation)	24.6 g/L	Phitsuwan et al. (2017)
	Bioethanol	Monterey pine slurry	Pretreatment with dilute sulfuric acid (2.2%, w/w) and sodium bisulfite (10%, w/w), hydrolysis with cellulase (15 FPU/g of substrate) at 50 °C, 200 rpm for 24 h	*S. cerevisiae*	Fermentation media containing glucose (200 g/L), yeast extract (1 g/L), peptone (2 g/L) incubated at 35 °C for 24 h (fed-batch, semi-solid-state fermentation)	82.1 g/L	Dong et al. (2018)

(continued)

Table 16.2 (continued)

Type of products	Products	Ligno-cellulosic waste	Pretreatment and hydrolysis methods	Microorganisms	Operation conditions	Product yield/ titre	References
Polyols	Xylitol	*Eucalyptus* leaves	Pretreatment with steam explosion, H_2SO_4 (5%, v/v) hydrolysis, pH adjustment	*Aspergillusniger* NCIM 1015	Hydrolysate containing xylose (1.030 g/L), peptone (20 g/L), yeast extract (10 g/L), $(NH_4)_2SO_4$ (2 g/L), initial pH adjusted to 5.0, inoculum size (0.2%, v/v) under 120 rpm for 96 h (batch)	0.300 g/L	Mudaliyar et al. (2011)
	Sorbitol	Rice bran	NA	*Lactobacillus plantarum* NCIM 2912	Substrate (75%, w/v), K_2HPO_4 (10 g/L), tween 80 (5 g/L), $MnCl_2$ (0.3 g/L), ammonium citrate (10 g/L) incubated at 37 °C under 150 rpm for 72 h (solid state fermentation)	5.3 g/L	Jan et al. (2017)
	Xylitol	Cocoa pod husk	Oven drying (until 3% humidity) and size reduction, pretreatment with H_2SO_4 (3.49%, v/v), pH adjustment, ion exchange resin with hydrolysate volume: resin mass of 2:1	*Candida boidinii* XM02G	Hydrolysate containing xylose (11 g/L), peptone (5 g/L), yeast ectract (5 g/L), $(NH_4)_2SO_4$ (2 g/L), inoculum size (0.05 g/L) for 96 h (batch)	0.52 g/g, 11.34 g/L	Santana et al. (2018)

Organic acids	Succinic acid	*Arundo donax*	Cellulase (140 U/g of wet biomass) hydrolysis	*Basfia succiniciproducens* BPP7	Hydrolysate with xylose (13–22 g/L), glucose (25–37 g/L), acetic acid (6–10 g/L), fur (20–50 mg/L), hydroxymethylfurfural (40–70 mg/L), vanillin (10–30 mg/L), 4 – hydroxic benzoic acid (10–30 mg/L), yeast extract (5 g/L) with pH 7.5 at 37 °C for 48 h (bioreactor)	17 g/L	Cimini et al. (2016)
	Lactic acid	*Phragmites australis* straw	Grinding to 20 mesh, pretreatment with 2% (w/v) H_2SO_4, hydrolysis with cellulase (20 FPU/g)	*Bacillus coagulans* IPE22	Inoculum size (5%, v/v), simultaneous saccharification and co-fermentation at 50 °C and 150 rpm	0.41 g/g	Zhang et al. (2018)

(continued)

Table 16.2 (continued)

Type of products	Products	Ligno-cellulosic waste	Pretreatment and hydrolysis methods	Microorganisms	Operation conditions	Product yield/titre	References
	Acetic acid	Pine apples waste	–	*S. cerevisiae* and *Acetobacter acetii*	17 g pine apple peel +20 g sugar + ammonium phosphate 6 g + 800 ml H_2O, pH 4.0, incubated at 25–28 °C for ethanolic fermentation with *S. cerevisiae* for 2 days and then for acetic acid fermentation with *A. acetii* for 11 days	4.77% (v/v)	Raji et al. (2012)
		Papaya peel	8 g papaya peel treated by steam at 121 °C for 20 min; 200 ml H_2O addition and boiled at 80 °C for 30 min + acid hydrolysis with 1% HCl v/v for 30 min. pH adjusted with CaO to 6–6.8	*S. cerevisiae* and *Acetobacter aceti*	100 ml papaya hydrolsate +1% glucose+1 gm dry yeast culture, incubated for ethanolic fermentation at 28 °C, 300 rpm for 2 h intially and then for anaerobic fermentation in dark for 48 hrs at room temperature. For acetic acid fermentation incubated with *A. aceti* for 3 days at 37 °C	5.23% (v/v)	Vikas and Mridul (2014)
					30 g pulp+70 ml H_2O, pH 6.5, ethanolic fermentation by *Saccharomycescerevisiae* in 52 hours at 36 °C producing 3.16% (v/v) ethanol; acetic acid fermentation by *A. aceti* in 48 hours at 34 °C	3.91% (w/v)	Chakraborty et al. (2015)

