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Dinesh K. Maheshwari
K. Annapurna *Editors*

Endophytes: Crop Productivity and Protection

Volume 2

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Preface

A better understanding of endophytic microorganisms may help to elucidate their functions and potential role in developing sustainable systems of crop production and their protection against abiotic and biotic stresses. Endophytes play a vital role in growth and health promotion of plant. Endophytic bacteria are of agrobiological interests because they create host–endophyte relationship having exciting prospects for newer biotechnological applications. Endophytes proved beneficial alternative for sustainable solutions for agrochemicals due to their role in biological control of pests and diseases. They reduce the burden of excess use of agrochemicals. On the other hand, endophytes are potential source of several secondary metabolites and several useful other metabolites such as alkaloids, enzymes, biosurfactants, biocontrol agents, and plant growth promoters. It is imperative that these products have industrial applications in the field of biotechnology, pharmacy, and agriculture.

The 'Endophytes: Vol. II Crop productivity and protection' is an endeavor to review the current developments in the understanding of microbial endophytes and their potential applications in the enhancement of productivity and disease protection. This book contains various chapters presenting state of knowledge on involvement of endophytes in crop productivity and soil health because of beneficial for agricultural and forest ecosystem. Endophytes contribute in nonnative crops, volatile organic compound production, and a remarkable source of biologically active secondary metabolites and enzymes, as lignin degrading fungi, in bioremediation, phosphate solubilization, agricultural productivity, and plant disease control. The chapters describe the strategies for crop improvement and production of useful metabolites and aromatic compounds, enzymes, and other metabolites. These chapters are described with advance information on endophytes for productivity and protection in sustainable plant ecosystem.

We are sure the book will be useful to botanists, microbiologists, biotechnologists, molecular biologists, environmentalists, and those working for the protection

of plant species of agricultural and medicinal importance. I am thankful to the contributors of these books for their cooperation and patience in the compilation of this task. I am also thankful to Springer team, particularly Drs. R. Valeria and Takeesha for their constant support in the publication of this work.

Haridwar, India — Dinesh K. Maheshwari
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Chapter 1
Endophytes as Contender of Plant Productivity and Protection: An Introduction

Dinesh K. Maheshwari, Shrivardhan Dheeman and K. Annapurna

Abstract Bacterial endophytes are versatile with impeccable mastery to occupy their niche in plant tissues, thus, experiences less competition than the other free-living rhizospheric inhabitants. These holds vast and extended scope of their utilization in plant health and growth promotion and contribution in sustainable agriculture as potent contender. This chapter introduces overview on the diverse role of endophytes for multidisciplinary benefits exclusively in plant productivity and protection.

Keywords Bioremediation · Bacterial metabolites · Invasive endophytes Native plants · Non-native plants · Forest ecosystem

1.1 Introduction

There is a great deal of interest in understanding the role of endophyte diversity in plants and their ecology, evolutionary biology and applied sciences research ranging from crop productivity to protection against abiotic and biotic stresses. During last decade, maximum numbers of papers on beneficial endophytes have been published from the USA followed by narrow difference between China and India. Top nine countries have published on different aspects. Whereas subject-wise

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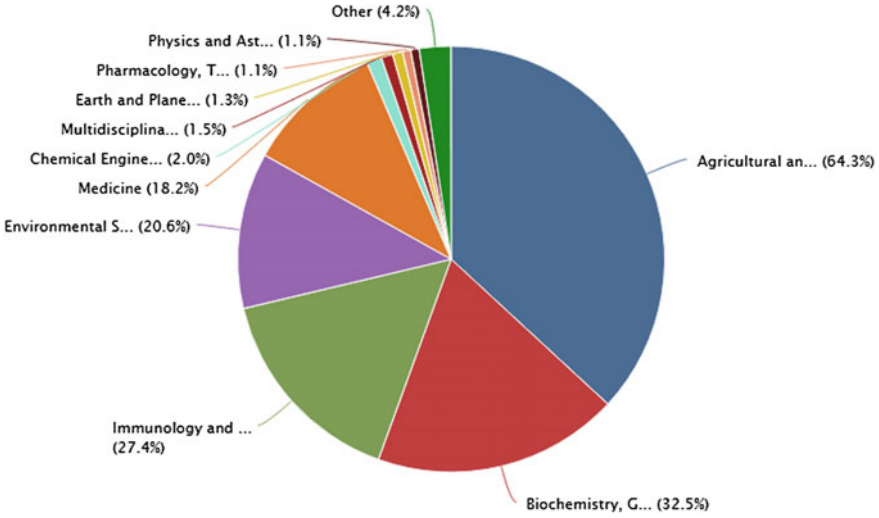

Fig. 1.1 Beneficial endophytes in different area (subject wise distribution). *Source* www.scopus.com/

maximum publications appeared on their beneficial role in both native and non-native crops and more particularly to that of agricultural benefits (Fig. 1.1).

With the growing need for increase food and bioenergy biomass but with a great understanding of the implications of conventional intensive agriculture, the time is right for a great emphasis on biological mechanisms for improvement of plant growth. Endophytes have an advantage since there would be less competition, when adding soil bacteria to the established rhizosphere communities. Endophytes with the ability to colonize internal host tissues has made them valuable microorganisms to improve crops performances as well as forest trees which are equally benefitted by using endophytes via seeds, seedlings, etc.

Almost whole plant, even the pollen and pistil are the sources of endophytic microorganisms but, present more in root than that of aerial plant tissues. Similarly, epiphyte microbial (leaf) populations (phyllosphere) are more numerous in comparison to that of endophytic populations (Beattie and Lindow 1999). It is interesting to note that fungal endophytes have bacteria and viruses make tritrophic endophytic interactions (Hoffman and Arnold 2010). Recently, Aeron et al. (2014) observed endophytic colonization of putative invasive non-rhizobia endophytes from *Clitoria ternatea* L. nodules; the bacteria that lack the ability to form nodules were also observed in the root nodules.

The majoring of reports deal with the culturable endophytes and for most of such nodule inhabiting bacteria, their endophytic nature is not yet proven. Since, they remain associated with plant adhering tissues, viz, nodules; these are now referred as putative endophytes. Various genera such as *Streptomyces*, *Agrobacterium*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Enterobacter*, *Paracoccus*, *Lysinibacillus*,

Staphylococcus, *Starkeya* and others exist or co-exist with or without a tree symbiont inside nodules.

Plant tissues colonized with diverse genera of microbes those persist as epiphytes and endophytes and historically, endophytes inherited from endotroph introduces concerns in relation with endomycorrhizal association (Frank 1885) and later used to define ferns colonized with algae as described by Campbell (1908). Endophytes have both beneficial and harmful effect to the associated plants. But more often, the endophytic microbes reduce herbivory (Koh and Hik 2007), induce plant growth and development (Hardoim et al. 2008), increase mineral uptake (Malinowski and Belesky 2000), fix nitrogen (Doty et al. 2009), suppress phytopathogens and diseases (Melnick et al. 2008) and induce plant defence Kloepper et al. 2004). As a matter of fact, their colonization in an ecological niche is similar to plant pathogens which might favour them as a potential biocontrol agent (Ramamorthy et al. 2001). The close association with plant tissues make them amicable and often a unique opportunity for their role in biological control. The endophytic microbes in biocontrol received lot of interest and suitably described in the present book.

Endophytes proved as a novel source of enzymes, antibiotics including other secondary metabolites of agro-biological and ecological significance. In addition, endophytes are often used in rhizoremedation. Reports on their ability and applications to degrade pollutants have now been possible (Doty 2008; Segura et al. 2009).

Next generation sequencing such as pyrosequencing, ROCHE sequencing, High throughput sequencing etc. can lead to discovery of new groups of microbes bioremediation of pollutants. Bacterial community from aerial part of plant bears plant growth promoting attribute to control diseases. The leaves harbour endophytic culturable bacteria beneficial to plant which can be used as bioinoculants for plant growth promotion thus for increasing their productivity (Malfanova 2013).

1.2 How Endophytes Are Beneficial for Agriculture System?

Similar to other bacteria endophytes are potential inhabitant in a wide variety of native and cultured crop plants. Their presence inside the host tissues undoubtedly exhibiting with diverse morphologies that ranges unicellular to filamentous forms. Their presence in both terrestrial and aquatic ecosystem, including marine environmental plants holds beneficial impacts via offering nutrient accumulation, secondary metabolite production, etc. Other than, rhizospheric benefits, actinobacteria are also involved in recycling of nutrients, decomposition of organic matter, degradation of agricultural and urban wastes, environmental pollutants, such as petroleum, dyes and other recalcitrant compounds which in turn corroborate the soil ecology and agro-ecosystem as discussed in Chap. 2.

1.3 Endophytes: A Part of Forest Ecosystem

Forest trees are providing unique ecological reservoir for bacterial endophytes. Of course, forests are important component to sustain environment and play significant role to keep integrity and sustainability of nature. Forests cover one-third of entire land on Earth, providing vital organic infrastructure for some of the planet's thickest and most diverse collections of life. Bacterial endophytes associated with tree species are rather limited but their importance should not be underrated. By virtue of beneficial endophytes associated with forest tree, wide range benefits can be harnessed in term raising potential future for forest trees so as to restore the density and sustainable existence of forest to keep earth green as reviewed in Chap. 3.

1.4 Endophytes in Native and Non-native Crops

The increasing introduction of non-native plants particularly improved germ-plasm of crops is utmost necessary for adequacy of food to human beings and feed to animals. Microbial invasion in plants has a considerable role to play in facilitating their growth and productivity besides biological control of deleterious phytopathogens causing diseases in non-native plants. To apply for beneficial relationships, endophyte-plant host interactions are suitable strategies that facilitates agricultural productivity. Beneficial endophytes of non-native crop host can be utilized in native or indigenous crop as reviewed in Chap. 4.

1.5 Endophytes Increase Microbial Activity in Tissues

The outer epidermal walls of plant cells are covered with mucilage and cuticle. The cell also secretes polysaccharides and their biopolymers. The organic and inorganic compounds in the cells cytoplasm are diffused out. This occurs probably due to unfavourable conditions and sometimes indirectly affect the aerial surface accumulate directly. In case of underground region, beneath the soil is root and loss of organic and inorganic compounds from its surface is known as root exudates. Inside the tissue, endophytes colonize and constitute a good base which is utilized by microorganism and release various metabolites multifarious in nature.

1.6 Endophyte as a Source of Potential Metabolites

These are member of volatile organic compounds as well as diffusible substances produces by endophytes. The low molecular weight hydrocarbons, aldehydes alcohol, lectones, peptides inorganic volatiles such as HCN are produced during

primary and secondary metabolism of these endophytes. Some of these chemicals are the source of signalling that facilitates the activity of other microorganisms present is the ecological niche prove beneficial in both raising productivity and protecting plants. Even few of the endophytes act as agents triggering plant immunity and enhancing plant growth and health support. Thus, impact to understand the bioconversion of cellulosic domain into liquid fuel, role of volatile organic compounds in biocontrol, etc. cannot be ruled out. The characterization and elucidation of these compounds, with suitable strategy in agricultural practices has been elaborated in Chap. 5.

New discovery of molecule is a continuous process in pharmaceutical industry because of development of new races and genera of resistance in microorganisms. Various genera such as *Escherichia*, *Salmonella*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Micrococci,* etc. belong to multidrug resistance and some *Enterococcus* spp. proved vancomycin resistance. There is no proper drug available to combat infections cause by these genera. Suitable strategies still need to establish for isolating potent biomolecules both from microorganism as well as plants (Table 1.1). Endophytes are ubiquitous in nature associated with different genera and tissues of diversify nature cellulosic versus non-cellulosic, pectolytic versus

Table 1.1 Showing the similar product of both endophyte and plant origin

Name of the metabolite	Plant/plant part	Microorganisms	References
Azadirachtin A	*Azadirachta indica* A. Juss	*Eupenicillium parvum*by	Kusari et al. (2012)
Camptothecine (CPT)	*Miquelia dentata* Bedd.	Endophytic bacteria	Shweta et al. (2013a)
Rohitukine	*Dysoxylum binectariferum* Hook.f	*Fusarium proliferatum* (MTCC 9690)	Kumara et al. (2012)
Paclitaxel (taxol®)	*Taxus brevifolia*	*Taxomyces andreanae*	Stierle et al. (1993)
Plant-derived bioactive compounds	–	Endophytic fungi	Zhao et al. (2011)
CPT, 9-methoxy CPT (9-MeO-CPT) and 10-hydroxy CPT (10-OH-CPT)	*Miquelia dentata* (Icacinaceae)	*Fomitopsis* sp. P. *Karst* (MTCC 10177), *Alternaria alternata* (Fr.)	Shweta et al. (2013b)
		Keissl (MTCC 5477) and *Phomposis* sp. (Sacc.)	
Taxol	*Taxus brevifolia*	*Taxomyces andreanae*	Stierle et al. (1993)
Camptothecin	*Nothapodytes foetida*	*Entrophospora infrequens*	Puri et al. (2005)

(continued)

Table 1.1 (continued)

Name of the metabolite	Plant/plant part	Microorganisms	References
Camptothecin	*Apodytes dimidiate*	*Fusarium solani*	Shweta et al. (2010)
Podophyllotoxin	*Sinopodophyllum hexandrum*	*Alternaria* sp.	Trivedi et al. (1970)
Podophyllotoxin	*Sabina recurva*	*Fusarium oxysporum*	Kour et al. (2007)
Vinblastine	*Catharanthus roseus*	*Alternaria* sp.	Li et al. (2004)
Vincristine	*Catharanthus roseus*	*Fusarium oxysporum*	Wang et al. (2006)
Hypericin	*Hypericum perforatum*	*Chaetomium globosum*	Kusari et al. (2008)
Diosgenin	*Paris polyphylla var. yunnanensis*	*Cephalosporium* sp.	Jin et al. (2004)
Azadirachtin	*Azadirachta indica*	*Eupenicillium parvum*	Kusari et al. (2011)

non-pectolytic as well as in tissues having various deposits. Screening of endophytic microbes for biologically active metabolites with promising medicinal and agricultural application may provide a suitable outcome from endophytes association as discussed in present volume.

1.7 Are Endophytes Remediating Pollutants in Ecosystems?

Most studies of wood-decaying fungi are based on advanced stages of wood degradation. However, some endophytic fungi could be involved in triggering the development of early stages of wood decay. In nature, endophytes inhabit asymptomatic plant tissues, living in symbiosis with their hosts. Thus it becomes necessary to explore the role of wood-inhabiting fungi and study their ligninolytic mechanistic strategies so as to exploit as alternative for degrading lignin or other recalcitrant compounds hazardous to environment. Technological application of these fungi could improve current technological performance of bioconversion processes as reviewed in Chap. 7.

Although phyto-extraction process affect many advantage to remediate heavy metal contaminated soil but it has several demerits mainly the process is economically non-viable (Succuro et al. 2009). The addition of microorganisms in the

plant rhizosphere is an established route to make the process more efficient. The microbial inducer improvement in the accumulation of the heavy metals in plant biomass are always coincident with enhances net phytoextraction (Pajuelo et al. 2007). Microbes in general and fungi in particular clean up environment and proved potential source for biodegradation of organic pollutant. Various genera of endophytic fungi developed a variety of tolerant mechanism toward host metabolites in order to increase their adaptability in environment and interconnection between different organisms further augment bioremediation potential of endophyte fungi in the management of toxic pollutant has suitably given in Chap. 8.

1.8 Factors Affecting Endophytic Colonization

Endophytic microbial colonization affecting by mass factors such as (a) temporary chilling of plant increases the release of amino acid from roots in sand soil (b) exudation induce under high intensity of light (capture by endophyte plant) and temperature (c) secondary metabolites of certain bacteria cause increase and in the presence of competitive synergative rhizobia; polygalactouronase is released from the roots resulting increase in polypeptide antibiotics thus increase the substantial leakage of both organic and inorganic compounds (Swamy et al. 2016). Root exudates are, therefore, bears induction of chemotaxis in bacteria towards the roots and the simultaneous conditioning of bacterial cells for host cell attachment. Thus, it is hypothesized that the capability of bacteria to condition for (plant) host cell attachment during chemotaxis is one of the most important factors for pathogenicity or colonization efficiency.

1.9 Conclusion and Suggestions

Endophytes in plants play significant role in microbial ecology, evolutionary biology, applied life sciences ranging from bioprospecting for genes and molecules to lead productivity enhancement and biocontrol for wide array of crop fungal pathogens. They are expected to control both endophytic fungi and epibiotic to other microorganisms of endophytic species as tools to manage plants disease, reproductive biology of plants. Biocatalysis and other biotechnological processes, new technologies and new crops with endophytes still have many areas open for future research. After consideration of all the chapters included in the present volume, some of the points have been summarized with few more interesting aspects being highlighted. More research on endophytes, yet to be cultivated on artificial culture media are required. This will be possible when a better knowledge of endophyte ecology and molecular interactions is attained.

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Chapter 2
Plant Growth Promotion by Endophytic Bacteria in Nonnative Crop Hosts

Akshit Puri, Kiran Preet Padda and Chris P. Chanway

Abstract Studies highlighting the colonization and plant growth-promoting ability of endophytic bacteria inoculated into nonnative plant hosts reviewed and presented in this chapter. Endophytic bacteria, especially those related to the genus *Bacillus*, *Burkholderia*, *Enterobacter*, *Gluconacetobacter*, *Herbaspirillum*, *Paenibacillus*, *Pseudomonas* have been reported to form endophytic colonies in roots and shoot of nonnative plant hosts. Marker genes like green fluorescent protein have also been used widely to view the sites of colonization in real time. Apart from colonizing a nonnative plant host, these endophytic bacteria are also involved in promoting host plant growth and acting as a biocontrol agent against pathogenic fungi. Such endophytes have a great potential in future for sustainable agriculture since they could be used in a range of environmental and biological conditions.

Keywords Endophytic bacteria · Nonnative crop hosts · Biological nitrogen fixation · Plant growth promoting bacteria · Diazotrophic endophytes

2.1 Introduction

When one considers both the expected worldwide population increase and the increasing environmental damage that is a result of ever-greater levels of industrialization, it is clear that in the next 10–20 years it will be a significant challenge to feed all of the world's people, a problem that will only increase with time. According to a report released by the United Nations in 2015, the world's population is set to rise to 9.7 billion by 2050 (United Nations 2015). Sadly, the threat of

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having inadequate food to feed all of the world's population in future is again in the news. At this point, our world is experiencing a variety of problems like climate change, food wastage, spoilage on an enormous scale, unequal distribution of food resources, and continuously growing population. There is certainly no time to lose and the world needs to act to feed this growing population. Although it is quite tempting to use chemical fertilizers to boost up the agricultural productivity, such a solution will have a detrimental effect on our environment. Agricultural scientists around the world are working round the clock to look for innovative ways to increase agricultural productivity sustainably, but it certainly represents a great challenge for them. The use of microorganisms with the objective of improving agricultural productivity is one of the most important sustainable practices (Freitas et al. 2007).