					Fibrous bed reactor	48% (w/w)/20.9 g/L	Wei et al. (2012)
		Corn husk	Pretreated with acid hydrolysis (0.4 M H_2SO_4 at 110 °C for 6 h)	*Clostridium tyrobutyricum*	Fibrous bed reactor at 37 °C for 2 days	39% (w/w)/21.8 g/L	Fu et al. (2017)
Others	Single cell protein	Wheat bran	NA	*Candida utilis, Rhizopusoligosporus*	Substrate (1 kg/L), chloramphenicol (0.4 g/L), $(NH_4)_2PO_4$ (40 g/L), inoculum size (10%, v/v), inoculum age (48 h) incubated at 30 °C for 96 h (solid state fermentation)	41.02%	Yunus et al. (2015)
	Vanillin	Aleppo pine cones	Pretreatment with conc. H_2SO_4 of pH 2.0, enzymatic hydrolysis with cellulase (30.37 U/mg)	*S. cerevisiae*	Fermentation hydrolysate (15 mL) containing yeast ectract (200 g/L), peptone (400 g/L) pH of 5.5 at 30 °C under 100 rpm for 96 h	162 μg/mL	Messaoudi et al. (2017)
	Microbial lipids	Hemicellulose	NA	*Rhodotorula toruloides* CBS 14	Crude glycerol (55 g/L), hemicellulose hydrolysate (100 g/L)	10.6 g/L	Chmielarz et al. (2021)

Note: *vvm* volume of gas per volume of liquid per minute, *FPase* Filter-Paper cellulase, *CMCase* Carboxymethyl cellulase, *FPU* Filter Paper Units, *XU* Xylanase Units

16.4.1 Production of Industrial Enzymes

Enzymes are proteinaceous biomolecules which enhances the rate of reaction by lowering the activation energy. Enzyme production is one of the central parts of global industrial biotechnology, and utilization of low-cost lignocellulosic biomass as substrate is an appropriate approach for production of proficient enzymes. Different bacteria (*Acidothermus, Acidobacterium, Bacillus, Clostridium, Cellulomonas, Corynebacterium, Enterobacter, Pseudomonas, Staphylococcus*), fungi (*Aspergillus, Cephalosporium, Fusarium, Humicola, Mucor, Neurospora, Penicillium, Rhizopus, Trichoderma*), and yeasts (*Candida, Cryptococcus, Debaryomyces, Hansenula, Kloeckera, Kluyveromyces, Monilia, Pichia, Rhodotorula, Saccharomyces, Torulopsis, Trichosporon*) are involved in the production of industrially important enzymes (Shahriarinour et al. 2011; Lugani et al. 2015; Sooch et al. 2019).

Actinomycetes constitute an important group of microbial population for production of novel enzymes having commercial potential due to their extreme stability, ability to decompose various materials, and unusual substrate specificity. Many commercially relevant enzymes like amylase, cellulase, chitinase, glucose oxidase, lipase, lipoxygenase, pectinase, phytase, peroxidase, protease, and xylanase have been reported to be produced from lignocellulosic biomass (Table 16.2) by different genera of actinomycetes including *Actinomadura, Cellulomonas, Microbiospora, Nocardiopsis, Streptomyces, Thermoactinomycees, Thermobifida, Thermomyces, Thermomonospora* (Prakash et al. 2013). Among all the enzymes, cellulases and hemicellulases have tremendous applications in different industrial sectors like agriculture, animal feed, brewery and wine, chemicals, food, fuel, laundry, paper, pulp, and textiles (Beauchemin et al. 2003).

The two major fermentation strategies used for production of enzymes are solid-state fermentation and submerged fermentation, and there is different genetic expression of microbes under different fermentation modes (Gonzalez 2012; Sirohi et al. 2018). Solid state fermentation leads to utilization of solid substrate for the production of industrial enzymes from different fungal species like ascomycetes, basidiomycetes, and deuteromycetes (Batche et al. 2014) and lignocellulosic materials are found suitable for production of many industrial enzymes. This method promises less downstream processing, minimum effluent production, high volumetric productivity, and increased product concentration (Singhania et al. 2010). However, there are several limitations also associated with this method like there is limited reproducibility of results, and it is not able to standardize process parameters such as temperature and aeration which may lead to enzyme inactivation (Holker and Lenz 2005).

The second fermentation strategy is submerged fermentation which utilizes aqueous medium to maintain uniform temperature, pH, aeration and agitation conditions within the vessel, and this method is commonly used for large scale enzyme production (Ravindran and Jaiswal 2016). There is strict control of different parameters within this system; hence, there are minimum chances of contamination, and less byproducts production like metal ions, butylated hydroxytoluene and hydrogen peroxide (Roukas 2016). Hence, submerged fermentation is commonly used for the fermentative production of different enzymes and bioactive compounds from bacteria, and filamentous fungi using lignocellulosic biomass. However, there are many reports which clearly revealed that submerged fermentation is not an economical mode of enzyme production at large scale because there is a need of sophisticated and large equipment in this mode. Previously, Zhang et al. (2012) showed that capital investment for submerged fermentation is 78% more than solid state fermentation. Research work of various enzyme producing industries is now focusing towards isolation of novel extremophilic and recombinant microbial strains with enhanced enzyme production from lignocellulosic biomass.

Different agricultural lignocellululosic materials like wheat straw (Sumantha et al. 2005), rice straw, rice bran (Virupakshi et al. 2005), coffee by-products (Murthy and Naidu 2010), oil palm empty fruit bunch (Shahriarinour et al. 2011), chocory Dahlia (El-Hersh et al. 2011), wheat bran (Kumar and Sushma 2012), pine apple (Thangaratham and Manimegalai 2014), banana waste (Dabhi et al. 2014), sugarcane bagasse (Kaur et al. 2015), corn cob (Barathikannan et al. 2016), sugarcane industrial waste (Ellila et al. 2017), and wastewater (Zouaoui and Bouziane 2018) were used as feedstocks for production of industrial important enzymes. Different lignocellulosic materials like bajra straw, *Trifolium* hay, sorghum straw, maize straw, oat hay, and wheat straw were screened for production of thermostable xylanase production from *Trichoderma viride*, and the maximum enzyme production was achieved with maize straw under submerged fermentation (Goyal et al. 2008). Detoxified sugarcane bagasse hydrolysate was used for production of xylose reductase from *Candida guilliermondii* FTI 20037, and the maximum enzyme production (2.5 U/mg of protein) was found under microaerobic conditions (Milessi et al. 2011).

Lignocellulosic feedstocks like date seeds, grasses and palm leaves were also tested for the production of different hydrolytic enzymes (α-amylase, pectinase, xylanase) under solid state fermentation from *Bacillus megatherium*, and wheat bran was found to be the best among all the substrates used for production of different hydrolytic enzymes (El-Shishtawy et al. 2014). In another study, carrot peel was used for pectinase production by *Bacillus mojavensis* I4 in submerged fermentation using two statistical methods i.e., Taguchi design (for key ingredients) and Box-Behnken design for optimizing different parameters, and maximum pectinase production of 64.8 U/mL were obtained under optimized conditions with 6.5% (w/v) carrot peel powder (Ghazala et al. 2015). In another study, among all the seven fungal strains tested for lipase production, the maximum enzyme activity of 5.12 ± 0.0059 U/mL was attained with *Aspergillus niger* at 30 °C after fermentation period of 72 h using olive oil as substrate (Mukhtar et al. 2015).

Different forms of cellulases i.e., endoglucanase, exoglucanase and β-glucosidase were produced by *Aspergillus flavus Linn* after utilizing freshly ripe tomato fruit under solid state fermentation (Damato et al. 2010), and by *Bacillus subtilis* from molasses sawdust and bagasse under submerged fermentation (Singh et al. 2016). Response surface methodology was adopted for production of protease from different substrates (corncob and coffee pulp waste) using Box-Behnken design and the maximum enzyme yield of 920 U/mL was attained with 3 g/L and 2 g/L of coffee pulp waste and corncob, respectively at 37 °C after 60 h of fermentation (Kandasamy et al. 2016). Extracellular lipase activity of 140 U/mL was also obtained from novel *Bacillus stratosphericus* using coconut dregs as feedstock in submerged fermentation, and the resultant enzyme was observed to be active under wide range of pH and temperature conditions in the presence of detergents (Zin et al. 2017).

16.4.2 Production of Bioethanol

Bioethanol is a clean, environment-friendly, and alternate fuel for future generation, which results in less emission of green house gases, and hence considered as an alternative strategy for reducing global warming (Joshi et al. 2011). Based on type of raw material, biofuels are classified into four generations i.e., first generation biofuels from edible agricultural crops, second generation biofuels from non-edible lignocellulosic feedstocks, third generation biofuels from algal biomass, and fourth generation biofuels from metabolically engineered algal strains (Meneses et al. 2017). Currently, sucrose and starch-based grains are utilized for industrial level bioethanol production (Asgher et al. 2013); whereas it may generate a competition between food production and fuel ethanol for future generations (Gnansounou 2011). Hence, to avoid such situation, one of the alternative potential approaches is utilization of different agricultural lignocellulosic residual materials for low-cost fermentation production (Iqbal et al. 2013; Lugani et al. 2019). The major steps involved in biofuel production are pretreatment, hydrolysis, fermentation and distillation (Mosier et al. 2005).

Different fermentation modes for bioethanol production are batch, fed-batch and continuous mode. The most traditional method of ethanol production is batch fermentation in which high initial substrate concentration is used (Olsson and Hagerdal 1996). In a continuous system, there is constant addition of nutrients and substrate with removal of biomass and metabolites, and the merits associated with this mode of fermentation are easy to control, less labor intensive, high productivity, and elimination of undesired products (Sanchez and Cardona 2008). Fed-batch system is a combination of batch and continuous process in which substrate is added intermittently, which results in shorter fermentation time, higher ethanol productivity, high dissolved oxygen and low toxicity of media components (Cheng et al. 2009). In another study, ethanol productivity of 0.44 g/L/h, and ethanol concentration of 53.3 g/L was obtained from wheat meal and wheat straw in fed batch system at 32 °C for 120 h under agitation conditions (300 rpm) using *Saccharomyces cerevisiae* TMB3400 (Erdei et al. 2012). The ethanol productivity of 3.8 g/L/h was obtained in a continuous system with a dilution rate of 0.131/h using nonlinear model predictive controller algorithm (Ajbar and Ali 2017).

Various previous reports have been published on ethanol production by utilizing different lignocellulosic feedstocks such as water hyacinth (Kumar et al. 2009), cassava powder (Choi et al. 2010), grass silage (Sieker et al. 2011), spent coffee grounds

(Mussatto et al. 2012), coffee processing waste (Kefale et al. 2012), poplar (Wang et al. 2012), grasses (Scordia et al. 2014), douglas fir (Inoue et al. 2016), rice straw (Wi et al. 2013; Phitsuwan et al. 2017), waste paper (Nishimura et al. 2017), pine needle (Vaid et al. 2018), and pine slurry (Dong et al. 2018). In one previous study, ethanol was produced from cotton hydrolysate using *Saccharomyces cerevisiae* var. *ellipsoideus* at 30 °C under agitation conditions (Nikolic et al. 2016). In a similar study, maximum ethanol production of 33.7 g/L was observed after 96 h of fermentation at 34 °C from corn stover pretreated with ethylenediamine using simultaneous saccharification and co-fermentation strategy (Qin et al. 2018). The ethanol yield is very less with wild microbial strains; hence many recombinant strains have been developed for improved production of ethanol (Cavalheiro and Monteiro 2013; Sar et al. 2017; Ko et al. 2018; Lopez-Hidalgo et al. 2021).