The soil is full of microscopic life including a diverse range of bacteria, fungi, protozoa, and algae. It is estimated that there are more than 94 million organisms in a single gram of soil, with most of them being bacteria (Glick 2015). The interaction between bacteria and plants could be beneficial, neutral, or detrimental to the plant. However, the effect that a particular bacterium has on a plant may change as the conditions change. For instance, a bacterium that facilitates plant growth by providing either fixed nitrogen (N) or phosphorus compounds that are often present in only limited amounts in many soils is unlikely to provide any benefit to plants when a significant amount of chemical fertilizer added to the soil (Glick 2012). This observed when a bacterial strain of *Paenibacillus polymyxa* (Bal et al. 2012) was inoculated into lodgepole pine (*Pinus contorta* var. *latifolia* Engelm. ex S. Watson). The bacterial strain fixed significant amounts of N directly from the atmosphere under N-limited conditions (Anand et al. 2013), but was unresponsive when sufficient amount of N was present in the soil (Yang et al. 2016, 2017).

2.2 Plant Growth-Promoting Bacteria (PGPB): Biofertilizers for Sustainable Agriculture

Bacteria that are able to provide a range of benefits to the plant also known as plant growth-promoting bacteria (PGPB). Bashan and Holguin (1998) proposed the term PGPB in the field of plant-microbe interactions. These bacteria are capable to affect plant growth via numerous independent or linked mechanisms for sustainable agriculture (Compant et al. 2010; Palacios et al. 2014). They counteract many stresses in plants (Kang et al. 2010; Kim et al. 2012), fighting against phytopathogens (Verhagen et al. 2004; Raaijmakers et al. 2009) and assisting in the recovery of damaged or degraded environments (Denton 2007; de Bashan et al. 2012). Nowadays, PGPBs are of great interest because of their applications in agriculture as biofertilizers, pesticides, and phytoremediation (Sturz et al. 2000; Berg 2009; Lugtenberg and Kamilova 2009; Weyens et al. 2009; Compant et al. 2010). Classification of PGPB based on their habitable niche presented in Fig. 2.1.

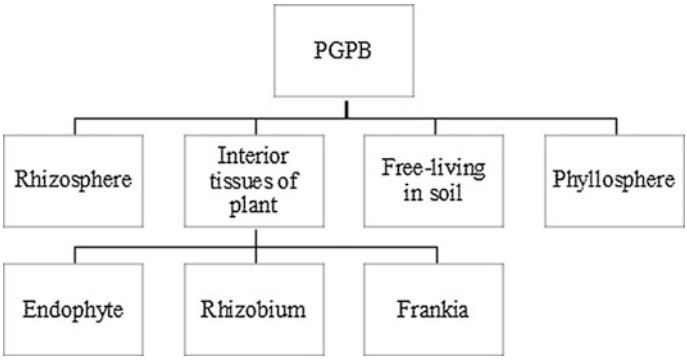

Fig. 2.1 Classification of plant growth-promoting bacteria (PGPB) based on their habitable niches

The rhizosphere is well explained and known to host a diversity of PGPB from more than 20 genera, including *Pseudomonas*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Paenibacillus*, *Azospirillum*, *Agrobacterium*, and *Azotobacter*. Several bacteria deriving from the rhizosphere not only colonize the rhizoplane but can also enter plants and colonize internal tissues and many of them have shown plant growth-promoting effects (Hallmann 2001; Sessitsch et al. 2004; Compant et al. 2005, 2008, 2010; Hallmann and Berg 2006; Anand et al. 2013; Puri et al. 2015; Padda et al. 2016a, b). Often not considered as PGPB, cyanobacteria are also renowned for their ability to promote plant growth indirectly by fixing carbon through oxygen photosynthesis and N through biological nitrogen fixation. They can survive in diverse ecological niches including but not limited to phyllosphere (Fürnkranz et al. 2008; Hamisi et al. 2013), rhizosphere (Karthikeyan et al. 2009; Prasanna et al. 2009) and plant interior (Tyagi et al. 1980; Krings et al. 2009).

2.3 Endophytic Bacteria: Microbial Life Inside the Plant

About 150 years ago the term, "endophyte" was first coined by de Bary (1866) for pathogenic fungi entering inside leaves. Since then, many authors have been redefining this term, but taken literally, the word endophyte means "in the plant" (endon = within; phyton = plant). Galippe (1887) was the first scientist to postulate that various vegetable plants host microbes within their interior and these microbes are soil habitant. This was later confirmed by di Vestea (1888), but well-known scientists at that time such as Pasteur, Chamberland, Fernbach, Laurent, and others claimed that plants are normally free of microbes and they indeed demonstrated contradictory results to prove that Galippe's hypothesis is wrong (Compant et al. 2010). However, it is now well accepted that plants generally host a wide range of phylogenetically distinct endophytes in various organs (Bacon and White 2000),

and that almost all of these microbes are derived from the soil environment (Rosenblueth and Martínez-Romero 2006; Hardoim et al. 2008; Ryan et al. 2008; Compant et al. 2010).

Since this chapter has key focus on endophytic bacteria, the term needs to redefine before starting a new discussion. Numerous definitions of the term "Endophytic Bacteria" could be found in the literature (Kado 1992; Quispel 1992; Beattie and Lindow 1995; Hallmann et al. 1997), but each has its own restrictions. In this chapter, we use the term "Endophytic Bacteria" to describe "the bacteria that can be detected at a particular moment within the tissue of apparently healthy plant hosts without inducing disease or organogenesis" (Chanway et al. 2014). It is believed that via rhizosphere colonization, endophytic bacteria become colonize in various plant parts/tissues such as roots, stem, leaves, flowers, fruits, and seeds (James et al. 2002; Sessitsch et al. 2002; Berg et al. 2005; Compant et al. 2005, 2008, 2011; Okunishi et al. 2005; Bal et al. 2012; de Melo Pereira et al. 2012; Anand and Chanway 2013a; Trognitz et al. 2014; Puri et al. 2015, 2016a, b). Endophytic bacterial population is extremely variable in different plant organs and tissues shown to vary in from as low as hundreds to as high as 9×10^9 of bacteria per gram plant tissue (Jacobs et al. 1985; Misaghi and Donndelinger 1990; Sturz et al. 1997; Hallmann et al. 1997; Chi et al. 2005; Padda et al. 2016a, b). In contrast to free-living, rhizosphere or phyllosphere microorganisms, bacterial endophytes are better protected from abiotic stresses such as extreme variations in temperature, pH, nutrient, and water availability as well as biotic stresses such as competition (Loper et al. 1985; Cocking 2003; Rosenblueth and Martinez-Romero 2006). In addition, endophytic bacteria colonize niches that are more conducive to forming mutualistic relationships with plants (Richardson et al. 2009), for example providing fixed N to the plant and getting photosynthate in return (Hallman et al. 1997; Reinhold-Hurek and Hurek 1998a, b; Santi et al. 2013). Primary mechanisms by which endophytic bacteria promotes plant growth are highlighted in Fig. 2.2.

2.3.1 *Diazotrophic Endophytes: Biological N-Fixers Living Inside the Plant*

For plants, N is an essential mineral required to survive and grow. It is a primary constituent of nucleotides, proteins, and chlorophyll (Robertson and Vitousek 2009). The availability of fixed N (nitrate or ammonium converted from dinitrogen) is seen by many as the most yield-limiting factor related to crop production (Muthukumarasamy et al. 2002). Although N is found in high abundance in the atmosphere, biologically available N in terrestrial ecosystems is in short supply. Root-nodulating bacteria, such as well-known rhizobia form a symbiotic association and provide biologically fixed N directly to leguminous plants. However, nonleguminous plants, including economically important crop species belonging to Poaceae family like sugarcane (*Saccharum officinarum* L.), corn (*Zea mays* L.),

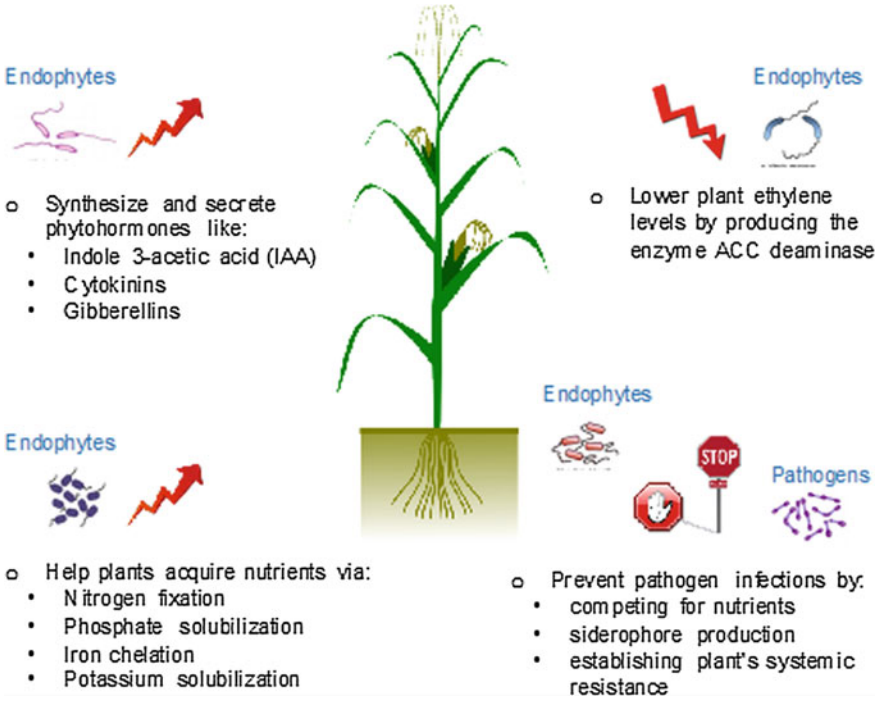

Fig. 2.2 Principal mechanisms of plant growth promotion exhibited by endophytic bacteria

wheat (*Triticum* spp.), and rice (*Oryza sativa*), do not have this type of symbiosis. Brazilian researchers were the first to report the presence of N-fixing bacteria (diazotrophs) in the rhizosphere and rhizoplane of a nonleguminous plant, sugarcane (Döbereiner and Alvahydo 1959; Döbereiner 1961). Initially, it was postulated that nitrogenase activity occurs in the rhizosphere soil but not in roots (Döbereiner et al. 1972; Ruschel 1981). In subsequent studies, various diazotrophs like *Azospirillum lipoferum*, *Azospirillum amazonense*, *Bacillus azotofixans*, *Enterobacter cloacae*, *Erwinia herbicola*, *Bacillus polymyxa* (Rennie et al. 1982; Magalhaes et al. 1983; Seldin et al. 1984; Baldani et al. 1986) were isolated from the rhizosphere of sugarcane. Later, it was determined that rhizospheric N-fixation does not occur at sufficient rates to facilitate high sugarcane yields. Cavalcante and Döbereiner (1988) reported the isolation of a diazotrophic bacterium from the stem and root tissues of sugarcane and postulated that this bacterium might be involved in fixing high amounts of N biologically. The isolated diazotroph was initially named as *Saccharobacter nitrocaptans* (Cavalcante and Döbereiner 1988) but was later changed to *Acetobacter diazotrophicus* (Gillis et al. 1989) and then renamed as *Gluconacetobacter diazotrophicus* (Yamada et al. 1997). This bacterium was able to form high endophytic populations and fix N at high sucrose concentrations (Boddey et al. 1991) and in low pH conditions (Boddey et al. 1991; Stephan et al.

1991) and these conditions are typically found in sugarcane tissues. This led to the suggestion that it can satisfy almost all of the sugarcane N requirements while living inside the sugarcane tissues. The term "endophytic diazotrophic bacteria" was then coined by Döbereiner (1992) to designate all diazotrophs able to colonize primarily the root interior of graminaceous plants, survive very poorly in soil and fix N in association with these plants (Baldani et al. 1998). Since the discovery of endophytic diazotrophic bacteria in sugarcane, other agronomically important crop species including rice (Baldani et al. 2000; Gyaneshwar et al. 2001; Hurek et al. 2002), corn (Olivares et al. 1996; Riggs et al. 2001; Roesch et al. 2008; Montañez et al. 2009; Puri et al. 2015, 2016b), canola (*Brassica napus* L.) (Germida and de Freitas 1998; Puri et al. 2016a; Padda et al. 2016a, b) and wheat (Sabry et al. 1997) have been postulated to receive significant amounts of fixed N in this way. Table 2.1 presents a brief list of prominent diazotrophic endophytes isolated from key agricultural crops.

Table 2.1 Prominent diazotrophic bacteria isolated from different crop species

Crop	Diazotrophic endophytes	References
Canola	*Bacillus polymyxa*	Germida and de Freitas (1998)
	Paenibacillus polymxa	Padda et al. (2016a, b), Puri et al. (2016a)
Corn	*Burkholderia tropica* sp.	Reis et al. (2004)
	Burkholderia silvatlantica sp.	Perin et al. (2006)
	Gluconacetobacter diazotrophicus	Eskin (2012)
	Herbaspirillum spp.	Olivares et al. (1996), Roesch et al. (2008)
	Ideonella spp.	Roesch et al. (2008)
	Klebsiella pneumoniae	Palus et al. (1996), Chelius and Triplett (2000)
	Paenibacillus polymyxa	Puri et al. (2015, 2016b)
	Pseudomonas spp.	Montañez et al. (2009)
Rice	*Alcaligenes faecalis* [now known as *Pseudomonas stutzeri* (Vermeiren et al. 1999)]	You and Zhou (1989)
	Azoarcus spp.	Egener et al. (1999), Engelhard et al. (2000), Hurek et al. (2002)
	Burkholderia spp.	Baldani et al. (2000), Rangjaroen et al. (2015)
	Herbaspirillum spp.	Baldani et al. (2000), Elbeltagy et al. (2001)
	Klebsiella sp.	Rangjaroen et al. (2015)
	Serratia marcescens	Gyaneshwar et al. (2001)
Sugarcane	*Azoarcus* spp.	Reinhold-Hurek et al. (1993)
	Azospirillum brasilense	Carrizo de Bellone and Bellone (2006)
	Burkholderia tropica sp.	Reis et al. (2004)

(continued)

Table 2.1 (continued)

Crop	Diazotrophic endophytes	References
	Burkholderia silvatlantica sp.	Perin et al. (2006)
	Herbaspirillum spp.	Baldani et al. (1992, 1996, 2002), Cavalcante and Dobereiner (1988), Muthukumarsamy et al. (1999)
	Gluconacetobacter diazotrophicus	Gillis et al. (1989), Boddey et al. (1991), Stephan et al. (1991), Cavalcante and Dobereiner (1988), Sevilla et al. (2001)
Wheat	*Azorhizobium caulinodans*	Sabry et al. (1997)
	Azospirillum brasilense	Schloter and Hartmann (1998), Rothballer et al. (2003)
	Klebsiella pneumoniae	Iniguez et al. (2004)
	Herbaspirillum hiltneri	Rothballer et al. (2006)

2.4 Foreign Associations: Endophytic Bacteria Promoting the Growth of Nonnative Crop Species

Plants are a complex micro-ecosystem which can only be colonized by foreign microbes having metabolic diversity. Foreign associations of endophytes are not unfamiliar to the scientific community and numerous studies have highlighted the ability of these microbes to associate with a diversity of hosts. Endophytic bacteria can colonize and provide benefits to a variety of foreign plant hosts ranging from monocots to dicots, gymnosperms to angiosperms and woody trees to herbaceous plants. Although the list of these endophytes is very long and include genera such as *Acetobacter, Arthrobacter*, *Azoarcus*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Burkholderia*, *Enterobacter*, *Flavobacterium*, *Frankia*, *Gluconacetobacter*, *Herbaspirillum*, *Paenibacillus*, *Pseudomonas*, *Rhizobacter*, *Rhizobium*, *Sinorhizobium*, *Streptomyces*, and *Xanthomonas*, only a few important ones have been discussed in this chapter. A brief informative list of key endophytes that have been reported to play an important role in growth promotion of nonnative hosts through direct or indirect mechanisms has been compiled in Table 2.2. In the sub-sections to follow, studies relating to endophytic colonization and plant growth promotion by six of the most important bacterial endophytes reported in foreign plant host species have been reviewed in detail.