There are some fermentation integrated strategies such as simultaneous saccharification and fermentation, simultaneous saccharification and co-fermentation and consolidated bioprocessing, which are adopted for bioethanol production to overcome the drawbacks of traditional fermentation systems. Simultaneous saccharification and fermentation system allows use of a single vessel for simultaneous hydrolysis and fermentation, which allows ease of process operation, less chances of contamination, and economical ethanol production in short duration. Simultaneous saccharification and co-fermentation method is one step complete simultaneous hydrolysis and fermentation of both pentoses and hexoses into ethanol in short duration (Nikolic et al. 2016). The most upgraded highly integrated approach is consolidated bioprocessing in which single microbial community brings all the processes in a single step including sugar production, enzyme hydrolysis and fermentation (Lynd et al. 2005).

Previously, waste newspaper was used as substrate for ethanol production in simultaneous saccharification and fermentation using thermotolerant yeast *Saccharomyces cerevisiae* KNU5377 at 50 °C, and the maximum ethanol production of 8.4% was attained after 72 h of fermentation (Park et al. 2010). 82.1 g/L ethanol was produced from 25% (w/w) undetoxified pile slurry in simultaneous saccharification and fermentation system (Dong et al. 2018). Simultaneous saccharification and co-fermentation system was used previously by many researchers for the production of ethanol (Liu and Chen 2016; Sharma et al. 2018; Qin et al. 2018). In a recent study, the maximum ethanol yield of 0.148 g/g after 72 h of fermentation was obtained from pine needle biomass using *Saccharomyces cerevisiae* and *Pichia stipitis* through consolidated bioprocessing (Vaid et al. 2018).

16.4.3 Production of Xylitol

Xylitol is an industrially important polyol sugar having tremendous applications in different industrial sectors such as food, bakery, confectionery, cosmetic, odontological, pharmaceutical, and medical sectors (Lugani and Sooch 2017, 2018, 2020; Lugani et al. 2017, 2020; Baptista et al. 2018). The unique properties of xylitol such as negative heat of dissolution, improvement of taste and color of food, and bakery products, absence of Maillard reaction, low calorie sugar, insulin independent metabolism, enhanced remineralization of carious lesions, and ability to retain moisture make this alcoholic sugar an attractive choice for industrial applications (Albuquerque et al. 2014), and the global market of xylitol is supposed to be USD 6.30 billion by 2022 (Markets and Markets 2016). Biotechnological methods (fermentative and enzymatic), being environment friendly, are currently explored to produce xylitol to meet its increasing global demand. Fermentation method, also known as microbial method, uses whole microbial (bacterial, yeast, and fungal) cells for xylitol production at ambient temperature, and pressure conditions with enhanced product yield and productivity, and there is minimum synthesis of by-products during this process (Tran et al. 2004; Lugani et al. 2021a).

Yeasts are the primary candidate of choice for xylitol production due to improved yield and productivity compared to bacteria and fungal strains, and *Candida boidinii, C. guillermondii, C. tropicalis, C. parapsilosis, C. boidinni, Pichia* sp., *Pachysolen* sp., *Kluyveromyces marxianus, Kloeckera* sp., *Saccharomyces* sp., *Rhodotorula* sp., *Hansenula* sp., *Torulopsis* sp., *Trichosporon* sp., *Cryptococcus* sp., and *Debaromyces hansenii* have been extensively utilized by many researchers for xylitol production from various agricultural waste materials (Cortez and Roberto 2014; Tamburini et al. 2015; Zahed et al. 2016; Dasgupta et al. 2016; Xavier et al. 2018). Various genera of bacteria such as *Acetobacter*, *Gluconobacter, Achromobacter, Actinomadura, Agrobacterium, Arthrobacter, Azotobacter, Brevibacterium, Corynebacterium, Erwinia, Flavobacterium, Micrococcus, Nocardia, Planococcus, Pseudomonas, Rhodococcus, Morganella,Streptomyces,* and *Gluconobacter* (Takeuchi et al. 2001; Sugiyama et al. 2001; Lugani and Sooch 2020), and fungi *Neurospora, Penicillium, Aspergillus, Rhizopus, Gliocladium, Byssochlamys, Myrothecium* (Sampaio et al. 2003; Mudaliyar et al. 2011) are also reported for xylitol production.

Different agricultural wastes such as walnut shell (Tran et al. 2004), sugarcane bagasse (Santos et al. 2005), spent brewing grain (Carvalho et al. 2005), rice straw (Zeid et al. 2008), sorghum straw (Sene et al. 2011), groundnut shells, glass straw (Mudaliyar et al. 2011), rice bran (Martinez and Santos 2012), coffee husks, moungbean hull, oat hull, peanut hull, corncob (Jeevan et al. 2011; Tada et al. 2012), sago trunk (Mohamad et al. 2013), coconut husk (Neeru et al. 2013), banana peel (Rehman et al. 2013), pomegranate peel (Barathikannan et al. 2016), oil palm empty fruit bunch (Kresnowati et al. 2016), beech wood, and cocoa pod husk (Santana et al. 2018) have been used as raw material of xylose for xylitol production. Two agricultural wastes such as beech wood and walnut shells were screened for xylitol production by *Candida tropicalis* IFO0618, and the maximum xylitol yield (50%) was obtained with beech wood hydrolysed solution mixed with glucose (1%, w/v) (Tran et al. 2004).

Agricultural wastes such as coconut husks, groundnut shells, grass straw, *Eucalyptus* leaves, *Eucalyptus* wood, and *Jambulina* leaves have been tested for production of xylitol by *Aspergillus niger*, and the maximum xylitol production (0.300 g/L) has been attained with *Eucalyptus* leaves (Mudaliyar et al. 2011). The maximum xylitol production of 35 g/L was obtained from corn cob hydrolysate (containing 40 g/L xylose) by *Pichia* sp. under stirring conditions (150 rpm) at temperature of 28 °C and pH of 6.0 after 72 h of fermentation (Jeevan et al. 2011). Xylitol was produced from cocoa pod husk hemicellulose hydrolysate by *Candida boidinii* XM02G, and the highest xylitol yield of 0.52 g/g was observed at the end of fermentation and the yeast strain was also observed to tolerate phenolic compounds present in hemicellulose hydrolysate up to 6 g/L (Santana et al. 2018). Recently, Lugani et al. (2021b) have utilized rice straw as feedstock for the production of xylose reductase (a xylitol producing enzyme), from novel isolated *Pseudomonas putida* BSX-46. Some attempts have been made for improved xylitol production using industrially efficient recombinant microbial strains produced by mutagenesis and recombination DNA technology (Peng et al. 2012; Pal et al. 2013; Zhang et al. 2015; Kogje and Ghosalkar 2017; Baptista et al. 2018). Other polyol like sorbitol is also produced fermentativly using rice bran from *Lactobacillus plantarum* NCIM 2912 with 5.3 g/L yield (Jan et al. 2017).

16.4.4 Production of Vinegar

Vinegar is a sharp and sour liquid produced by double fermentation of sugar containing solution and is used as condiment and preservation of food. According to Food and Drug Administration, USA, vinegar is produced by two step fermentation process i.e., splitting of sugar into alcohol followed by bioconversion of alcohol into acetic acid. Different processes which have been adapted for production of acetic acid from past decades are Orleans/generator process, submerged fermentation and quick process, and the last two processes are used presently for commercial production of vinegar. Different steps involved in commercial vinegar production are fermentation, filtration, clarification, distillation, pasteurization, and bottling (Oyetoro et al. 2017). Traditionally, different types of raw materials like apples, berries, fruit juices, grapes, grains, honey, plant extracts, rice, sugars, whey have been utilized for production of different types of vinegar. However, in the recent past, different agricultural materials like banana peels (Bazirake et al. 2014), decomposed fruits (Diba et al. 2015), pineapple wastes (Raji et al. 2012; Krusong and Vichitraka 2010; Roda et al. 2014), star fruit juice (Minh 2014), and wood (Donald et al. 2009) have been utilized as substrate for vinegar production.

16.4.5 Other Products

Synthesis of various value-added products like vanillin and gallic acid from lignin-based materials by *Phanerochaete chrysosporium* was reported in literature (Ribbons 1987). Vanillin has potential applications in different industrial products like anti-foaming agents, drugs, herbicides, and household products (Priefert et al.

2001). Furfural is another value-added product produced by bioconversion of xylose containing lignocellulosic feedstock, and it is used for manufacturing of furfural-phenol plastics, pesticides and varnishes (Peleteiro et al. 2016).

The other products produced by lignocellulosic materials are antibiotics, methane, phenolic compounds, polysaccharides, and single cell proteins (Howard et al. 2003). In a recent study, a novel yeast strain *Candida intermedia* FL021 was reported to produce single cell protein by consuming lignocellulosic hydrolysate (Wu et al. 2018). Some organic chemicals such as benzene, ethylene, propylene, toluene, and xylene are also produced by lignocellulosic materials (Pothiraj et al. 2006), and these chemicals are in turn required for the synthesis of different chemical products like resins and polymers (Coombs 1987). Lignocellulosic waste is also utilized for the synthesis of high value byproducts like amino acids, organic acids, vitamins, bacterial and fungal polysaccharides such as xanthan (Pothiraj et al. 2006). In a previous study, L-lysine was produced by both wild and engineered strains of *Corynebacterium glutamicum* using acid pretreated detoxified biorefinery waste stream (Christopher et al. 2016). Chemicals such as 2,3-butanediol (2,3-BD) (Okonkwo et al. 2017), 1,3-propanediol (1,3-PD) (Vivek et al. 2018), and organic acids like lactic acid (Zhang et al. 2018; Juturu and Wu 2018), fumaric acid (Liu et al. 2017), itaconic acid (Saha et al. 2017), butyric acid (Wei et al. 2012; Fu et al. 2017; Xiao et al. 2018) gluconic acid, xylonic acid (Zhang et al. 2016b) and succinic acid (Cimini et al. 2016; Alexandri et al. 2016) have been reported to be produced by microbial fermentation by utilizating lignocellulosic feedstocks. The biodegradable polymer poly-3-hydroxybutyrate was also produced previously by utilizing xylose rich lignocellulosic wastes (Raposo et al. 2017).

Various valuable components such as polysaccharides, vitamins and trace elements like Ca, Fe, Mg, Zn, produced by lignocellulosic materials, improved digestibility of feedstuffs in animals (Zhu et al. 2012). Some previous authors have utilized lignocellulosic materials like corncob, corn stover, eucalyptus, rice straw, spent grain, and sugarcane bagasse for the synthesis of renewable and marketable biochemicals like benzene, biphenyls, catechol, cyclohexane, guaiacols, phenols, syringaldehyde, vanillin and vanillic acid (Messaoudi et al. 2017; Ji et al. 2012; Varanasi et al. 2013). Apart from bioethanol, other environment friendly clean biofuels, such as bio-butanol (Nilsson et al. 2015; Maiti et al. 2018) and bio-hydrogen (Abdul et al. 2013; Sen et al. 2016) are also produced by lignocellulosic biomass. During the past few years, some research has been directed towards development of bio-based composites with different functionalities of interest from lignocellulosic waste residual materials (Bajpai et al. 2013), and production of medium-density fiber board is one of the successful attempts in this research (Li et al. 2013).