2.4.1 Arthrobacter

In 1947, Conn and Dimmick established a new genus "*Arthrobacter*" in the world of Microbiology (Conn and Dimmick 1947). By far more than 70 species have been included in this genus (Fu et al. 2014). Bacterial species belonging to this genus are

Table 2.2 List of important endophytic bacteria reported to colonize and promote growth of nonnative plant hosts

Genus	Strain	Isolated from	Inoculated into	Benefits provided to the nonnative host
Arthrobacter	*A. humicola* YC6002	Korean turf grass (Chung et al. 2010)	Radish (Chung et al. 2010)	Weed management
Azoarcus	*A.* sp. BH72	Kallar grass (Reinhold-Hurek et al. 1993)	Rice (Hurek et al. 1994)	Increases biomass and total protein
Bacillus	*B. subtilis* EDR4	Wheat (Qiao et al. 2006)	Rapeseed (Chen et al. 2014)	Biocontrol against pathogenic fungus
	B. licheniformis CHM1	Rice (Wang et al. 2009a)	Cole (Wang et al. 2009a)	Increases fresh weight and chlorophyll content
	B. subtilis FL and *B. atrophaeus* NRRLNRS-213	Japanese honeysuckle (Zhao et al. 2015)	Wheat (Zhao et al. 2015)	Increases seedling biomass and length
	B. subtilis EPC8	Coconut (Rajendran et al. 2008)	Tomato (Prabhukarthikeyan et al. 2014)	Increases plant length and fruit yield
Burkholderia	*B. gladioli* 3A12	Corn (Shehata et al. 2016)	Creeping bentgrass (Shehata et al. 2016)	Biocontrol against common crop pathogens
	B. cenocepacia 869T2	Vetiver grass (Ho et al. 2015)	Banana (Ho et al. 2015)	Biocontrol against fungus that causes Panama disease of Banana
	B. phytofirmans PsJN	Onion (Frommel et al. 1991)	Potato (Frommel et al. 1991)	Enhances root growth and plant lignin content
			Potato (Frommel et al. 1993)	Enhances root growth and overall yields
			Grapevine (Compant et al. 2005, 2008)	Increases seedling length and fresh weight

(continued)

Table 2.2 (continued)

Genus	Strain	Isolated from	Inoculated into	Benefits provided to the nonnative host
Enterobacter	*E. asburiae* JM22	Cotton (McInroy and Kloepper 1995)	Cucumber and bean (Quadt-Hallmann and Kloepper 1996)	–
	E. sp. strain 35	Sugarcane (Tanaka et al. 2006)	Cultivated rice and wild rice (Zakria et al. 2008)	Nitrogen fixation
	E. cloacae 344	Cacao tree (Leite et al. 2013)	Cucumber, corn, common beans (Moreira et al. 2015)	–
Gluconacetobacter	*G. diazotrophicus* spp.	Sugarcane (Youssef et al. 2004)	Wheat (Youssef et al. 2004)	Nitrogen fixation
	G. diazotrophicus PAL5	Sugarcane (Bertalan et al. 2009)	Rice (Alquéres et al. 2013)	–
			Rice (Rouws et al. 2010)	–
			Arabidopsis thaliana (Rangel de Souza et al. 2016)	Increases photosynthetic rate and water-use efficiency
	Acetobacter diazotrophicus (now known as *G. diazotrophicus*) PA15	Sugarcane (Gillis et al. 1989)	Rice (Sevilla and Kennedy 2000)	Increases plant height in N-limited conditions
Herbaspirillum	*H. seropedicae* strains ZAE94 and ZAE67	Sorghum (Baldani et al. 1986)	Rice (Baldani et al. 2000)	Fixes nitrogen and increases biomass
	H. seropedicae strain LR15	Corn (Baldani et al. 1986)	Corn, sorghum, rice (Roncato-Maccari et al. 2003)	Nitrogen fixation
	H. frisingense strain GSF30^T	*Miscanthus sacchariflorus* (Kirchhof et al. 2001)	Barley (Rothballer et al. 2008)	IAA production and ACC utilization

(continued)

Table 2.2 (continued)

Genus	Strain	Isolated from	Inoculated into	Benefits provided to the nonnative host
Paenibacillus	*P. polymyxa* strains 1D6, 4G12 and 4G4	Wild maize (teosinte) (Johnston-Monje and Raizada 2011)	Modern maize (corn) hybrid (P35F40) (Mousa et al. 2015)	Reduce Gibberella ear rot disease severity
	P. polymyxa IAM 13419 and *P. ehimensis* IFO15659	Japanese honeysuckle (Zhao et al. 2015)	Wheat (Zhao et al. 2015)	Increase seedling length, biomass and chlorophyll content
	P. polymyxa P2b-2R	Lodgepole pine (Bal et al. 2012)	Corn (Puri et al. 2015, 2016b)	Fixes nitrogen and increases seedling length and biomass
			Canola (Puri et al. 2016a; Padda et al. 2016a, b)	
			Tomato (Padda et al. 2016a)	
Pseudomonas	*P.* sp. Ph6	Clover (Sun et al. 2014)	Ryegrass (Sun et al. 2014)	Degrades phenanthrene, a toxic metabolite that enters plant
	P. aeruginosa PM389	Pearl millet (Gupta et al. 2013)	Wheat (Gupta et al. 2013)	Increases root and shoot length and vigor index
	P. brassicacearum YC5480	*Artemisia* sp. (Chung et al. 2008)	Radish (Chung et al. 2008)	Counteracts inhibitory effect of a pathogenic fungus on seed germination and shoot growth
	P. aeruginosa PW09	Wheat (Pandey et al. 2012)	Cucumber (Pandey et al. 2012)	Increases seedling biomass under biotic and abiotic stresses

Gram-positive obligate aerobes commonly found in soils. They are rod-shaped during the stationary growth phase and cocci-shaped during stationary phase. Members of *Arthrobacter* genus can survive in a variety of environmental conditions, including but not limited to water, air, human skin, oil, sludge, tobacco leaves, soil (Ding et al. 2013; Fu et al. 2014). Studies have shown that members of this genus can be helpful in many ways in agriculture. For instance, they fix atmospheric N, solubilize sulfur and phosphorous in soil and degrade heavy metals in polluted sites (Singer et al. 2000; Jiang et al. 2004; Postma et al. 2010). One of the most important aspects of plant growth promotion is deriving N from the atmosphere. *Arthrobacter* sp. HS-G8 was isolated from compost in Japan's Okinawa prefecture that possessed N-fixing ability (Jiang et al. 2004). In another study, two endophytic strains, *Arthrobacter nitroguajacolicus* A18 and A34, originally isolated from corn leaves possess nitrogenase reductase gene *nif*H indicating that these strains could fix atmospheric N (Pisarska and Pietr 2012). These strains successfully colonized and fixed N in different cultivars of corn thereby promoting the growth of a nonnative host (Pisarska and Pietr 2012). An endophytic bacterial strain, *Arthrobacter humicola* YC6002, from surface-sterilized root tissues of Korean turf grass (*Zoysia japonica*) reported by Chung et al. (2010). This bacterial endophyte successfully colonized internal tissues of a nonnative host, radish (*Raphanus sativus*), and could be used in future for weed management due to its ability to produce phytotoxic compounds like 3-phenylpropionic acid (Chung et al. 2010).

2.4.2 *Bacillus*

The history of genus *Bacillus* dates back to 1835 when Christian Gottfried Ehrenberg isolated a bacterium (*Vibrio subtilis*, now known as *Bacillus subtilis*) belonging to this genus (Ehrenberg 1835). Later, in 1872, Ferdinand Cohn proposed a new genus "*Bacillus*" and renamed *Vibrio subtilis* to *Bacillus subtilis* (Cohn 1872). Bacteria of this genus are Gram-positive, endospore-forming and rod-shaped that could be either obligate aerobes or facultative anaerobes. Genus *Bacillus* is one of the most diverse group of bacteria that is well known for its many agricultural and industrial applications. In agriculture, bacteria of this genus are widely used as an effective biocontrol agent for numerous crop species. The commercial success of *Bacillus thuringiensis* exemplified as a biocontrol agent worldwide. Other bacterial isolates of this genus having biocontrol and plant growth-promoting (PGP) properties have also been widely studied and successfully used commercially in agriculture. Endophytic colonization in plant species by bacteria has also been reported (Wang et al. 2009b; Lee et al. 2012; Liu et al. 2014; Khalifa and Almalki 2015). Biocontrol of pathogens like *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Botrytis cinereapers*, *Gibberella zeae*, *Dothiorella gregaria*, *Colletotrichum gossypii*, *Phytophthora capsici*, *Pythium myriotylum*, *Athelia rolfsii*, *Magnaporthe oryzae*, *Ralstonia solanacearum, and*

Xanthomonas axonopodis pv. *punicae* by *Bacillus* in non-native plants has been reported over the years (Maheshwari 2013).

Stem rot disease of rapeseed (*Brassica napus* L.), caused by a pathogenic fungus *Sclerotinia sclerotiorum*, is a major problem faced worldwide by many countries. Chen et al. (2014) tested the ability of an endophyte, *B. subtilis* EDR4, to inhibit the growth of this pathogen in vitro and in vivo in rapeseed under greenhouse and field conditions. *B. subtilis* EDR4 was initially isolated from root tissues of wheat (Qiao et al. 2006) and subsequently reported to inhibit the growth of the fungal pathogen, *Gaeumannomyces graminis* var. *tritici*, of wheat (Liu et al. 2007). In the in vitro experiments, germination rate and hyphal growth of *S. sclerotiorum* were significantly inhibited by *B. subtilis* EDR4 and the results of in vivo experiment conducted under greenhouse and field conditions were no different. Scanning electron microscopy revealed that EDR4 causes leakage in the cytoplasm, shrinking of hyphae and irregular swelling of tips of the fungus. In another study related to *Brassica napus*, an endophytic strain *B. licheniformis* CHM1 was isolated from stem tissues of rice and tested for biocontrol activity and plant growth promotion in cole (*Brassica napus*) (Wang et al. 2009a). Strain CHM1 colonized stem/leaf tissues and significantly promoted the growth of cole seedlings (increasing the fresh weight of seedlings by 72% and chlorophyll content by 61%). This bacterial strain also inhibited the growth of common fungal pathogens like *F. oxysporum*, *R. solani*, *B. cinereapers*, *D. gregaria*, *G. zeae* and *C. gossypii* in in vitro experiments. In in vivo experiments, it provided 60% protection against *R. solani* in horse bean (*Vicia faba*) and 70% protection against *Bipolaris maydis* in corn. In a more recent study, wheat plant growth was significantly promoted by two endophytic strains (135 and 170) belonging to the genus *Bacillus*, isolated from stem and root tissues of a medicinal plant, *Lonicera japonica*, native to eastern China (Zhao et al. 2015). In in vitro experiments, it was found that these two strains possess many PGP traits that could increase wheat growth. Results of in vivo experiment were consistent with results of in vitro experiment since inoculation with these strains significantly increases fresh weight, dry weight and length of wheat seedlings along with the chlorophyll content. These strains also showed in vitro antifungal activity against common pathogenic fungi like *Magnaporthe grisea* (rice blast fungus), *F. oxysporum* (usually affects wheat and rice crops) and *Alternaria alternate* (causes leaf spot disease). Based on the results of physiological and biochemical tests, and the sequencing of 16S rRNA gene and phylogeny analysis, it was revealed that strains *Bacillus* spp. 135 and 170 are very closely related to *B. subtilis* FL and *B. atrophaeus* NRRLNRS-213^{T}, respectively. This study was also important in establishing the fact that strains belonging to genus *Bacillus* are potentially capable of colonizing and promoting the growth of a completely distinct host (wheat, a monocot) as compared to the host species from which it was isolated (*Lonicera japonica*, a eudicot).

In a completely different approach to combat with pathogens and increase plant yield, Prabhukarthikeyan et al. (2014) used a bioformulation containing a mixture of an entomopathogenic fungus, *Beauveria bassiana* B2, known for its ability to control a wide range of agriculturally important insect pests and an endophytic

strain of *B. subtilis* (EPC8) against *Fusarium* wilt (*F. oxysporum* f. sp. *lycopersici*) and fruit borer (*Helicoverpa armigera*) disease in tomato (*Solanum lycopersicum* Mill.). It should be noted that *B. subtilis* EPC8 was initially isolated from root tissues of coconut (*Cocos nucifera*) (Rajendran et al. 2008). Bioformulation of B2 and EPC8 suppressed these pathogens in in vitro experiments and under glasshouse and field conditions when tomato plants were treated with this mixture. The combination of B2 and EPC8 was better than the pesticide control (carbendazim + quinalphos) against both *Fusarium* wilt and fruit borer in glasshouse study and was equally good in field conditions. Interestingly, it was also observed that such bioformulation promotes tomato growth by increasing the plant height and fruit yield under both glasshouse and field conditions. Recently, Munjal et al. (2016) reported that an endophytic biocontrol agent, *Bacillus megaterium* BP17, initially isolated from root tissues of black pepper (*Piper nigrum*) (Aravind et al. 2009) can colonize ginger plant (*Zingiber officinale*). Ginger roots were successfully colonized by this bacterial strain with population size ranging from 2.5 to 2.8 $\log_{10}$ cfu/g. It was also reported that this bacterial strain is capable of releasing antimicrobial chemical compounds. In an interesting study, colonization pattern of three nonnative host species by an endophytic *Bacillus* strain under sterile and non-sterile conditions was reported by Moreira et al. (2015). *Bacillus amyloliquefaciens* 629 was initially isolated from *Theobroma cacao* (Leite et al. 2013) and was inoculated into three distinct host species namely, cucumber (*Cucumis sativus* cv. Marketmore 76), corn (cv. BRS Caatingueiro) and common bean (*Phaseolus vulgaris* cv. BRS Notável). Strain 629 successfully colonized stem and leaf tissues of cucumber, root and stem tissues of common bean, and root, stem and leaf tissues of corn plant under both sterile and non-sterile conditions significantly. It is important to note that the population size of endophytic bacteria was 3 times lower under non-sterile conditions in all plant species as compared to the sterile conditions. It could be concluded that indigenous endophytic bacteria and fungi pose a competition to the nonindigenous endophytes. Thus, the foreign association and establishment of an endophyte within a nonnative host is a formidable task.

2.4.3 *Burkholderia*

The genus '*Burkholderia*' was first proposed by Yabuuchi et al. (1992) for the RNA homology group II of *Pseudomonas* genus. Seven species of this group were transferred to the new genus *Burkholderia* and renamed as *B. caryophylli*, *B. cepacia*, *B. gladioli*, *B. mallei*, *B. pickettii*, *B. pseudomallei,* and *B. solanacearum*. Currently, there are close to 100 species in this genus that are known to inhabit diverse ecological niches, ranging from contaminated soils to the respiratory tract of humans. *Burkholderia* species are renowned for their ability to promote plant growth through various mechanisms including, N-fixation (Gillis et al. 1995; Cruz et al. 2001; Estrada-De Los Santos et al. 2001) and biocontrol of pathogens (Hebbar et al. 1998; Heungens and Parke 2000; Parke and Gurian-Sherman 2001). The

majority of species are soil bacteria that are generally found in the rhizosphere or as free-living microbes in the soil but there are some species that can colonize internal tissues of plants and form beneficial interactions (Caballero-Mellado et al. 2004; Pandey et al. 2005; Park et al. 2005; Mendes et al. 2007; Ho et al. 2015). The interactions of some endophytic species of *Burkholderia* genus seem to be restricted to only one type of host, whereas other species have a diverse host range (Coenye and Vandamme 2003).

In a recent study, three strains belonging to the *B. gladioli* species were isolated from roots and seeds of ancient and wild maize plants (Shehata et al. 2016). In vitro studies revealed that these strains can inhibit fungal pathogen *Sclerotinia homoeocarpa* and their interaction was also visualized on microscope slides by staining with Evans blue. These strains were also successful in inhibiting the growth of other common crop pathogens. The ability of these strains to act as a biocontrol against *S. homoeocarpa* was also tested in vivo with creeping bentgrass (*Agrostis stolonifera*) in two greenhouse trials and the results were no different from the in vitro studies. The endophytic ability of one of the strains, *B. gladioli* 3A12, was also tested in a nonnative host, creeping bentgrass, by tagging the strain with green fluorescent protein (GFP) and examining under a confocal microscope. It was found that GFP-tagged 3A12 strain successfully colonized shoots of creeping bentgrass. The authors concluded that wild cultivars of agricultural crops might possess an unexplored reservoir of bacterial endophytes having biocontrol traits against a wide range of pathogens. In a study conducted a few years back, an endophyte, *B. cenocepacia* 869T2, was isolated from root tissues of vetiver grass (*Chrysopogon zizanioides*) (Ho et al. 2015). In vitro, strain 869T2 was able to inhibit the mycelial growth of *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (Foc TR4), a pathogenic fungus that causes Panama disease in banana (*Musa acuminata*), showing 44% antifungal efficiency. When this endophytic strain was inoculated into banana plantlets (Cavendish cv. Pei-Chiao), it developed stable endophytic population in pseudostem tissues, thus showing endophytism in a distinct host. The in-field experiment revealed that inoculation of banana plantlets with strain 869T2 not only reduces the disease symptoms of Foc TR4 but also promotes growth by increasing the plant height and pseudostem girth significantly. This strain of *B. cenocepacia* can be used as an effective biocontrol agent in susceptible banana cultivars. Species of *Burkholderia* MSSP inhabit root nodule of *Mimosa pudica* capable for N fixation along with antagonism against *Rhizoctonia solani,* and *Sclerotinia sclerotiorum* has been reported by Pandey et al. (2005).

A remarkable endophytic bacterial strain (PsJN) was isolated by Dr. Jerzy Nowak as a contaminant from surface-sterilized onion (*Allium cepa* L.) roots infected with fungal pathogen *Glomus vesiculiferum* (Frommel et al. 1991; Sessitsch et al. 2005). This strain has shown outstanding ability over the years to endophytically colonize a wide range of plant hosts. The strain PsJN was initially classified as a *Pseudomonas* sp. (Frommel et al. 1991), but was later reclassified as a *B. phytofirmans* sp. (Sessitsch et al. 2005). Endophytic colonization by PsJN in a nonnative host was first reported in potato (*Solanum tuberosum*) (Frommel et al. 1991). By using light and electron microscopy Frommel et al. also reported that

endophytic population of PsJN strain is present in the epidermal layers of root and in the xylem tissues of the stem. They also found that inoculation significantly promotes the growth of potato plantlets by increasing root dry weight, secondary root branching, root number, haulm dry weight, stem length, leaf hair formation, and total lignin content of the plant. They also laid out a preliminary hypothesis that growth promotion by the strain PsJN is due to the production of phytohormones. In a subsequent study (Frommel et al. 1993), the ability of this strain to colonize internal root tissues and promote plant growth in field conditions was reported with the same cultivar of potato as was used in Frommel et al. (1991). In-field, it stimulated plant emergence, root development, and overall yields of the potato plant. Another report about the endophytic colonization of a nonnative host by strain PsJN was published in 1997, in which the effect of inoculum density, temperature, and genotype on colonization and growth promotion of tomato (*Lycopersicon esculentum* L.) seedlings was evaluated (Pillay and Nowak 1997). In this study, the inoculum range that promoted shoot and root interior colonization also best-promoted plant growth of tomato cultivars. Endophytic colonization patterns of strain PsJN were reported for the first time by Compant et al. (2005) inside grapevine (*Vitis Vinifer* L.). The strain PsJN was tagged with GFP or *gusA* and visualized under the desired microscope to examine internal tissue colonization. Colonization of grapevine plantlet started with the bacterial strain gaining entry through the sites of the emergence of lateral root or through the root tips, then accumulating near the cell wall of the rhizodermis cells followed by intercellular colonization of cortical cells. PsJN bacterial cells moved up through the xylem vessels colonizing the fifth internode and leaf internal tissues. It was also observed that the strain PsJN secretes cell wall-degrading enzymes, endoglucanase, and endopolygalacturonase thus supporting the findings of microscopy studies. In a subsequent study with grapevine, GFP-tagged PsJN strain could also be visualized as an endophyte inside young berries (Compant et al. 2008) and was able to thrive inside and outside the plantlet even when grown under non-sterile conditions (with the presence of other microorganisms). Analysis of the complete genome of a microorganism can reveal a lot about its properties and behavior in diverse ecological niches. Although, the complete genome of *B. phytofirmans* PsJN was sequenced and reported earlier (Weilharter et al. 2011), the analysis of the genome was carried out by Mitter et al. (2013). As reported by Mitter et al. PsJN strain in many aspects is outstanding because it has a large genome which is well-equipped with genes that can degrade complex organic compounds (plant cell walls). It also possesses a high number of cell surface signaling and secretion systems and has a 3-OH-PAME quorum-sensing system that might be helping this bacterium to switch from free-living to symbiotic lifestyle. In another interesting study, the ability to fix N was successfully transferred from a known N-fixing bacterium, *B. phymatum* STM 815, to *B. phytofirmans* PsJN through horizontal gene transfer (Lowman et al. 2015). The new strain was named PsJN+, which outperformed the wild-type strain PsJN in terms of promoting the growth of switchgrass plant even under low N conditions. *B. phytofirmans* PsJN is a unique and completely outstanding endophyte that has been shown wide spectrum of endophytic lifestyles in

diverse host species ranging from monocots to dicots since its isolation from onion roots (Frommel et al. 1991, 1993; Liu et al. 1995; Pillay and Nowak 1997; Sharma and Nowak 1998; Nowak et al. 2004; Compant et al. 2005, 2008; Sun et al. 2009; Poupin et al. 2013; Naveed et al. 2014a, b) and could be used as an effective commercial biofertilizer in agriculture production.

2.4.4 *Gluconacetobacter*

The genus *Gluconacetobacter* was proposed by Yamada et al. (1997) in an attempt to reclassify and include the bacterial species *Acetobacter diazotrophicus* into a new genus. Although there are currently 24 species in this genus but the most widely studied species is *Gluconacetobacter diazotrophicus*. *G. diazotrophicus* is a renowned diazotrophic endophyte found frequently in tissues of sugarcane and other grasses, known for its ability to provide significant amounts of N to the plant directly from the atmosphere. Studies about this bacterial species, including earliest isolation, endophytism, and N-fixing trait have already been discussed in Sect. 2.3.1. The studies highlighting the association of this bacteria with diverse host species are discussed here. *A. diazotrophicus* (now known as *G. diazotrophicus*) strain PA15 isolated from sugarcane roots (Gillis et al. 1989) was tagged with three different reporter genes, *uidA*, GFP and *cobA* to evaluate the colonizing ability of this bacterial strain in three different crops namely wheat, corn and rice (Sevilla and Kennedy 2000). Strain PA15 heavily colonized corn kernels, primary root, and root hairs in just two days after inoculation. Rice seeds were not as heavily colonized as corn but lateral roots and root hairs of rice were colonized heavily. Colonization pattern in wheat was similar to rice. Plant growth promotion by strain PA15 was observed only in rice seedlings and was thought to be due to the bacteria's N-fixing ability since mutants of PA15 with *nif* gene removed were not able to promote rice growth. In another study, diazotrophic isolates belonging to the genus *Gluconacetobacter* were isolated from internal tissues of sugarcane growing in ancient agricultural fields of the Nile Delta (Giza) (Youssef et al. 2004). It was observed that these *Gluconacetobacter* spp. were able to form colonies in the stem (xylem vessels) and roots (cortex and vascular cylinder) of 21-day-old wheat seedlings when studied by using scanning electron microscopy. Apart from endophytically colonizing a diverse host species (wheat) these isolates were able to increase the stem and root dry weight significantly, thus increasing the overall plant biomass of wheat. Another study, *G. diazotrophicus* strain PAL5 (Bertalan et al. 2009) isolated from sugarcane was shown to colonize rice shoot and root endophytically with a population size of 10^4 cfu/gm fresh tissue. To visualize the endophytic colonies in rice, this strain was tagged with GFP and observed by using confocal laser microscopy. Microscopy experiment revealed that bacterial cells of PAL5 initially gather near the sites of lateral root emergence and at junctions between root cap and root axis in the vicinity of the apex and then enter the roots through these different openings (Rouws et al. 2010). In a subsequent study,

Alquéres et al. (2013) also indicated the endophytic colonization of rice roots by strain PAL5 through GFP-tagging. Secretion of reactive oxygen species (ROS) is a typical defense response activated by the plants in response to a pathogen attack. This study also established that strain PAL5 secrets ROS-scavenging enzymes that play a key role in the endophytic colonization of rice. Further, endophytic colonization pattern of strain PAL5 in *A. thaliana* root was studied by tagging it with a red-fluorescent protein (Rangel de Souza et al. 2016). Inoculation by this strain significantly promoted shoot and root fresh weight, shoot and root dry weight, total leaf area, the number of leaves. Whole canopy gas exchange was also evaluated in this study by using a portable photosynthesis system and the results revealed that inoculation by PAL5 significantly increases net photosynthetic rates, lowers transpiration rate and increases water-use efficiency in *A. thaliana*. These studies clearly establish the ability of *G. diazotrophicus* PAL5 to endophytically colonize a range of plant hosts and promote plant growth through different mechanisms. Although, *G. diazotrophicus* bacterium grows well in high sucrose environments like internal tissues of sugarcane and has been associated most of the time with sugarcane either as an endophyte or as a beneficial rhizospheric microbe, but this bacterium can also endophytically colonize a variety of plant species and promote their growth mainly through N-fixation.

2.4.5 *Paenibacillus*

The genus *Bacillus* was very heterogeneous containing phylogenetically diverse bacterial species. To reclassify some facultative anaerobes into a new genus (particularly *B. polymyxa* and some of its close relatives; rRNA group 3 of Ash et al. (1991, 1993) created the genus *Paenibacillus* (meaning: almost a *Bacillus*). Bacterial species belonging to this genus are low (mol% G + C contants) in DNA, Gram-positive, neutrophilic, peri-flagellated heterotrophic, endospore-forming facultative anaerobes. There are currently more than 180 species in this genus, most of them discovered within the last decade (http://www.bacterio.net/paenibacillus.html). The type species of this genus, *Paenibacillus polymyxa*, is well known for its ability to fix N (Guemouri-Athmani et al. 2000; Anand et al. 2013; Anand and Chanway 2013b; Bal and Chanway 2012a, b), promote plant growth (Timmusk et al. 1999; Puri et al. 2015; Puri et al. 2016a, b; Padda et al. 2016a, b) and suppress plant pathogens (Dijksterhuis et al. 1999; Ryu et al. 2006; Choi et al. 2007; Haggag and Timmusk 2008; Timmusk et al. 2009). *P. polymyxa* is known to colonize diverse ecological niches like soil, rhizosphere, intercellular and intracellular spaces of plant tissues, marine environments, fermented food products (Lal and Tabacchioni 2009). Endophytic colonization of plant tissues by this bacterial species has been reported time and again by various scientists (Bent and Chanway 1998; Shishido et al. 1999; Chanway et al. 2000; Bal et al. 2012; Pu et al. 2015; Yang et al. 2016; Tang et al. 2017).

An interesting study about the invasion of plant roots and endophytic colonization by *P. polymyxa* suggests that it form biofilms on the surface of the roots to gain entrance into the plant (Timmusk et al. 2009). Biofilms are communities of bacterial cells covered in a self-produced extracellular matrix, that are surface-attached and highly structured (Costerton 1995). GFP-tagging of *P. polymyxa* and visualization under confocal laser microscope has revealed that this bacterium can colonize both intercellular and intracellular spaces of stem and root tissues, which was significant in establishing its endophytic nature (Timmusk et al. 2009; Anand and Chanway 2013a). Zhao et al. (2015) isolated several endophytic strains from a medicinal plant, *Lonicera japonica*, generally grown in eastern china. Two of the isolated strains belonged to genus *Paenibacillus* (*P. polymyxa* and *P. ehimensis)* and possessed many plant growth-promoting characteristics including siderophore production, phosphate solubilization, IAA production, aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, and cellulase and pectinase activity. Apart from that, these strains were able to suppress the growth of common crop pathogens. These *Paenibacillus* strains endophytically colonized a nonnative host, wheat, and promoted its growth by significantly increasing shoot and root length, seedling fresh and dry weight, and chlorophyll content. In another recent study, several endophytic strains were isolated from wild maize (teosinte) believed to harbor beneficial endophytes that could provide resistance to common crop pathogens (Mousa et al. 2015). After initial in vitro screening against fungal pathogen, *Fusarium graminearum*, causative agent of Gibberella Ear Rot (GER) in modern corn, three antifungal endophytes identified as *P. polymyxa* were tested for their ability to suppress GER in modern corn seedlings. GFP-tagged *P. polymyxa* endophytic strains colonized internal tissues of modern corn plants and suppressed the growth of *F. graminearum* pathogen in vivo. It was concluded that wild relatives of modern crops might have a reservoir of endophytes that could be used as biocontrol against pathogens that lead to extensive crop loss.

Chris P Chanway and his group have been working with *P. polymyxa* since 1988 and have published significant reports about the role of this bacterium in promoting plant growth and health in both agricultural and forest ecosystems. In 2012, the group reported the existence of an endophytic diazotroph, *P. polymyxa* P2b-2R, living in stem tissues of a gymnosperm, lodgepole pine (*Pinus contorta*), naturally regenerating at a site located in Williams Lake, BC, Canada (Bal et al. 2012). P2b-2R was able to grow on N-free media, combined carbon medium (CCM; Rennie 1981), and consistently reduced significant amounts of acetylene in the acetylene reduction assay (ARA) (Bal et al. 2012). By using a more accurate method of determining the amount of N fixed (^{15}N foliar dilution assay), Anand et al. (2013) discovered this bacterial strain's remarkable ability to derive up to 79% of N from the atmospheric pool. In a subsequent report, it was observed that strain possesses *nif* genes, required to fix atmospheric N (Anand and Chanway 2013c). GFP-tagged P2b-2R strain was constructed to evaluate the endophytic colonization sites in lodgepole pine and it was reported to colonize both intercellular and intracellular spaces of lodgepole pine interior tissues (Anand and Chanway 2013a). First reports about P2b-2R's ability to colonize a nonnative host came out in 2012

and 2013 when this bacterial strain was found to colonize internal tissues of stem and root of another gymnosperm tree species, western red cedar (*Thuja plicata*) (Bal and Chanway 2012b; Anand and Chanway 2013b). P2b-2R significantly enhanced seedling length and biomass of western red cedar and also fixed considerable amounts of N from the atmosphere (Anand and Chanway 2013b). Subsequently, Puri et al. (2015) hypothesized that this bacterial strain could provide similar benefits to angiosperms, especially the crop species, by colonizing them endophytically. Their hypothesis was evidenced and P2b-2R colonized internal root tissues of corn seedlings with a population size of 10^5 cfu/g fresh tissue weight in just 10 days. P2b-2R also fixed up to 20% of N from the atmosphere, increased seedling length by 35% and biomass by 30% in 30-day long trials (Puri et al. 2015). P2b-2R's ability to colonize diverse host species was ascertained, when it successfully colonized interior tissues of an important oilseed crop species, canola (Puri et al. 2016a) and vegetable crop species, tomato (Padda et al. 2016a). Similar benefits were provided by P2b-2R to these crop species indicating that P2b-2R can symbiotically associate with a broad range of hosts (see Table 2.3). Padda et al. (2017) reported an astonishing discovery with the GFP-tagged P2b-2R (P2b-2R*gfp*) constructed by Anand and Chanway (2013a), where P2b-2R*gfp* inoculation significantly enhanced corn seedling growth (length and biomass) as compared to the wild-type P2b-2R inoculation. This was the first report in literature where GFP-tagging of a bacterial strain related to the *Bacillus* (and *Paenibacillus*) genus enhanced its growth-promoting abilities. A similar discovery about the enhancement of PGP abilities by GFP-tagging was reported in *Azospirillum brasilense* a

Table 2.3 Nitrogen fixation and plant growth promotion of important agricultural crops by *Paenibacillus polymyxa* P2b-2R

	Days after inoculation	Corn	Canola	Tomato
%Ndfa[a]	20	6.52	8.08	10.0
	30	10.9	12.9	12.3
	40	15.7	16.2	18.1
	90	30.2	27.1	–
% seedling length promotion[b]	20	28.4	17.8	40.6
	30	24.1	20.5	36.5
	40	24.7	28.4	24.9
	90	51.9	70.7	–
% seedling biomass promotion[c]	20	17.2	57.0	56.1
	30	34.1	53.7	69.0
	40	28.4	37.1	93.0
	90	52.7	100.9	–

[a]Percent nitrogen derived from the atmosphere (%Ndfa)
[b]Percent seedling length promoted by inoculation with *P. polymyxa* P2b-2R
[c]Percent seedling biomass promoted by inoculation with *P. polymyxa* P2b-2R. These parameters were calculated using the formulas described in Puri et al. (2016b). [Data provided in the table has been compiled from [Padda et al. (2016a, b, 2017); Puri et al. (2016b)]

decade ago (Rodriguez et al. 2006). The ability of P2b-2R*gfp* to perform better than the wild-type strain was also confirmed in canola and tomato (Padda et al. 2016a). Benefits of inoculating this PGP endophytic strain and its GFP-tagged counterpart in a long-term trial were also evaluated and the results were even better than the previous studies which were of shorter duration (Puri et al. 2016b; Padda et al. 2016b). Thus, it can be concluded that *P. polymyxa* strain P2b-2R is an ideal endophytic strain that is able to colonize a variety of host species that are completely different physiologically and botanically.

2.4.6 Pseudomonas

Pseudomonas genus was first identified and described in the late nineteenth century (Migula 1894). The history of this genus from the time when it was first discovered till now has been described in great detail by Palleroni (2010). It is a diverse genus containing more than 230 species (http://www.bacterio.net/pseudomonas.html). Most of these species have a wide range of metabolic and catabolic capabilities. Bacterial species can be found in diverse ecological niches and could be plant growth and health-promoting bacteria, plant pathogens, or disease-causing human and animal pathogens (Preston 2004; Miller et al. 2008). *Pseudomonas* spp. are known to promote plant growth through a variety of mechanisms like biocontrol of pathogens, stimulating induced systemic resistance, N-fixation, phosphorus solubilization, and secreting phytohormones like auxins and cytokinins (Miller et al. 2008). Many studies have reported the ability of *Pseudomonas* spp. to associate endophytically with a variety of plant hosts, such as Peanut (Gupta et al. 2006), Sesame (*Sesamum indicum* L.) (Kumar et al. 2009), Mustard (Aeron et al. 2011), potato (Andreote et al. 2009), olive (*Olea europaea*) (Prieto et al. 2009; Maldonado-González et al. 2013), poplar (*Populus deltoides*) (Weyens et al. 2010, 2012), and wheat and cucumber (Pandey et al. 2012). Due to the diversity of *Pseudomonas* spp., many scientists have reported about their ability to colonize a range of nonindigenous plant hosts.

A diazotrophic endophyte, *P. aeruginosa* PM389, was isolated from an important forage crop, pearl millet (*Pennisetum glaucum*), widely grown in the Indian subcontinent, South America, USA and Australia (Gupta et al. 2013). It was observed that PM 389 has the ability to fix N, solubilize mineral phosphate, produce siderophores, inhibit the growth of bacterial and fungal pathogens. Looking at its plant growth-promoting abilities, Gupta et al. (2013) inoculated this bacterial strain into wheat and observed that it successfully colonizes the wheat seedlings and significantly enhance root and shoot length, and vigor index. In another study, another strain of *P. aeruginosa* originally isolated from wheat stem successfully shielded cucumber seedlings from various biotic and abiotic stresses (Pandey et al. 2012). Biomass of *P. aeruginosa* PW09-inoculated cucumber seedlings increased significantly as compared to the controls when grown under biotic stress (treated with pathogenic fungus, *Sclerotium rolfsii*) and abiotic stress (NaCl treatment). In a

subsequent study, another strain PaBP35, belonging to this bacterial species, isolated from stem tissues of black pepper and tagged with GFP to visualize the endophytic colonization sites in a nonnative host, tomato (Kumar et al. 2013). GFP-tagged PaBP35 colonized interior tissues of the root, stem, and leaves of a 14-day-old tomato with high population densities, thus confirming its ability to form endophytic colonies in a nonnative host. Effective root colonization is a prerequisite attribute for the success of PGPR in plant growth and yield promotion. Colonization by fluoresent *Pseudomonas* in sesame rhizosphere promotes growth and proved effective as indigenous microflora over nonindigenous microflora (Aeron et al. 2010). Recently, a phenanthrene-degrading endophytic *Pseudomonas* strain was isolated from clover (*Trifolium pratense* L.) (Sun et al. 2014). Phenanthrene is a polycyclic aromatic hydrocarbon, which is a toxic metabolite found in some soils and can be taken up by the plants through roots. It can enter the food chain and cause serious harm to human health. Sun et al. (2014) investigated the ability of *Pseudomonas* strain Ph6 to colonize ryegrass (*Lolium multiflorum* Lam.) and degrade phenanthrene. GFP-tagged Ph6 colonized root, stem, and leaf tissues internally when visualized under fluorescence microscope. Heavy colonization of root and shoot tissues by GFP-tagged Ph6 was observed with population density ranging from 10^3 to 10^5 cfu/g fresh tissue weight. Inoculation of ryegrass with Ph6 led to a significant decrease in the concentration of phenanthrene in shoot and roots. Along with that the overall accumulation of phenanthrene in roots and shoot was also significantly reduced with inoculation, possibly due to the degrading mechanism of Ph6 strain (Sun et al. 2014).

P. fluorescens and *P. putida* are the most commonly studied PGPB known to associate with many different plant host species and colonize them both internally and externally. In a study conducted on phosphate solubilizing *P. fluorescens* strains, L132 and L321, isolated from *Miscanthus giganteus* leaf tissues (Keogh 2009) were tested for their ability to promote pea (*Pisum sativum* L.) growth (Oteino et al. 2015). It was observed that inoculation with these endophytic strains significantly increased fresh weight as well as the dry weight of the pea seedlings possibly due to the phosphate solubilizing abilities of these endophytes since mean soluble phosphorous levels were also observed to be higher in inoculated plants as compared to the controls. Another endophyte related to *Pseudomonas* genus was isolated from internal root tissues of *Artemisia* sp. (Chung et al. 2008). The strain was identified as *P. brassicacearum* YC5480 and was observed to demonstrate antifungal activity against common pathogens like *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, and *Phytophthora capsici*. When colonized into a different host, radish, treated with *C. gloeosporioides*, the bacterial strain YC5480 counteracted the inhibitory effects of this pathogenic fungus. Therefore, it can be concluded that *Pseudomonas* spp. have the ability to cross-infect plant species other than their native host and have a broad application as a PGP agent in the agricultural industry.