Agricultural lignocellulosic wastes like cassava peel, coconut oil cake, corn cob, corn husk, corn steep waste, peanut shells, rice husk, rice straw, sugarcane bagasse, sugarcane molasses, and wheat bran flour have been utilized previously for the production of various antibiotics like cephalosporin, cyclosporine A, neomycin, oxytetracycline, rifamycin A, and tetracycline using solid state fermentation (Kaur et al. 2014). In a previous study, corn stover was utilized to produce succinic acid from *Basfia succiniproducens* and a final productivity of 0.43 g/L/h was obtained

(Salvachua et al. 2016). In another study, *Actinobacillus succinogenes* and *Basfia succiniproducens* was used for biotransformation of spent sulphite liquor into succinic acid production and 39 g/L yield was achieved. Along with succinic acid, lignosulphonates (32.4 g/L), and phenolic rich extract (1.15 g) was also produced after fractionation (Alexandri et al. 2016). Many industrial valuable products such as astaxanthin, biohydrogen, β-carotene, docosahexaneoic acid, eicosapentaneoic acid, lutein, lycopene, phycobiliproteins, and zeaxanthin are produced by microalgal biomass (Bhalamurugan et al. 2018).

16.5 Improved Strategies for the Production of Industrial Products

The yield and productivity of industrially important valuable products is very less with wild microbial strains, and hence these methods can't be adapted at commercial level for production of energy and cost-effective bio-based products. Hence, the focus of researchers is towards development of different strategies like genetic and metabolic engineering, immobilization methods and *in silico* computational techniques to reduce the cost of products produced by utilization of lignocellulosic biomass.

16.5.1 Development of Genetically-Modified Strains

Major techniques used to produce recombinant microorganisms are genome-based strain reconstruction, metabolic engineering, protein engineering, genome-wide transcript expression analysis, molecular breeding, genome mining, recombination DNA technology, whole genome shuffling and genome mining (Adrio and Demain 2010; Yang et al. 2017; Yang et al. 2018). One of the emerging fields of recombinant DNA technology is metabolic engineering to improve the production of novel products, accelerating rate determining step, engineer enzyme activities and shift metabolic flux towards synthesis of desired product by modulating transport of sugar, gene regulation and enzyme expression. In this process, there is introduction of heterologous genes or regulatory elements for developing novel metabolic configuration (Joshi et al. 2011). Various attempts have been made for adopting genetic engineering techniques for development of industrially efficient microbial strains which can show enhanced gene expression, produce improved product yield and tolerate high temperature and low pH.

The genetically engineered strains of *S. cerevisiae* and *Zymomonas mobilis* have been employed by DuPont Dannisco Cellulosic Ethanol and Butalco for commercial production of ethanol (Weber et al. 2010). Protein engineering is another advance in biotechnology for construction of tailored biocatalysts with desired functions using directed evolution or rational design strategies. The other techniques of protein engineering are semi-rational design (Lutz 2010), truncation and fusion (Yang et al. 2014) and structure-based designs using site-specific or non-specific chemical modifications (Davids et al. 2013). Various genetically modified fungal strains have been developed which possess the ability for production of large-scale hydrolytic enzymes such as cellulose, hemicellulase and xylanase and fermentable sugars from agricultural waste materials such as corn stover, straw, sugarcane bagasse, and switchgrass (Deswal et al. 2014).

However, genetically modified microorganisms possess several environmental and public health risks when used in large-scale fermentation systems (Limayem and Ricke 2012). Hence, to avoid such risks associated by exposure of genetically modified microorganisms, microbial risk assessment modeling approach is used for risk assessment of microbial dissemination in four steps namely hazard identification, hazard characterization, exposure assessment and risk characterization (Haas et al. 2004). Nanotechnology is one of the emerging fields of industrial biotechnology for generation of recombinant microbial strains by site-specific insertion of desired gene (Chekol and Gebreyohannes 2018).

16.5.2 Immobilization Strategies

Biocatalysts show tremendous applications to produce industrial important products from lignocellulosic materials by accelerating the rate of reaction at optimum conditions. However, there are some limitations for their use at commercial scale like easy solubility in aqueous media, high cost, less stability, and availability in small amounts (Sarrouh et al. 2012). Immobilization is one of the attractive approaches as an alternate which makes the enzyme immobilize onto insoluble matrix for retaining its geometry and economic reuse under stabilized conditions. Enzymes can be immobilized on supports either reversibly using adsorption, affinity, ionic and metal binding or irreversibly using entrapment and covalent binding. Different immobilization techniques used to bind the enzymes are shown in Fig. 16.3.

Enzymes bind to different carrier support materials through various functional groups (amine, alcoholic, carboxylic, guanidine, imidazole, phenol, thioester and thiol) by physical (hydrophobic and van der Waals interactions), covalent or ionic interactions. Covalent interactions are observed to be strongest and ionic interactions are weakest among different types of enzyme-carrier interactions (Ali et al. 2017; Kauldhar et al. 2016). Adsorption, cross-linking, encapsulation and entrapment are traditional immobilization strategies, whereas, protein engineering, nanotechniques and affinity tags are considered as modern strategies for biocatalyst immobilization (Ali et al. 2017). Protein engineering for enzyme immobilization is done by site-directed protein modification techniques such as enzymatic modification, native chemical ligation, auxotrophic expression and nonsense suppression.