2.5 Conclusion

Since their discovery, endophytic bacteria have been considered to play a crucial role in survival and growth of plants. By living inside the plant they are better protected from various biotic and abiotic stresses as compared to the rhizobacteria and free-living bacteria in soil. They have been reported to occupy almost every part of the plant, including intracellular and intercellular spaces. Due to the unique metabolic diversity of selected endophytes, they have been reported to colonize many nonindigenous plant host species and promote growth through direct or indirect mechanisms. Special mentioning deserves the endophytic bacteria belonging to the genus *Burkholderia* and *Paenibacillus*. Species belonging to these two genera have been frequently reported to endophytically colonize a variety of important agricultural crops, promote their growth in greenhouse and field conditions, and inhibit the growth of common crop pathogens in vitro as well as in vivo. These endophytic bacteria could potentially be the future commercial biofertilizers and biocontrol agents that can be used with many different crops and in various growing conditions, thus promoting sustainable agriculture.

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Chapter 3
Endophytic Fungi Bioremediation

Yelugere L. Krishnamurthy and B. Shankar Naik

Abstract Fungal endophytes are isolated from almost every host plant studied so far. The relationship between endophytes and host plants involves both mutualism and antagonism. Plants have many mechanisms to limit the growth of endophytes which include producing a variety of toxic metabolites such as terpenoides. But over a long period of co-evolution, endophytes have gradually formed a variety of tolerant mechanisms towards host metabolites by producing exo enzymes and mycotoxins. These enzymes include pectinase, cellulase, lipoidase, proteinase, phenol oxidase and lignin catabolic enzymes. When host plants die the fungi utilize the carbon source, plant residues such as glucose, oligosaccharides, cellulose, hemicelluloses, lignin, keratin, pectin, lipids and proteins and decomposes effectively. These enzymes may also degrade macromolecule compounds into small molecules or convert more toxic substances into less toxic in order to increase their adaptability. The use of fungi to clean up environmental pollutants has gained momentum in the past few years. However, most studies have focussed on white rot fungi and use of endophytic fungi might be a novel and important source for degradation of toxic pollutants including hydrocarbons, polychlorinated biphenyl's (PCBs), polyaromatic hydrocarbons (PAHs), radionuclides, and metals. Phytoremediation is another important bioremediation aspects of endophytic fungi in soils contaminated with hydrocarbons and heavy metals. Depolymerisations is one of the most efficient methods of plastic waste management by endophytic fungal enzymatic action. Complex polymers disintegrate into short chains of oligomers, dimers and monomers which can act as a source of carbon and energy. The enzymes produced by the microbes vary with the species even between strains of the same species. Enzymes are very specific in their action on substrates so that different enzymes help in the degradation of various types of enzymes.

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3.1 Introduction

Endophytes are bacterial or fungal microorganisms that colonize healthy plant tissues intercellular and/or intracellular without causing any apparent symptoms of disease (Wilson 1995). Almost every host plant studied so far is associated with some microorganism (Arnold et al. 2000; Shankar Naik et al. 2008). Symbioses between a fungus and a plant a wide spread phenomenon in nature and plays a major role in structuring plant communities by affecting colonization, competition, co-existence and soil nutrient dynamics (Clay and Holah 1999; Lemons et al. 2005; Krishnamurthy et al. 2009; Ghimire et al. 2010; Shankar Naik et al. 2014). The relationship is noted for balanced antagonism between endophytic virulence and plant defensive response (Schulz and Boyle 2005).

Plants have several mechanisms to limit the growth of endophytes including producing a variety of toxic metabolites (Muciarelli et al. 2007; Shankar Naik et al. 2006), but over a long period of co-evolution, the host endophyte may develop genetic systems allowing for the transfer of information themselves. Thus, endophytes have gradually formed a variety of tolerant mechanisms towards host metabolites by producing exo enzymes and mycotoxins (Costa et al. 2000; Schulz et al. 2002). Several workers have reviewed that endophytes produce diverse secondary metabolites related to terpenes, flavonoides, alkaloids, quinines, cyclohexanes and hydrocarbons. Many of these compounds showed antimicrobial, antioxidant, antineoplastic, anti-leishmanial and anti-proliferative activity, cytotoxicity and also fuel production (Shankar Naik et al. 2006; Wei et al. 2007; Chomcheon et al. 2009; Shankar Naik and Krishnamurthy 2010; Wang and Dai 2011) (Table 3.1). The enzymes produced by endophytic fungi may degrade macromolecule compounds into small molecules which could allow them to survive and reproduce despite plant defence mechanisms (Zikmundova et al. 2002). The extra cellular enzymes include pectinase, cellulase, lipoidase proteinase, phenol oxidase and lignin catabolic enzymes (Oses et al. 2006; Tan and Zou 2001; Bischoff et al. 2009). Generally fungal endophytes have the ability to utilize various organic compounds (carbon) which enables them in degradation of structural components such as glucose, oligosaccharides, cellulose, hemicelluloses, lignin, keratin, pectin, lipids and proteins (Lumyong et al. 2002; Urairaj et al. 2003; Tomita 2003; Kudanga and Mwanje 2005) present in leaf, litter and wood (Osono and Takeda 2001; Urairaj et al. 2003). In addition they have potential to decompose environmental pollutants and improve the soil micro environment (Wang and Dai 2011).

Few recent studies revealed that endophytes affect litter decomposition rates (Purahong and Hyde 2011) and stimulate soil carbon sequetraton and alter the flux of greenhouse gases (CO_2 and N_2O) from soil to the atmosphere (Iqbal et al. 2013; Saikkonen et al 2015). With the increasing industrialization of the global economy

Table 3.1 Therapeutic compounds from endophytes for various hosts

Natural product	Endophyte	Host plant	Activity	References
Taxol	*Taxomyces andreanae*	*Taxus brevifolia*	Anticancer	Stierle et al. (1993)
Cryptocandin	*Cryptosporiopsis quercina*	*Tvipterigeum wilfordii*	Antifungal	Strobel et al (1999)
Cryptocin	*Cryptosporiopsis quercina*	*Tvipterigeum wilfordii*	Antifungal	Li and Strobel (2001)
Pestaloside	*Pestalotiopsis microspore*	*Torreya taxifolia*	Antifungal	Lee et al. (1995)
Torreyanic acid	*Pestalotiopsis microspore*	*Torreya taxifolia*	Anticancer	Lee et al. (1996)
Subglutinols A&B	*Fusarium subglutinols*	*Taxus cuspidata*	Immunosuppressive	Kim et al (2004)
Campothecin	*Entrophospora infrequens*	*Nothapodytes foetida*	Antineoplastic	Puri et al. (2005)
Naptha-y-pyrone	*Aspergillus niger*	*Cynodon dactylon*	Antitumor	Song et al. (2007)
Vincristine	*Fusarium oxysporum*	*Cantharanthus roseus*	Anticancer	Kumar et al. (2013)
Peniprequinolone	*Penicillium janezewskii*	*Prumnopitys andina*	Nematicidal	Hirschmann et al. (2005)
Podophyllotoxin	*Alternaria* sp.	*Sabina vulgaris*	Antiviral	Eyberger et al. (2006)
Vinblastin	*Alternaria* sp.	*Catharanthus roseus*	Anticancer	Guo et al. (1998)
Volatile hydrocarbons	*Gliocladium roseum*	*Eucryphia cordifolia*	Mycodiesel	Stinson et al. (2003)
Volatile hydrocarbons	*Hypoxylon* sp.	*Persea indica*	Mycodiesel	Tomsheck et al. (2010)
Volatile hydrocarbons	*Muscodor albus*	*Ginkgo biloba*	Mycodiesel	Banerjee et al. (2010)

over the past century, a wide variety of pollutants such as (PHC, PAHs, halogenated hydrocarbons, pesticide solvents, salt and heavy metals have been introduced into the environment and cause environmental problems (Rajkumar et al. 2010; Ma et al. 2011).

New technology such as phytoremediation and bioremediation using microbes especially endophytes are gaining considerable momentum (Li et al. 2012; Weyens et al. 2009; Germaine et al. 2009). In this review, we attempted to discuss the role of endophytic fungi and their potential in bioremediation of natural and anthropogenic toxic pollutants.

3.2 Enzymes in Endophytic Fungal Remediation

Endophytic fungi produce enzymes such as amylases, lipases, proteases, etc., as part of their mechanism to overcome the defence of the host against microbial invasion and to obtain nutrients to their development (Sunitha et al. 2012; Torres et al. 2003). Naturally, endophytic fungi play an important role in global carbon and nitrogen cycling by promoting the bioconversion of organic matter through enzymatic and non-enzymatic systems. In forest region, the endophytes breakdown wood polysaccharides using a combination of enzymes which break glycosides linkages between B-D-xylopyranosyl and glucopyranosyl residues using cellulase system consists of three classes of enzymes, i.e. 1,4-β-D-glucan cellobiohydrolases, endo-1,4-β-D-glucanases and 1,4-β-D-glucosidase (Rodrigues et al. 2011).

Phenol oxidase enzymes which include peroxidases, laccases and tyrosinases degrade lignin which is a hydrophobic polymer that fills up the space between the cellulose micro fibrils and laccases are the copper containing oxidases that have the ability to oxidize substrates with high redox potential in the presence of synthetic mediators which allow the degradation of non-phenolic lignin. Lignin peroxidase and manganese peroxidases are described as true ligninases because of their high redox potential. Some researchers stated that on the role of Xylariaceous endophytic fungi simply waiting for their host to senesce to begin the decomposition of the host cell wall material (Petrini and Petrini 1985; Rodrigues et al. 2011). Several endophytic fungi are known to produce lignocellulolytic enzymes (Suryanarayanan et al. 2009). Lignin is a heterogenous and irregular arrangement of phenyl propanoid polymer protects cellulose from chemical or enzymatic degradation. Fungi produce extracellular enzymes to cleave the aryl –α-carbon bond or bond between the α- and β-carbons of the alkyl chain radical in lignin (Karsten 2008). Shi et al. (2004) demonstrated that adding endophytic fungi *Phomopsis* sp. to scantly decompose straw by degrading lignin. In another study, laccase and peroxide produced by endophytic fungi contribute directly to the decomposition of litter lignin (Dai et al. 2010).

Nutritional and environmental stress may induce fungal relative enzymatic gene express, and then change endophytic fungal metabolic pathway (Chen and Dai 2013). The synergetic metabolism of endophytic (Basidiomycetes) and soil fungi transform stable polymers to other simple compounds such as CO_2, humus substance and glycoproteins (Granit et al. 2007; Talbot et al. 2008).

Rodrigues et al. (2011) reported that a basidiomycete and a deuteromycete corresponding to mycelia sterile isolated from the Chilean native trees *Prumnopity sandina* and an unidentified basidiomycete and mycelia sterile from *Drimys winteri* had lingo-cellulolytic activity thus promoted the wood biodegradation. Researchers reported that the lignocellulosic materials were degraded by fungal enzymes on two systems (a) hydrolytic system consisting xylanases and cellulases and (b) unique oxidative ligninolytic system comprises laccases, ligninases and peroxidases (Correa et al. 2014).

On the other hand, amylases (α amylase, β-amylases and glucoamylases) which are the amylolytic enzymes convert starch into different sugar solutions also contained in endophytic fungi. Fungal amylases especially glucoamylases are widely used in industries. *Aspergillus* sp. and *Rhizopus* sp. are often used as sources for the production of glucoamylases (Pandey et al. 2000). In a study endophytic *Fusicoccum* sp. showed strong amylolytic activity under in vitro conditions (Champreda et al. 2007). Similarly, the endophytic *Cylindro cephalum* sp. isolated from medicinal plant *Alpinia calcarata* (Haw.) Roscoe found to produce amylase under 30 °C and at pH 7.0 in the presence of maltose and sodium nitrate sources (Sunitha et al. 2012). In another study, enzymes of endophytic strains belonged to *Gibberella pulicaris*, *Acremonium* sp., and *Nodulisporium* sp. hydrolysed raw sagostarch to produce solely glucose whereas amylases of *Synnematous* sp. produced glucose and maltose (Marlida et al. 2000).

Another import enzyme secreted by a group of endophytic fungi are lipases which are hydrolytic enzymes that in vivo break the ester bond of triacyl glycerol releasing free fatty acids and glycerol being then classified as a special class of esterases (Oliveira et al. 2012). They also catalyze interesterification, alcoholysis, acidolysis, esterification and aminolysis reactions under proper conditions (Damassoet al. 2008). Endophytic *Rhizopusoryzae* isolated from Mediterranean plants found to be producers of membrane bound lipases (Torres et al. 2003). Similarly, protees are enzymes which hydrolyse peptide bonds of proteins, they are also called proteolytic enzymes or proteinases. Few year back, a novel fibrinolytic enzyme was discovered from endophytic *Fusarium* sp. isolated from *Chrysanthemum* stems (Wu et al. 2009).

Fungi are the major decomposers of lignocelluloses in several ecosystems and play an essential role in cycling of carbon and other nutrients. The main hydrolytic enzymes involved in lignocelluloses degradation are exo and endoglucanases, β-glycosidase, exo and endoxylanases and β-xylosidases (Dyk and Pletschke 2012). Correa et al. (2014) reported that for complete degradation of lignocellulose materials, laccases, manganese peroxidise and lignin peroxidise (oxidative enzymes) and additional hemicelluloses (e.g., acetyl esterase, β-glucuronidase, endo-1, 4-β-mannanase, α-galactosidase) and oxidoreductases (aryl alcohol oxidase, glucose-1-oxidase, glyoxal oxidase, pyranose-2-oxidase) are also needed.

3.3 Endophytic Fungi and Nutrient Cycling

The importance of phyllospheric endophytic fungi to ecosystem functioning via soil processes has aroused increasing interest during the past decade. The endophytes may affect plant litter quality, organisms that control litter decomposition, and the availability of nutrients in plant communities. Endophytes are likely to affect the decomposition of plant litter and soil nutrient trans formations at least in three ways (i) by acting as saprophytes in abscised plant parts and aiding their decay (ii) by affecting the amount and/or quality of plant litter (iii) by affecting the abundance

richness and composition of decomposer organisms (Saikkonen et al. 2015). The non-systemic endophytes from grasses and other plants are also survive in decomposing plant litter as saprotrophs and endophytes, as they can play a role in leaf senescence (Purahong and Hyde 2011). Systemic grass endophytes can increase growth, reproduction and stress resistance of their host plant (Clay and Hollah 1999) and thereby increase the amount of litter produced by the host. They can affect the quality of plant litter by modulating the foliage quality of the host plant.

The endophyte plant symbiosis produces various alkaloids such as pyrolizidines (Lolines), ergot alkaloids, indolediterpenoides (including lolitrems), and the pyrrolopyrazine alkaloids (Peramine) (Saikkonen et al. 2010; Schardl 2010) and alter the concentration of sugars, water and modulates their oxidative balance, phytohormone signalling and other metabolic pathways (Liu et al. 2011; Saikkonen et al. 2013).

The host plants induce responses to invaders and attackers by two evolutionary conserved phytohormone signalling pathways, i.e., by the salicylic acid (SA) and jasmonic acid (JA) pathways (Pieterse and Dicke 2007). Plant defence responses to bio trophic pathogens are mediated by SA pathways (Thaler et al. 2012). Endophytes have both positive and negative effects of decomposer organisms (Lemons et al. 2005). Saikkonen et al. (2013) proposed that endophytes similar to that of the parasites likely induce SA pathway, thus suppressing the mutually antagonistic JA pathway, which is mainly involved in the defence system against pathogens and herbivores. Alternatively, the negative effects can arise prior to colonization of the leaf litter and competitive exclusion of the saprophytic fungi. The allelopathic chemicals produced by endophytes toxic to both microbial and invertebrate decomposers would also lead to negative effects (Saikkonen et al. 2015). Endophytic fungi occur in various plant organs and have a close relationship between hosts and soil (Sun et al. 2008; Chen and Dai 2013). Compounds released as a result of endophytic plant symbiosis could decompose organic matter or inhibit other microbial growth (Suberkropp and Weyers 1996). Endophytic fungi colonizing the host roots could affect soil productivity by promoting soil nutrition through decomposition and reduces soil heavy metal toxicity (Chen and Dai 2013). Endophytic fungi also play an important role in the degradation of plant debris. Oses et al. (2006) found that endophytic fungi belonged to basidiomycetes isolated from Chilean tree species *Drimys winteri* and *Prumnopitysandina* were able to degrade the wood similar to white rot fungi. Endophytes from spruce needles shown to pioneer decomposers in lab experiments (Muller et al. 2001). In a study lignocellulolytic activity was observed from *Alternaria, Phoma* and *Phomopsis* isolated from surface sterilized pods of *Colophospermum mopane* (Jordaan et al. 2006; Wang and Dai 2011). A strain of *Phomopsis* sp., isolated from the inner bark of *Bischofia polycarpa* was able to decompose pea nut straw (Shi et al. 2004). A number of strains belonged to endophytic fungi such as *Xylaria, Geniculosporium, Coccomyces, Monotospora* produced lignicellulolytic enzyme activity (Koide et al. 2005; Osono and Takeda 2001).

3.4 Endophytic Fungi in Phytoremediation

Phytoremediation is one of the approaches in which living green plants *in situ* are used for bioremediation. They have the ability to decrease and/or removing contaminants from soil, water, sediments and air. Numerous recent studies have demonstrated that endophytic microorganisms can accelerate these processes effectively by interacting closely with their host plants (Li et al. 2012). Endophytic *Neotyphodium coenophialum* and *Neotyphodium uncinatum* both were found to be successfully removed PAH and TPH from rhizosphere by two grass species *Festucaarun dinacea* Schreb and *F.pratensis* Hude (Soleimani et al. 2010). Espinosa et al. (2005) demonstrated that phytoremediation of hydrocarbon contaminated soil with *Cyperuslaxus* inoculated with endophytic fungi. Similarly, phytoremediation efficiency of wheat, mungbean and eggplant grown in hydrocarbon contaminated soil was reported by Rabie et al. (2005). Recently Cruz-Hernandez et al. (2013) demonstrated the removal of polyaromatic hydrocarbons by *Festuca aruninaceae* from both perlite and soil by endophytic *Lewia* sp. The inoculated plants exhibited higher phenanthrene degradation (100%) as compared to non-inoculated plants in perlite and soil.

Fungi are highly resistant to heavy metal pollution (Jordan and Lechevelier 1975) and play very important role in element cycling and mineral transformations (Gadd 2007). The mechanism of metal tolerance in fungi includes metal adsorption and immobilization, complexing and quanti-valence changing (Collin et al. 2003; Gadd 2007). Fungal cell wall contains various active groups such as hydrosulphide carboxyl hydroxyl which could respond to heavy metal ions and precipitate on the surface of the cell wall (Shen et al. 2006). It was found that dark septate endophytic fungi tolerant to environmental metal pollution and accumulates heavy metal in vitro (Zhang et al. 2008; Ruotsalainen et al. 2007). Generally, endophytes which live in host roots are similar to mycorrhizal fungi in many aspects (Brundett 2006).

The fungal endophytes have been shown to ameliorate metal toxicity for their plant hosts by restricting the uptake of toxic metals and improving the supply of essential elements (Likar et al. 2011) in red plant biomass despite higher Cu and Zn accumulation in plant roots through expression of certain genes together with increased free and conjugated polyamine levels (Cicatelli et al. 2010). Endophytic fungi may increase host tolerance towards biotic and abiotic stresses. The plants inoculated with endophytic fungi exhibited higher biomass production and higher potential to accumulate Cd in roots and shoots than fungi free plants (Soleimani et al. 2010).

In another study, endophytic *Trichoderma* sp. associated with *Acacia auriculoformis* produced more fresh weight than control plants and also increased the translocation factors and metal bio concentration on growth of mustard, (*Brassica juncea* L.) grown on Cd and Ni contaminated soils (Jiang et al. 2008). In a phytoremediation study, the endophytic *Mucor* sp. enhanced the phytoremediation potential of rape roots grown in soil contaminated with Pb and Cd. Deng et al. (2013) proposed an efficient method of phytoremediation by constructing mutant by

protoplasm fusion of endophyte *Mucor* sp. in rape roots contaminated the soil with Pb and Cd.

Although heavy metals are toxic to plants, it has been demonstrated that many plants are metal tolerant and some of them are metal hyper accumulators (Rosa et al. 2004; Li et al. 2012). Many metal resistant endophytes were isolated from hyperaccumulating plants. These fungi belonged to various taxa include *Microsphaeropsis*, *Mucor, Phoma, Alternaria, Pyronellaea, Steganosporium* and *Aspergillus*. Soleimani et al. (2010) demonstrated that endophytic fungi were helpful in phytoremediation of aged petroleum contaminated soil and that these fungi improved host plants roots and shoot biomass and created higher levels of water soluble phenols and dehydrogenase activity in the soilTCE, Naphthalene, BTEX, catechol and phenol could be degraded by endophytes which decreased the contaminant phytotoxicity and improved plant growth (Weyens et al. 2010; Ho et al. 2009; Li et al. 2012).

3.5 Endophytic Fungi in Bio Degradation of Pollutants

Fungi are known to utilize a wide range of organic compounds for nutrition and energy generation through extracellular enzymes. These organic compounds include cellulose, pectin, lignin, lignocelluloses, chitin and starch and anthropogenic substances such as hydrocarbons, pesticides and other xenobiotics. The white rot fungi like *Phaenerochate chrysosporium* can degrade several xenobiotics such as aromatic hydrocarbons, chlorinated organics, poly chlorinated biphenyls, nitrogen containing aromatics and many other pesticides, dyes and xenobiotics (Gadd 2007; Harvey and Thursten 2009).

The use of fungi to clean up environmental pollutants has gained the momentum in past few years, however most studies have focussed on white rot fungi (Marco-Urrea et al. 2008; Nikiforova et al. 2009) and the use of endophytic fungi might be a novel approach and important source for degradation of toxic pollutants which includes hydrocarbons, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), radionuclides and metals.

Fungi are known to degrade PAHs in surface of soil. These fungi produce extracellular enzymes with lower substrate specify which enable degrade aromatic compounds including PAHs (Leonardi et al. 2007; Farnet et al. 2009). In a study, manganese peroxidase was found to be the dominant ligninolytic enzyme in the degradation PAH (Tian et al. 2007; Dai et al. 2007). Researchers found that endophytic fungi might be a novel and important resource for the degradation of polycyclic aromatic hydrocarbons (PAHs). An endophyte fungal strain *Ceratobasidium stevensii* isolated from the plant of *Euphorbiaceae* was found to metabolize phenanthrene effectively (Dai et al. 2010). Tian et al. (2007) demonstrated the degradation of phenanthrene by endophytic *Phomopsis* sp., with rice plant. In a study, endophytic *Xylariaceae* strains isolated from healthy tropical native plants of Thailand found to be the producers of ligninolytic enzymes

(Urairaj et al. 2003). Russel et al. (2011) demonstrated that the ability of endophytic fungal degradation of synthetic polymer polyester polyurethane (PUR) by the production of serine hydrolases. The *Pestalotiopsis microspora* isolate was uniquely able to grow on PUR as the sole carbon source under both aerobic and anaerobic conditions. Recently endophytic *Fusarium* sp., isolated from the leaves of *Pterocarpus macrocarpus* Kurz.was able to degrade benzo(a)pyrene (BAP), a five ring polycyclic aromatic hydrocarbon produced by the incomplete combustion of organic materials (Juhasz and Naidu 2000).

3.6 Conclusion

Fungal involvement in element cycling has important implications for living organisms and human health. Hence, better understanding of fungal activities, complexity of heterogeneous environment and interactions between different organisms helps to formulate further effective bio remedial strategies. In this review, we have reported the bioremediation potential of endophyticfungi and discussed the role of endophytic fungi in the management of toxic pollutants in future.

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Chapter 4
Endophytic Bacteria: Role in Phosphate Solubilization

Abhishek Walia, Shiwani Guleria, Anjali Chauhan and Preeti Mehta

Abstract The worldwide need to increase agricultural and horticultural production from a consistently diminishing and degraded land resource has set remarkable strain in light of agro biological systems. The current methodology is to keep up and enhance agricultural and horticultural productivity only by means of the utilization of chemical fertilizers and pesticides. Despite the fact that the utilization of chemical fertilizers is credited with almost fifty percent of increase in agricultural production yet they are closely associated with environmental contamination and health problems in human beings and animals. Microbial assorted qualities in the soil are viewed as critical for keeping up for the manageability of agriculture and horticulture systems. Nonetheless, the connections between microbial differences and environmental processes are not surely known. Rhizosphere soil strongly affects a range of procedures influencing crop yield. Rhizobacteria that are present inside plant roots, framing more close associations, are known as endophytes. These endophytes are likewise called intracellular plant growth-promoting rhizobacteria (PGPR) microorganisms dwelling inside plant cells, producing nodules and being present inside these specific structures. These incorporate an extensive variety of soil microorganisms framing less formal relationship than the rhizobia-legume advantageous interaction called symbiosis, endophytes may empower plant development, directly or indirectly and incorporate the rhizobia. In this review, we essentially concentrate on the plant development by Phosphate solubilization furthermore by different means. Phosphorus is normally lacking in most characteristic soils since it is settled as

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insoluble iron and aluminum phosphates in acidic soils or calcium phosphates in soluble soils. Phosphate-solubilizing bacteria (PSB) as inoculants have the ability to convert insoluble forms of phosphorus to an usable form for high plant yields. This chapter mainly focuses on endophytic P-solubilizing bacteria, mechanism of P-solubilization, genetic diversity of P-solubilizers, and mass production of inoculants inoculant production and response of the crop to P-solubilizers bioinoculants.

Keywords Endophytes · Phosphate solubilization · PGPR · Bioinoculants Genetic diversity

4.1 Introduction

Microbial diversity in soil is viewed as critical for keeping up for the manageability of horticulture/agriculture creation frameworks. Notwithstanding, the connections between microbial diversity and ecosystem processes is not surely understood (Stark et al. 2007; Jha et al. 2014). Rhizosphere soil strongly influences the range of processes impacting crop yield. Numerous microorganisms are pulled in by supplements oozed from plant roots and this "rhizosphere impact" was initially depicted by Hiltner (1904). He observed higher number and activity of microorganisms in the region of plant roots. These microbes gain profit by the nutrient exudates by the plant roots, which ultimately advantageously impact the development of plants.

As of late, the interest in soil microorganisms has expanded, as they are a key component in supplement cycling and the support of soil fertility. Phosphorus is one of the essential macronutrient for plant growth and development In average soils, the P-content is about 0.05% (w/w) but only 0.1% of the total P is available to plants (Scheffer and Schachtschasel 1992; Otieno et al. 2015), since it is fixed as insoluble iron and aluminum phosphates in acidic soils or calcium phosphates in alkaline soils. These precipitated forms cannot be absorbed by plants, this leads to excessive and repeated application of P fertilizer to cropland (Sharma et al. 2013).

The capacity of a few soil microorganisms to change over insoluble types of phosphorus (P) to an accessible form is an imperative attribute in plant growth-promoting bacteria (PGPR) also known as P-solubilizing microorganisms (PSM). The utilization of PSM as inoculants enhances the P uptake by plants thus increasing plant yields (Ahemad and Khan 2010; Jain and Khichi 2014). Because of the negative ecological effects of compound composts and their expanding costs, the utilization of PSM is considered as a supplementary method for reducing the utilization of chemicals in agribusiness/cultivation (Welbaum et al. 2004; Hameeda et al. 2006; Mehta et al. 2013c; Walia et al. 2013a).

4.2 PSB and Their Hosts: Endophytic Region

For P-solubilizing PGPR to have an impact on plant development by means of an increment of the nutrient status of their host, there evident should be an intimate relationship between the phosphate-solubilizing bacteria (PSBs) and the host plant.

In any case, the level of closeness between the PSBs and the host plant can differ contingent upon where and how the PSBs colonizes the host plant. Connections between PSBs and their hosts can be ordered into two levels of complexity (1) Rhizospheric (2) Endophytic.

4.2.1 Endophytic Region

Rhizobacteria that build up and spends the entire piece of its life cycle inside plant roots, exhibit no outside contamination or negative impact on their host and forming more intimate associations, are endophytes or intracellular PGPR-(iPGPR). Perotti (1926) was the first to portray the event of nonpathogenic organisms in root tissues. Endophytic microorganisms have been considered to originate from the outside environment and enter the plant through stomata, lenticels, wounds, emergence of lateral roots and germinating radicals (Gaiero et al. 2013). Endophytic microbes can effectively or inactively colonize plants locally or systemically and both intercellularly and intracellularly. The endophytic niche provides protection from the environment for the colonizing bacteria that establish *in planta.* Subsequently, to be biologically effective, endophytes that infect plants from soil must be able to root colonizers. Despite the fact that, it is, for the most part, accepted that numerous bacterial endophyte groups are the result of a colonizing process started in the root zone (Compant et al. 2010), they might likewise begin from other sources than the rhizosphere, for example, the phyllosphere, the anthosphere, or the spermosphere. Lytic proteins created by root colonizing bacteria may likewise add to more effective penetration and colonization. The deliverable of endophytes like cellulolytic and pectinolytic catalysts are being considered for certain types of infection process, cell wall degrading chemicals, endogluconase and polygalacturonase causes infection of *Vitis vinifera* by *Burkholderia* sp. (Hallmann et al. 1997; Compant et al. 2005).

Endophytic microorganisms inhabiting vast assorted qualities of plants was looked into by Sturz et al. (2000) and Posada and Vega (2005). Rhizosphere is considered as a hot spot for P-solubilizing bacteria suggesting that these bacteria proliferates both in rhizosphere soil and root endosphere (Hui et al. 2011). But apart from that, population of endophytic bacteria is at the lower site as compare to rhizospheric bacteria or any other bacterial pathogens (Feng et al. 2013). Although, many researchers have confirmed the occurrence of least amount of endophytes in rhizosphere but Mehta et al. (2015) had given a strong evidence in their study with perpetually higher P-solubilizing bacteria in apple rhizosphere than those in roots endosphere (Table 4.1). The most acceptable reason for a higher population of rhizospheric bacteria could be due to high level of carbon fluxes creating the'rhizosphere impact' used to sustain bacterial growth (Reyes et al. 2006; Mittal and Johri 2007).

The population of PSB is always higher around the rhizosphere and around roots as compare non-rhizosphere. The high concentration of PSB around the roots

Table 4.1 Comparative data on P-solubilizing bacterial population in rhizosphere soil and roots of apple trees at different sites

Location	Percent P-solubilizers	
	Rhizosphere soil bacterial population	Root endophytic bacterial population
Chamba	79.2	38.8
Kinnaur	35.2	15.6
Shimla	29.2	18.7
Kullu	48.3	11.3
	T = 2.06	

occurs because of the presence of high levels of nutrients exuded from the roots of most plants that can support bacterial growth and metabolism (Glick 2003; Sharma et al. 2007). Higher the population of P-solubilizers is of direct significance to the plants as it helps in mobilization of insoluble P near the root, especially in P-deficient soils (Chatli et al. 2008; Gulati et al. 2008; Aranda et al. 2011).

In plant tissue, in general, endophytic P-solubilizing bacterial populations have been reported between 10^2 and 10^4 viable bacteria per gram (Sobral et al. 2004; Piromyou et al. 2010; Patel et al. 2012; Kumar et al. 2013a, b; Saini et al. 2015). Mehta et al. (2015) isolated one hundred and four and 85 of total 200 soil and root samples of apple trees harbored P-solubilizing bacteria. They observed that the proportion of rhizosphere soil and root endophytic P-solubilizing bacteria among culturable one varied greatly with respect to sampling sites, ranging from 0–79.2% to 0–60.6%, which was in agreement with previous study that showed large variation from 3 to 67 $\times$ 10^6 cfug^{-1} (Kundu et al. 2009). A large variation within and amongst different sites in population of P-solubilizing bacteria indicated their wide distribution within the crop and place of sampling. The poor population of P-solubilizing bacteria could be attributed to their meager natural population as a result of environmental factors along with physiochemical properties of the soil. Variation in the population of P-solubilizing bacterial status of samples within the sites is possible due to the collection of samples from a different point and an uneven population of competitive P-solubilizing bacteria.

Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species (Ryan et al. 2008; Mehta et al. 2015). The variation in endophytes occurrence might be a function of the different maturation stages specific to each plant, sampling time and environment condition, which contribute higher impact on different types and amounts of root exudates (Vendan et al. 2012). The presence of large population of bacteria isolated from all the sites unequivocally suggests the hypothesis that natural plant genotypic variants of a single species have a special choice for selection of specific microbiota consortia as a result of their unique root exudates profile (Micallef et al. 2009; Aranda et al. 2011).

4.2.2 Role of Phosphorus Solubilizing Microorganisms

The term microorganisms broadly encompass bacteria and fungi including other mini-creature only observed by microscope. Among the microorganisms, bacteria and fungi are more versatile to facilitate phosphate solubilization.

4.2.2.1 Phosphorus Solubilizing Bacteria and Fungi

PSM consist predominantly the bacteria and fungi among ectorhizospheric strains, *Pseudomonas*, *Bacillus,* and endosymbiotic rhizobia have been served as effective phosphate solubilizers (Igual et al. 2001). The vast majority of fungi are non-Phosphate solubilizers except for species of *Aspergillus* and *Penicillium* (Sagervanshi et al. 2012; Sahoo and Gupta 2014). Villegas and Fortin (2002) identified microorganism viz., *Rhizobium*, *Klebsiella*, *Mesorhizobium*, *Acinetobacter*, *Erwinia*, *Achrobacter*, *Enterobacter*, *Micrococcus*, *Pseudomonas* and *Bacillus* isolated from different soils as efficient P solubilizing strains. Majority of Gram-positive soil bacilli almost 95% belong to the genus *Bacillus* (Garbeva et al. 2003) and are capable to form endospores and for this reason survive beneath detrimental conditions; some species are diazotrophs along with *Bacillus subtilis* (Timmusk et al. 1999), while others have specific PGPR capacities (Kokalis-Burelle et al. 2002; Barriuso and Solano 2008). From rhizobial strains, two species of nodulating chickpea, *Mesorhizobium mediterraneum* and *Mesorhizobium ciceri*, are known for their high phosphate-solubilizing efficiency (Rivas et al. 2006). But, it is recognized that each aspect of nodule formation is limited due to the supply of P. legumes like alfalfa and clover displaying a positive effect in response to P supplementation (Gyaneshwar et al. 2002), however most of the supplemented P become unavailable when its reacts with soil components. The extracellular oxidation of glucose to gluconic acid via the quinoprotein glucose dehydrogenase results in efficient phosphate-solubilizing phenotype in Gram-negative microorganism (Otieno et al. 2012). Numerous soil microorganisms have the ability to solubilize this unavailable P through their metabolic activities exudating organic acids, which directly dissolve the rock phosphate, or chelating calcium ions that release P to the solution.

4.2.3 Microbes in Biogeochemical Cycle of P in Soil

Microorganisms are fundamental to the biogeochemical cycle of phosphorus and as such play crucial role in mediating the availability of phosphorus to flora (Richardson et al. 2011; Jain and Khichi 2014). Biogeochemical cycling of phosphorus is essential for various reasons. Every living cell requires phosphorus for nucleic acids, lipids, and a few polysaccharides. In soil, phosphorus exists in both

inorganic and organic forms. Inorganic phosphorous complexes without problems with cations (includes iron, aluminum, and calcium) in the environment as it is negatively charged. These compounds are relatively insoluble, and their separation is pH dependent, being accessible to plants and microorganisms between pH 6 and 7. Under such conditions, these organisms rapidly convert phosphate to its organic form in order that it becomes available to animals. A significant percentage of culturable bacterial and fungal communities were being accounted for inorganic P solubilizing activity (Barraquio et al. 2000; Chen et al. 2008; Ashrafuzzaman et al. 2009). The form of phosphorus found in biomass and materials such as humus and organic compounds is known as organic phosphorus. This organic phosphorus is recycled by microbial activity that involves transformation of simple orthophosphate (PO_4^-), with +5 valence state into more complex forms. These include the polyphosphate seen in metachromatic granules in addition to greater acquainted macromolecules.

4.2.4 P-Solubilizer as Biofertilizers

Microbial inoculants have provided a worth biological alternative to compensate agro chemicals and to sustain environment-friendly crop production (Dobbelaere et al. 2003; Musarrat and khan 2014). Phosphorus solubilizing microorganisms proved as an effectual approach for imparting balanced nutrition (Martins et al. 2004) and have recently attracted the attention of agriculturalists as soil inoculums to enhance the plant growth and yield (Fasim et al. 2002; Otieno et al. 2015).