The tags used for affinity immobilization are His (Histidine), GST (glutathione-S-transferase), FLAG (epitope tag), HA (hemagglutinin), myc, biotin and DNA

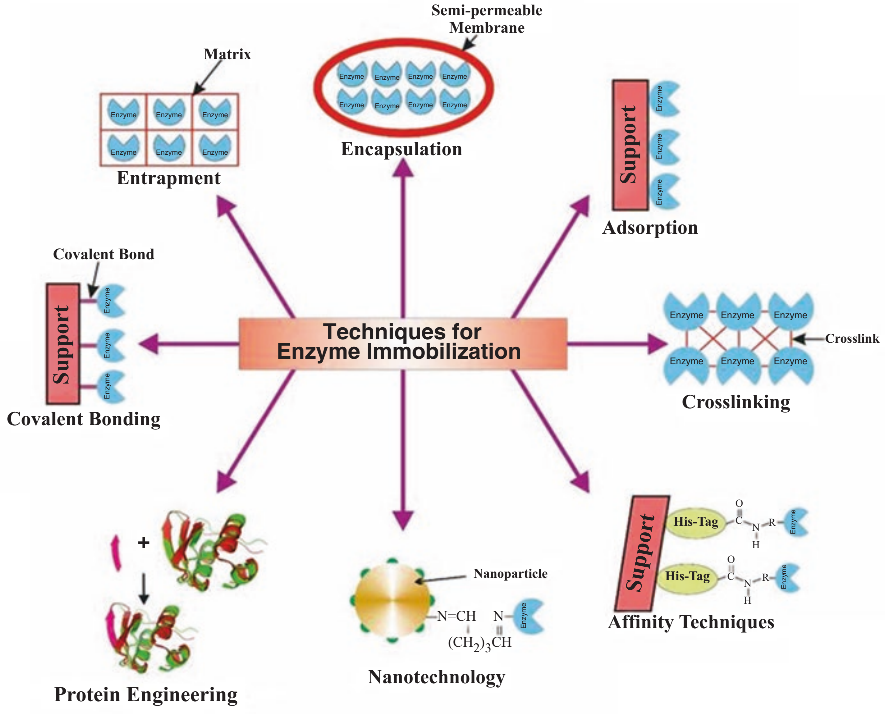

Fig. 16.3 Techniques for enzyme immobilization

mediated tags (Redeker et al. 2013). Some attempts have already been made by previous authors for development of cost-effective immobilization system for production of various bio-based products from lignocellulosic biomass; still minimum success has been achieved from previous studies for generation of commercial immobilization system. Development of multi-enzyme immobilization system is one of the promising alternate strategies which can be utilized for production of valuable products from lignocellulosic biomass. Hence, further studies and research is required to develop enzyme-based immobilization systems by understanding properties of proteins, their stability, conformational changes under different conditions and interactions involved in their immobilization.

16.5.3 Computational Strategies

Several genome sequence databases such as GenBank, European Nucleotide archive and *Saccharomyces* genome database, and protein sequence databases such as Swiss-Prot, Universal Protein Resource, TrEMBL, Protein Information Resource, worldwide Protein Data Bank are available for *in silico* computational studies. Other commonly used databases for protein-protein interactions, membrane transporters and carbohydrate-modifying enzymes are Molecular Interaction database, Transporters Classification Database and Carbohydrate-Active enzyme database. Molecular interactions are studied using Docking, AUTODOCK, High Ambiguity Driven Protein-Protein Docking and GRID (Mehmood et al. 2014). COMPOSER (Sutcliffe et al. 1987), 3D-IIGSAW (Bates and Sternberg 1999) and MODELLER (Sali and Blundell 1994) are used for molecular modelling of proteins.

For evaluation of potential stabilizing mutations, computational high-throughput screening methods have been observed to attract more attention (Goldenzweig et al. 2016). Molecular dynamics and quantum mechanics studies are used to understand the effect of every single amino acid on protein structure and function (Kaushik et al. 2016). Computational approaches have been integrated with metabolic engineering for developing models for improved prediction of metabolic fluxes and their regulation by metabolite concentration, gene expression and protein expression (Strauer et al. 2009). Different methods such as pathway-based approaches, optimization-based approaches, kinetic modeling approaches and many other bioinformatics tools are being used for the production of desired products from microorganisms (Reed et al. 2010; Sooch et al. 2016). Computational tools have also been used for development of engineered enzymes having novel and improved activities (Damborsky and Brezovsky 2014). Machine learning is one of the advanced techniques of metagenome analysis and this technique can be used in future for the selection of efficient wild and genetically modified microbial strains producing large amount of industrial important compounds in considerable good amount from lignocellulosic biomass.

16.6 Current Status of the Green Technology

The global market of industrial important enzymes like amylase, cellulase, protease, lipase and phytase is found to be increased by 6.30 billion USD by 2022 (Industrial Enzymes Market 2018).The major players in global market for production of industrial enzymes are Advanced Enzyme Technologies Ltd., Aumgene Biosciences, PAC Bio Fungbact Pvt. Ltd. (India), Amano Enzyme Inc., Asahi Kasei Pharma Corporation, Hayashibara Company (Japan), AB Enzyme GmbH, BASF SE, Direvo Biotech AG (Germany), BioResource International, Inc., Codexis, Inc., DuPont Nutrition and Health, Enzymatic Deinking Technologies LLC, Enzyme Innovation, Thermo Fischer Scientific, Inc. (USA), Chr. Hansen A/S, Novozymes A/S (Denmark), Royal DSM (The Netherlands) (Research and Markets 2018).

The major companies producing bioethanol in India from biomass are Aatmiya Biofuels Pvt. Ltd., D1 Oil Plc, Godrej Agrovet, Emami Group, Gujarat Oelo Chem Ltd., Jain Irrigation System Ltd., Nova Bio Fuels Pvt. Ltd., Reliance Industries Ltd., Sagar Jatropha Oil Extraction Pvt. Ltd. The main focus of their research work is towards establishment of cost-effective biofuels (butanol, ethanol, dimethylether, hydrogen, biodiesel and hydrocarbons). Different research centers which have been developed in India with prime interest towards biofuel generation using renewable resources are located in Delhi (International Centre of Genetic Engineering, Biotechnology Centre for Advanced Bioenergy Research), Faridabad (Centre for Advanced Bioenergy Research: Indian Oil Corporation Ltd. and Department of Biotechnology Institute), Mumbai (Institute of Chemical Technology) and Indian Institute of Technology located at Mumbai, Kharagpur, Guwahati, Roorkee and Jodhpur (Lali 2016).

The market of petroleum and natural gas sector was found to be USD 7 billion in June 2018 (IBEF 2018). Xylitol is one of the important industrial products which has shown tremendous applications in various industrial sectors and its global market is expected to be USD1 billion by 2020. The major companies of xylitol production are CSPC Shengxue Glucose Company Limited, Roquette Freres, Cargill Incorporation, Novagreen Incorporation, DFI Corporation, S2G Biochem, Dupont Nutrition and Health, Ingredion Incorporation, Shandong Futaste Company Limited, O'laughlin Industries Company Limited, Xylitol Canada Incorporation, Mitsubishi Shoji Foodtech Company Limited, Shandong Longlive Bio-Technology Company Limited, Shandong Lujian Biological Technology Company Limited, Thomson Biotech Company Limited, Zichem Incorporation, Zhejiang Huakang Pharmaceuticals Company Limited. DuPont Dansico is one of the global leading players of xylitol production with three production plants in USA, China and Finland with their sweetener business of Danisco USA Incorporation, Danisco Sw. Anyang Company Limited and Danisco Sweeteners Oy (Markets and Markets 2016).

The key global manufacturers of vinegar are AcetificiItaliani Modena, Australian Vinegar, Bizen Chemical, Fleischmann's Vinegar, Krafet Heinz, Mizkan and Shanxi Shuita Vinegar (Marketers Media 2018). Deloitte Touche Tohmatsu Ltd. has published a report in 2017 entitled 'The chemical multiverse 4.0' on market size of chemical industry, and in this report ethylene capacity is observed to be enhanced in 2018 by US and China. The maximum chemical mergers and acquisitions activity is found in US followed by China, UK, Germany, India, France, Brazil and Switzerland. In the same report *in silico* experimentation and machine learning are found to be two major computational techniques for development of novel chemicals in this competitive market (Deloitte 2017). United States Department of Agriculture has given a report on bio-based chemicals containing commodity chemicals (6–10%), polymers (10%) and fine chemicals (45–50%) in 2025 (USDA 2008).

The sales of DuPont's industrial biotechnology products increased to USD 1 billion in 2015 from USD 200 million in 2009. A joint venture was developed between DuPont and Tate & Lyle which was focused on production of propanediol from corn and other feedstocks by fermentation (www.duponttateandlyle.com). The global players of single cell proteins market are NOW Food Health LLC., Willows Ingredients, Devenish Nutrition Ltd., BIOMIN Holding GmbH, PRO SOLO SPA, Aumgene Biosciences, BIO-CAT, Novozymes, Alltecch Inc., Nutreco N.V. (Market Research 2018), and the market size of single cell proteins is expected to be USD 8.7 billion by 2023 with commercial success of *Chlorella* and *Spirulina* (P & S Market Research 2018).

16.7 Conclusion

It has been concluded from the present studies that lignocellulosic biomass can be exploited to produce numerous industrial important value-added products namely ethanol, vinegar, xylitol, industrial enzymes and chemicals (organic solvents, olefins and plastics), drink softner solvents and fermentable sugars. Lignocellulosic biomass is an important renewable source present in abundance in nature and its use for production of value-added products provides various environmental benefits. The major bottleneck for production of value-added chemicals at commercial scale is chemical complexity of lignocellulosic biomass, complex metabolic pathways of microorganisms, less product yield from wild strains and presence of inhibitors in lignocellulosic biomass hydrolysate. Development of improved pretreatment strategies, fermentation processes and recovery of value-added products by efficient methods may reduce their capital and operating costs.

Media and process engineering through statistical tools is one of the alternate methods to improve economic production of different products. System biology combines experimental and computational research, and it involves integration of many disciplines such as biology, engineering, computer science, chemistry and physics for understanding complex biological systems and network behavior between gene, protein and informational pathways. Hence, system biology can be adapted in future to understand microbial metabolic pathways for development of commercial biological systems which can carry out all the required steps (pretreatment, hydrolysis and fermentation of both pentose and hexose sugars) in a single pot.

Solid state fermentation is also a promising approach over submerged fermentation to use lignocellulosic biomass for the production of industrial products. Therefore, by adapting combinational advanced techniques like biodiversity studies, meta-genomics, system biology, protein engineering, developing research for regulating different media and process parameters, high automation for regulation of parameters during operation, it will become a reality in near future to produce renewable and pollution free value-added fuels and chemicals by utilizing cost effective lignocellulosic materials.

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