The inorganic phosphates solubilization in soil by microorganisms and making them available to plants is the well-known mechanism (Bhattacharya and Jain 2000; Chen et al. 2006) and organisms responsible for this are referred as phosphate solubilizers. Population count of phosphate-solubilizing microorganisms is at the concentrated form in the rhizosphere, and they are metabolically more active than other sources (Vazquez et al. 2000). It is well known that both groups of microorganisms including phosphate-solubilizing bacteria and fungi are equally important to enhance plant growth by using solubilization mechanism and their acquisition to plant production via synthesis of plant growth-promoting substance and organic acid (Yadav et al. 2011).

The improvement of soil health in terms of fertility is one of the most common ways to increase agricultural production for which biological nitrogen fixation is considered to be the most important. After biological nitrogen fixation, phosphate solubilization is equally essential, as phosphorus (P) is significant key macronutrients for biological growth and development. Microorganisms provide a biological rescue system that enables to solubilize the insoluble inorganic P of soil and make it available to the plants. The ability of a few microorganisms to convert insoluble phosphorus (P) to an available form, like orthophosphate, is a critical trait in a PGPB for improving soil fertility and plant yields. Thus, the rhizospheric

phosphate-solubilizing microorganism can be a promising source for plant growth-promoting agent in agriculture (Rodriguez et al. 2006).

Using phosphate-solubilizing microorganism as inoculants will increase the P uptake through plants (Chen et al. 2006). The production of bioinoculants on a commercial scale and their acceptance by farming communities are closely linked as it is not easy. Furthermore, environmental variables including salinity, pH, moisture, temperature and climatic conditions of the soil largely affect the establishment and performance in field cum demonstrations trials of these PSM inoculants developed under laboratory conditions. Hence, there is a great need for proper development of suitable technology for the isolation of effective inoculants of PSM based biofertilizers for their adoption under farmer's fields. Current approach and developments in our understanding of the functional diversity, rhizosphere colonizing ability, mode of actions and judicious application are likely to facilitate their use as reliable components in the management of sustainable agricultural systems (Zaidi et al. 2009a).

4.3 Mechanism of P-Solubilization

Organic acid production by soil microorganisms is predominant mechanism of phosphate solubilization. Organic acids result in a decrease in pH of microbial cell and its surroundings (Halder et al. 1990; Khan et al. 2014a) (Fig. 4.1). In soil, phosphorus is present in the organic and inorganic form. Soil microorganisms release phosphorus by organic and inorganic P solubilization. Organic P solubilization is mineralization process (Richardson and Simpson 2011). Numerous mechanisms are opted by soil microorganisms in order to perform P solubilization such as lowering of pH, organic acid production, chelation and exchange reactions (Gerke 1992). Microorganisms secrete different types of organic acids during solubilization and lower the pH of rhizosphere and consequently dissociate the bond form of phosphates like $Ca_3(PO_4)$ (Tri Calcium Phosphate) in calcareous soil. Furthermore, these microorganisms also serve as a sink for P in the vicinity of labile C. Soil microorganisms immediately immobilize it even in low P soils. Ecological changes, for example, freezing–thawing or drying–rewetting, can bring about flush-events, a sudden increment in accessible P because of high extent of microbial cell lysis (Butterly et al. 2009).

The major processes employed by microorganisms for soil P solubilization summarized here:

(1) Secretion of mineral dissolving compounds e.g. organic acid anions, protons, hydroxyl ions, CO, siderophores
(2) Biochemical P mineralization by release of extracellular enzymes and
(3) Biological P mineralization by liberation of P during substrate degradation

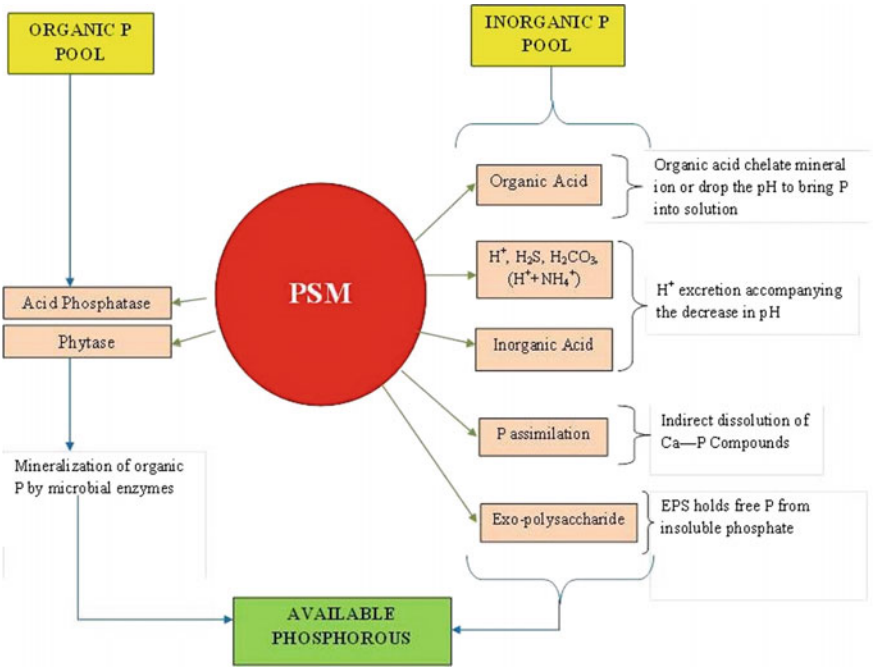

Fig. 4.1 Schematic representation of P solubilization/mineralization by various organic/inorganic substances produced by PSM

As described by Sims and Pierzynski (2005), the major processes of the soil P cycle that affect soil solution P concentrations are biologically mediated conversions of P between inorganic and organic forms, i.e., mineralization–immobilization; interactions between P in solution, and soil solid surfaces, i.e., sorption–desorption and dissolution–precipitation, i.e., mineral equilibria.

4.3.1 Different Microbial Mechanisms of P-Solubilization

Microorganisms are observed as proprietor of diverse mechanism to solubilize both organic and inorganic phosphate.

4.3.1.1 Organic P-Solubilization

Mineralization of organic phosphorus constituting 4–90% of the total soil P is referred as Organic P solubilization (Khan et al. 2009). Each organism can act in one or multiple ways to bring about the solubilization of insoluble P in soil. One of

which is use of enzyme, i.e., Non-specific acid phosphatases (NSAPs), phytases, C–P lyases, and Phosphonatases.

Non-specific acid phosphatases (NSAPs) have a capacity of dephosphorylate phosphoanhydride or phospho-ester bonds of organic matter. Among the different classes of phosphatase enzyme released by PSM, most studied and abundant class is Phosphomonoesterases (often called phosphatases) (Nannipieri et al. 2011). Depending upon the pH optima, phosphatases are further divided into acid and alkaline phosphomonoesterases (Jorquera et al. 2008). These enzymes (acid and alkaline phosphatases) are produced by plant roots as well as by PSM. Differentiation between phosphatases on the basis of their production source is very difficult (Richardson et al. 2009). However, plant roots can only produce large quantities of acid phosphatases. There are evidence proposing that phosphatases released from microbes have higher affinity for Po compounds as compared to phosphatases produced from plant roots (Chen et al. 2003), but still, there is not much understanding regarding the relationship between phosphatase activity of inoculated PSM and the subsequent mineralization of Po.

Phytases have a specific capacity of phytate degradation and cause P release. Phosphorous is stored in plant seeds and pollen in form of phytate. In the plant, it is primary inositol source. The key driver of regulation of phytate mineralization in soil is microorganisms. In spite of the fact that the capacity of plants to get P specifically from phytate is exceptionally restricted, but the vicinity of PSM inside of the rhizosphere provide an opportunity to plants to take up P directly from phytate (Richardson and Simpson 2011).

C–P lyases and phosphonatases are enzymes that act mainly in the breakdown of the C–P bond in organophosphonates (Rodriguez et al. 2006).

4.3.1.2 Inorganic P-Solubilization

Organic Acid Production

The major reason of inorganisc phosphorous solubilization is organic acid production by PSM. Primarily following organic acids are produced, i.e., acetic, citric, fumaric, glycolic, lactic, melonic, oxalic, propoionic, succinic acid, tartaric, etc. (Ahmad and Shahab 2011). Among all, the principal organic acid involved in inorganic P solubilization is gluconic acid. PSBs which produce abundant amount of gluconic acid are *Burkholderia cepacia*, *Erwinia herbicola, Pseudomonas* sp and *Pseudomonas cepacia* (Goldstein et al. 1994). However, sulphuric and nitric acids producing PBMs, i.e., *Thiobacillus* and *Nitrosomonas* species were also reported to solubilize phosphate compounds (Azam and Memon 1996).

HPLC (high-performance liquid chromatography) and enzymatic methods are mostly employed for the detection of organic acids produced by PSM (Whitelaw 2000). Mehta et al. (2013a) detected six different organic acids in culture filtrate of

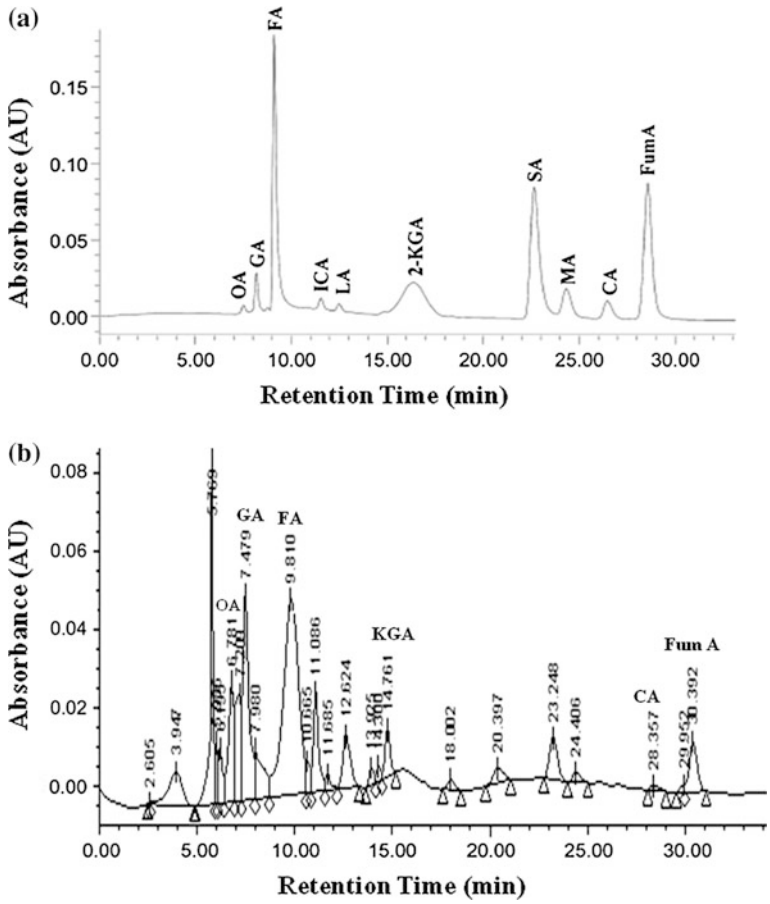

Fig. 4.2 HPLC chromatograms of authentic organic acids (**a**) and culture supernatant of *Bacillus subtilis* CB_8A grown for 3 days in PVK broth (**b**). Adopted from Mehta et al. (2013a). *OA* Oxalic acid; *GA* gluconic acid; *FA* formic acid; *ICA* isocitric acid; *LA* lactic acid; *2-KGA* 2-ketogluconic acid; *SA* succinic acid; *MA* maleic acid; *CA* citric acid; and *FumA* fumaric acid

Bacillus subtilis CB_8A by HPLC (Fig. 4.2). Six organic acids produced by *Bacillus subtilis* CB_8A are oxalic acid, gluconic acid, formic acid, 2-ketogluconic acid, citric acid, and fumaric acid. Out of these, major organic acids were gluconic acid (1.43%) and citric acid (0.67%) (Fig. 4.2). The reason of P solubilization by organic acid production may be: decrease in the pH; complex formation with metal ions of insoluble P (calcium phosphate, iron phosphate) and finally, P release; by competing with P for sites on the soil.

4.3.1.3 Important Facts of P-Solubilization by Organic Acid Production

i. Organic acids responsible for P-solubilization are the microbial metabolic product such as the product of fermentation of organic carbon sources (e.g., glucose) or oxidative respiration (Trolove et al. 2003).
ii. There is release of organic acids from the outer face of cytoplasmic membrane of P-solubilizing microorganisms which is the site of direct oxidation pathway. This organic acid release into the medium result in a decrease in pH (Zaidi et al. 2009b).
iii. PSM strains acidify the surrounding environment by synthesis and discharge of organic acid. Organic acids have the ability to chelate cations, i.e., Al, Ca, and Fe linked with P or they can result in exchange of acid anion with phosphate anion (Omar 1998).
iv. According to the abiotic study of Whitelaw et al. (1999), it was proved that HCl and gluconic acid can solubilize P. On the basis of above fact, solubilization of colloidal Al phosphate might be due to chelation of Al^{3+} by gluconic acid.
v. There is the presence of soluble inorganic phosphate i.e. H_2PO_4 at low pH. However, divalent and trivalent inorganic phosphate, i.e., HPO_4^{-2} and HPO_4^{-3} arise with the increase in soil pH.

However, acidification does not appear to be the main system of solubilization, as the capacity to decrease the pH at times did not associate with the ability to solubilize mineral P (Subba Rao 1982). The phosphate-solubilizing activity was ascribed both to reduction and to chelation processes.

4.3.1.4 Excretion of Proton

One of the major aspects responsible for P solubilization is pumping out of protons from cell (Krishnaraj et al. 1998). Some microorganisms release proton during NH^{4+} assimilation as the sole mechanism to promote P solubilization (Parks et al. 1990). Illmer and Schinner (1995) reported the absence of organic acids in culture solution by HPLC during P-solubilization by *Pseudomonas* sp. They also reported the probable reason of P-solubilization in culture solution, i.e., release of protons accompanying NH^{4+} assimilation or respiration. Participation of H^+ pump mechanism in P solubilization is also reported in *Penicillium rugulosum* (Reyes et al. 1999). Different mechanisms of proton release have been followed by different species. However, for P solubilization, only a few depends upon the presence of NH^{4+} ion (Carrillo et al. 2002).

4.3.1.5 Role of Siderophores and Exopolysaccharides in P-Solubilization

Siderophores are small, iron chelating molecules that bind with ferric ion and transport it to a cell. As, ligand exchange by organic acid anion is not a dominant P-solubilizing mechanism as compared to mineral dissolution. On the basis of this fact, the role of siderophores in enhancing P-solubilization is considered (Parker et al. 2005). There are various reports in the literature regarding the release of siderophores from PSM (Vassilev et al. 2006; Hamdali et al. 2008).

Microbial exopolysaccharides may play role in P-solubilization. Exopolysaccharides, secreted outside the cell by bacteria and fungi are mainly carbohydrate polymers. They are of different types, i.e., homo polysaccharides and heteropolysaccharides and may additionally contain a number of extraordinary organic and inorganic substituents. The role of microbial polysaccharides in P solubilization has been assessed by Yi et al. (2008). They reported significant production of EPS by highly efficient P-solubilizing bacteria, i.e., *Arthrobacter* sp. (ArHy-505), *Azotobacter* sp. (AzHy-510), *Enterobacter* sp. (EnHy-401), and *Enterobacter* sp. (EnHy-402).

4.3.1.6 Other Mechanisms

It has been suggested that processes such as sulphur oxidation, carbon monoxide, and nitrate production result in the formation of inorganic acids like sulphuric acid are a consequence of microbial phosphate solubilization (Swabyand Sperber 1958). The reaction between H_2S and ferric phosphate result in the formation of ferrous sulphate along with the simultaneous release of phosphate. So, production of H_2S can be one of the P-solubilization mechanisms.

4.3.1.7 Genetic Basis of Inorganic P-Solubilization

One of the major mechanism of P-solubilization is the production of organic acids, i.e., MPS. Therefore, understanding of the genetics behind MPS phenotype is necessary (Goldstein and Liu 1987). This assumption has been supported by cloning of PQQ gene responsible for gluconic acid production.

Pyrroloquinoline Quinone (PQQ) [(4,5-dihydro-4,5-dioxo-1H-pyrrolo-[2,3-] quinoline-2,7,9 tricarboxylic acid), aromatic, tricyclic ortho-quinone], belongs to the family of quinone cofactors. It serves as the redox cofactor for several bacterial dehydrogenases such as methanol dehydrogenase and glucose dehydrogenase (Fig. 4.3). PQQ-dependent glucose dehydrogenase (GDH) resides in the cytoplasmic membrane, can oxidize glucose to gluconate GDH, which needs PQQ for the holoenzyme. PQQ is derived from tyrosine and glutamic acid. It is characterized as a third class of redox cofactors following pyridine nucleotide and flavin-dependent cofactors (Houck et al. 1991).

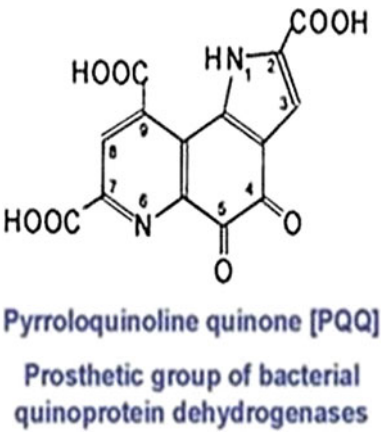

Fig. 4.3 Prosthetic group of bacterial quinoprotein dehydrogenases

Glucose dehydrogenase (GDH) Glucose dehydrogenase (GDH) is a quinoproteins which has the ability to oxidize glucose into gluconic acid. During catalytic reaction, GDH needs pyrroloquinoline quinone (PQQ) and metal ions such as Ca^{+2} or Mg^{+2} (in vitro). Membrane GDHs (m-GDHs) are monomeric proteins of 88 kDa with an N-terminal hydrophobic and large conserved PQQ-binding C-terminal domains. This C-terminal domain has catalytic activity (Yamada et al. 1994). However, N-terminal hydrophobic domain (residues 1–150) anchors the protein to the membrane. It consists of five trans-membrane segments which play a major role in anchoring the protein (Yamada et al. 1994). GDH plays a regulatory key in bioenergetic role in the bacteria. Uptake of exogenous compounds such as amino acids is due to trans-membrane proton motive force (PMF). Protons produced during oxidation participate directly in the generation of trans-membrane proton motive force (PMF). Therefore, this oxidative glucose pathway might be important for the survival of bacteria.

Very little is known regarding genetic or biochemical mechanisms involved in the synthesis of the GDH-PQQ halo enzyme. The possible inducers of halo enzyme are manitol, glucose, gluconate, and manitol (Van Schie et al. 1987). However, among several bacterial species, the difference in their constitutive and inducible phenotypes is observed (Goldstein 1994).

4.3.2 Genetic Diversity and Role of Genetic Engineering in P-Solubilization

4.3.2.1 Genetic Diversity of Phosphate-Solubilizing Microorganisms

Rhizosphere comprises of a huge microbial population of bacteria, fungi, protozoa, and algae. Bacteria are the most copious among them. The selection and

colonization of bacteria with in plant is based on their contribution to the fitness by releasing organic compounds through exudates (Lynch 1990), and therefore low diversity, selective environment is created (García et al. 2001). Since bacteria profusely colonize the rhizosphere and to the greater extent influence plants physiology, mainly taking into consideration of their competitiveness in root colonization (Antoun and Kloepper 2001).

The genotypic and phenotypic characteristic analysis of indigenous rhizobacteria can elucidate the mechanisms of interaction between them and plant roots. Studies on bacterial diversity are much more complex at taxonomic, functional and genetic levels in comparison to eukaryotes owing to the minute working scale and a large number of different bacterial species present in the environment.

Molecular basis behind phosphate solubilization by microorganisms is still limited and inconclusive (Rodriguez et al. 2006). Complete study of genes involved in P-solubilization and development of genetically engineered microbes is important not only for understanding their ecological role in the natural environment but also for their biotechnological application. As far as soil health is concerned exhaustive efforts are being made to explore indigenous soil microbial diversity with nutrient acquisition and mobilization potential with a special understanding of their distribution and behavior in soil habitats as well their influence on the quality of plant and soil health after introducing them as bioinoculants (Kumar et al. 2015).

A substantial number of phosphate-solubilizing culturable bacterial communities apart from genera *Pseudomonads* and *Bacilli*, there are some efficient P-solubilizing fungi that do not lose the P dissolving capacity even on repeated sub culturing under laboratory conditions as it occurs with most of the P-solubilizing bacteria (Kucey 1983). Generally, the release of organic acids by P-solubilizing fungi than bacteria consequently exhibit greater P-solubilizing activity. Among filamentous fungi that solubilize phosphate more efficiently belongs to genera *Aspergillus* and *Penicillium* (Reyes et al. 2002) although strains of *Trichoderma* and *Rhizoctonia solani* (Jacobs et al. 2002) have also been reported as good P solubilizers. Very few studies have been conducted in case of yeast to gauge their phosphate-solubilizing ability, these include *Yarrowiali polytica*, *Schizosaccharomyces pombe* and *Pichia fermentans* (Vassilev et al. 2001).

4.3.2.2 Genetic Engineering of PSM

High agricultural yield depends upon plant growth and nowadays it is achieved by employing high cost as well as environmentally hazardous phosphate fertilizers. To overcome this, an ecofriendly approach is to develop bacterial strains that can convert the form of phosphorus present in the soil to soluble forms which can be easily taken up by plants. Various attempts for developing such strains were made in past but failed due to incomplete knowledge of the phosphate-solubilizing genes, as well as the failure of the survival of bacterial strains under plant root environment. To deal with these challenges, this is desired to discover novel genes and pathways underlying solubilization of phosphorus sources which can be done by

the use of genome wise mutagenesis of phosphate-solubilizing bacteria. Validation of such novel genes and functions in *E. coli* is possible through advanced synthetic biological approaches which ultimately transfer novel phosphate-solubilizing capabilities associated with plant rhizosphere bacterium.

Several genes are isolated and characterized which are involved in mineral and organic phosphate solubilization. Cloning and expression of such genes in selected rhizobacterial strains through molecular biotechnology and genetic engineering have made a promising perception for obtaining recombinant strains with improved phosphate-solubilizing capability for agricultural purpose. Insertion of phosphate-solubilizing genes into microorganisms that lack P-solubilization trait may avoid the current need of using more than one strain of PGPR or consortia, when used as bioinoculants. The foremost success in cloning of a gene involved in mineral P solubilization in Gram-negative bacteria *Erwinia herbicola* was first time done by Goldstein and Liu (1987). The expression of this gene allowed the phosphate solubilization activity in *E. coli* HB101. *E. coli* can synthesize GDH, but not PQQ, thus it does not produce GA. This gene contributed in the synthesis of enzyme pyrrolo quinoline quinone (PQQ) synthase which was investigated through sequence analysis. For the synthesis of holoenzyme glucose dehydrogenase (GDH)-PQQ, PQQ is required which is a cofactor whose synthesis is directed by the enzyme pyrrolo quinoline quinone (PQQ) synthase. Formation of gluconic acid from glucose through direct oxidation pathway is catalyzed by glucose dehydrogenase (GDH)-PQQ. Sub cloning of the specific gene encoding mineral phosphate solubilization was done in a broad host range vector (pKT230). The recombinant plasmid expressed in *E. coli*, and further transferred to plant growth-promoting strains of *Burkholderia cepacia* and *Pseudomonas aeruginosa*, using tri-parental conjugation.

4.3.2.3 Mineral Phosphate-Solubilizing Genes for Strain Improvement

Genetic background, presence of number of copies of plasmids as well as metabolic interaction of recipient strains could highly influence the expression of an MPS gene in a different host. Thus, genetic transfer of any isolated gene involved in MPS to stimulate phosphate-mobilizing aptitude in PGPB strains, is an attention-grabbing approach.

Kim et al. (1998) reported the expression of MPS genes isolated from *Ranella aquatilis* which when cloned in *E. coli* boost a high-level production of gluconic acid (GA) and hydroxyapatite dissolution as compared to donor strain. It was suggested that different genetic regulation of the MPS genes might occur in both species. In another case study, an increase in exudation of organic acids as well as phosphate availability to plants was observed by the expression of bacterial citrate synthase gene when expressed in tobacco roots. More yield of leaf and fruit biomass was observed in citrate overproducing plants when grown under phosphate limiting conditions along with low P-fertilizer doze which depicted the putative role of organic acid synthesis genes in P uptake in plants.

Table 4.2 Microorganisms encoding phosphatase genes for P-solubilization

Microorganisms	Gene or plasmid	Features	References
Serratia marcesence	pKG3791	Produce gluconic acid and solubilizes P	Krishnaraj and Goldstein (2001)
Rahnella aquatilis	pKIM10	Solubilize P and produce gluconic acid in *E. coli* DH5α	Kim et al. (1998)
Enterobacter agglomerans	pKKY	Solubilize P in *E. coli* 109, does not lower pH	Kim et al. (1997)
Pseudomonas cepacia	Gab Y	Solubilize P and produce gluconic acid in *E. coli* JM 109	Babu-Khan et al. (1995)
Erwinia herbicola	*Mps*	Solubilize P and produce gluconic acid in *E. coli* HB 101, probably involve in synthesis of PQQ	Goldstein and Liu (1987)
Bacillus subtilis CB_8A	*Gdh*	Solubilise P and produce gluconic acid	Mehta et al. (2013c)

Most of the bacterial phosphatase-encoding genes were isolated by means of expression cloning systems entirely based on histochemical based screening of genomic libraries (Table 4.2). These procedures not only allow quick recognition of clones harboring, but also the expression of enzymatic activity.

Riccio et al. (1997) developed a selection system based upon indicator medium consisted of phosphatase substrate phenolphthalein diphosphate (PDP) and methyl green (MG) stain, resulted in green putative colonies with phosphatase positive phenotype (pho1) whereas, phosphatase negative (pho2) clones were grown as unstained colonies. This system offers an imperative approach for the isolation of several bacterial phosphatase-encoding genes from different species, such as *Providencia sturatii, Providencia rettgeri* and *Morganella morganii*.

Another important system for the expression of cloning of bacterial phosphatase-encoding genes (*phoC*) used by Pond et al. (1989) consists of Luria Agar amended with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) which was used for cloning of an acid phosphatase-encoding gene from *Zymomonas mobilis.* The transformant colonies were of dark blue which makes its easy direct selection on indicator plates.

Groisman et al. (1984) cloned the structural gene for the pH 2.5 acid phosphatase (*appA*) of *E. coli* for direct amplification of higher para-nitrophenyl-phosphate (pNPP) hydrolysis (phosphatase activity) responsible genes as a result acid phosphate colonies appeared yellow. Thaller et al. (1994) classified a non-specific phosphohydrolases into three different families: class A, class B, and class C phosphatases based on the cloning of phosphatase genes sequence analysis with other important parameters. Rossolini et al. (1998) studied the sequence level high homology in case of class A phosphatase genes from *M. morganii* and *P. stuartii*, which signifies that these genes are vertically derived from a common ancestor. A number of other phosphatase genes from *Escherichia coli* include: *ushA*, which encodes a 59-nucleotidase (Burns and Beacham 1986) *agp*, which encodes an acid

glucose-1-phosphatase (Pradel and Boquet 1988) and *cpdB*, encoding the 29–39 cyclic phosphodiesterase (Beacham and Garrett 1980).

Sharma et al. (2013) had suggested the application of genetically modified PSM as a potential candidate mover transgenic plants for improving plant performance: (1) with current technologies, a bacterium is much more easier to modify than complex higher organisms, (2) Multiple beneficial plant growth-promoting attributes can be introduced into a single organism, which could minimize the application of multi-strain bio-inoculant (3) Instead the engineering of crop by crop, a single, engineered inoculant can be used for several crops, especially when using a non-specific genus like *Azospirillum* (Rodriguez et al. 2006).

Gene recombination though an important conclusive approach but there are some barriers that needs be resolved first in order to achieve success, such as difference at the metabolic level and regulatory mechanisms between the donor and recipient strains. Despite many constraints and difficulties, significant and consistent progress are being done step by step in this field of molecular biology by genetically engineered microorganisms for sustainable and improved agriculture (Armarger 2002). On the whole, further advance studies on this aspect of PSM will provide key information in future for the better use of these PSM in diverse ecological conditions.

4.4 Phosphate-Solubilizing Bacteria as Plant Growth Promoters

P-solubilizers colonize plant roots and employ valuable effects on growth of plant and enhancement by a prevalent mechanism. To be an efficacious P-solubilizer, microorganisms need to set up itself in the rhizosphere at concentrations adequate to deliver the beneficial impacts. In this way, plant inoculation by P-solubilizer microorganism at a much higher rate than that regularly present in soil is important to exploit the property of phosphate solubilization for plant yield enhancement. There have been various reports on plant development and enhancement by microorganisms that can solubilize inorganic and/or natural P from soil after their inoculation in soil or plant seeds (Mehta et al. 2011; Kumar et al. 2015). The exact mechanism by which P-solubilizer stimulate plant growth is not clearly recognized, although several assumptions such as production of phytohormones, i.e., indoleacetic acid production, activation of P-solubilization, siderophore production, suppression of deleterious organisms, and promotion of the mineral nutrient uptake are usually accepted to be involved (Kumar et al. 2012; Walia et al. 2013b; Mehta et al. 2013a, b, c).

The P-solubilization capacity of the microorganisms is considered to be one of the most essential traits related with plant P-nutrition (Walia and Shirkot 2012). These PSMs render insoluble phosphate into available forms by the process of acidification, chelation, and exchange reaction (Pankaj and Sa 2008). This method

not only compensates the higher cost of industrial fertilizers but also mobilizes the fertilizers supplemented to the soil. In any case, at present, there is proof supporting the part of this component in plant development upgrade. For instance, a few soil microorganisms, including microbes, enhance the supply of P to plants as a result of their ability for inorganic or natural P solubilization (Lifshitz et al. 1987; Richardson 1994; Mehta et al. 2011). Considering that P accessibility is a restricting progress in plant sustenance, this confirmation proposes a basic assurance of phosphate-solubilizing microorganisms to plant nourishment and, consequently increase the performance of plant growth development. Mehta et al. (2013a, b) and Sharma et al. (2015) exhibited plant growth development of apple and tomato by a few microorganisms fit for mineral phosphate solubilization. There are so many strains indicating no indoleacetic acid production, however showing critical mineral phosphate solubilization and adequate movement of phosphatase has enhanced the yield of tomato, cauliflower, capsicum, apple, apricot, etc., among different cultivars, in field experiments.

Besides, a few illustrations of synchronous development and expansion in P uptake by plants as the consequence of phosphate-solubilizing microbial inoculations have been accounted for. Inoculation with two strains of P-solubilizers, i.e., *Rhizobium leguminosarum* has been showed to enhance root colonization and development advancement and to increase essentially the P application in tomato and apricot (Mehta et al. 2013c; Chauhan et al. 2014; Guleria et al. 2014a, b). Chabot et al. (1996) presumed that the P-solubilization impact of Rhizobia and other PSMs is by all accounts the most vital system of plant development advancement in reasonably rich and extremely fruitful soils. Then again, a strain of *Pseudomonas putida* too strengthened the development of roots and shoots and expanded 32P-named phosphate uptake in canola (Lifshitz et al. 1987). Inoculation of rice seeds with *Azospirillum lipoferum* strain 34H and tomato plants with *Bacillus subtilis* strain CKT1 expanded the phosphate particle content and brought about a huge change of root and shoot length and dry weights (Murty et al. 1988; Walia et al. 2013a). Concurrent expansions in P uptake and harvest yields have likewise been seen after inoculation with *Bacillus methylotrophicus* CKAM (Mehta et al. 2014), *Bacillus polymyxa* (Gaur and Ostwal 1972), *Bacillus subtilis* (Sharma et al. 2015), *Bacillus subtilis* CKT1 (Walia et al. 2013a) and *Bacillus circulans* (Mehta et al. 2013c), and others.

Another approach for the utilization of PSMs as microbial inoculants is the utilization of mixed or co-inoculation with different microbes. A few studies exhibit the useful impact of consolidated inoculation of P-solubilizing microbes and *Azotobacter* on yield, and in addition to nitrogen (N) and P accumulation in various crops (Kundu and Gaur 1984). Co-inoculation of *Pseudomonas striata* and *Bacillus polymyxa* strains demonstrating phosphate-solubilizing capacity, with a strain of *Azospirillum brasilense*, brought about a noteworthy change of grain and dry matter yields, with an increase in N and P uptake (Alagawadi and Gaur 1992). Likewise, phosphate-solubilizing *Agrobacterium radiobacte*r coinoculated with nitrogen fixer *Azospirillum lipoferum* showed enhanced grain yield as contrasted to single inoculations in pot and field tests (Belimov et al. 1995). These authors explained that

mixed inoculants gave more adjusted sustenance to the plants, and that the change in N and P uptake was the real mechanism involved. This proof focuses to the upside of the mixed inoculations of PGPR strains including PSMs.

Then again, it has been proposed that some PSMs act as mycorrhizal assistant microbes (Garbaye 1994). In such manner, a few studies have demonstrated that PSMs cooperate with vesicular arbuscular mycorrhizae (VAM) by discharging phosphate particles in the soil, which causes a synergistic connection that takes into consideration better use of ineffectively solvent P sources (Ray et al. 1981). It is likely that the phosphate solubilized by the microbes could be all the more effectively taken up by the plant through a mycorrhizae-intervened span in the middle of roots and encompassing soil that permits supplement translocation from soil to plants (Jeffries and Barea 1994). These authors concluded that the inoculated rhizobacteria could have released phosphate particles from insoluble rock phosphate and other P sources, and were then taken up by the outer VAM mycelium. Commercial biofertilizers affirming to experience phosphate solubilization utilizing mixed bacterial cultures have been produced. Extensive confirmation boosts the particular part of phosphate solubilization in the improvement of plant development by phosphate-solubilizing microorganisms. In any case, not all research center or field trials have offered positive results. For instance, an inoculant utilizing *Bacillus megaterium* var. phosphoricum, was used effectively in the previous Soviet Union and India, yet it did not demonstrate the same effectiveness in soils in the United States (Smith et al. 1962). Also, there are some deleterious species of bacteria present in the rhizosphere that have the potential to influence seed germination, plant growth, and crop yields significantly. These bacteria affect the plant growth through production of phytotoxins (Kumar et al. 2013a, b). Remarkably, in the study conducted by Walia et al. (2013a), a few isolates were found to significantly inhibit seed germination as demonstrated by a reduction in per cent of seed germination over uninoculated control, apparently by producing volatile metabolites. When studied, these deleterious bacterial isolates showed no HCN activity in vitro. Therefore, it is probable that some other gaseous metabolites produced by the bacteria under these conditions have repressed seed germination. This statement is supported by the increase in per cent seed germination by isolate N_{11} which otherwise produced HCN under in vitro conditions (Walia et al. 2013a, b; Alstrom and Burns 1989). Without a doubt, the productivity of the inoculation changes with the soil type, particular cultivar, and different parameters. The P substance of the soil is likely one of the critical elements in deciding the viability of the item.

4.4.1 Production of Phosphate-Solubilizing Microorganism Inoculants

Effective PSM cultures are mass-produced for supply to the agriculturists as microphos. The generation of microphos, i.e., a preparation containing

microorganisms with phosphate-solubilizing action, incorporates three stages: the main concerns choice and testing of phosphate-solubilizing strains; also, inoculant readiness, including determination and handling of the material carrier and mass culture of PSM; and thirdly, quality control methodology and dispersal. For microphos generation, peat, farmyard compost (FYM), soil and dairy animals waste cake powder have been recommended as suitable carriers (Kundu and Gaur 1981). For storage of cultures, these are packed in polybags for around three months at 30 ± 2 °C. In India, a microbial development termed Indian Agricultural Research Institute (IARI) microphos society (Gaur 1990; Khan et al. 2014b) was formed that contained two proficient phosphate-solubilizing microscopic organisms (*Pseudomonas striata* and *Bacillus polymyxa*) and three phosphate-solubilizing growths (*Aspergillus awamori*, *A. niger* and *Penicillium digitatum*).

4.4.2 Technology of Bioinoculants Production

Advancement of an effective inoculant includes a few basic components, for example, strain determination, choice of a carrier, mass duplication, detailing of the inoculant, and bundling and promoting. Stringent quality certification at different strides of generation guarantees the creation of reliably excellent inoculants. By and large, not long after the microbes are brought into the soil, the bacterial populace decays logically (Van Elsas et al. 1986; Bashan and Levanony 1988). This wonder might keep the development of an adequately vast microbial populace in the rhizosphere to acquire the expected plant reaction. The key snag is that the soil is a heterogeneous and flighty environment, even on the little scale (Van Elsas and Van Overbeek 1993). The inoculated microorganisms must contend with the frequently better adjusted local microflora. A noteworthy part of inoculant plan is to give a more suitable microenvironment to keep the fast decay of presented microorganisms in the soil. Although quite a bit of it is thought about the survival of microorganisms inside of the defensive environment of an inoculant transporter, little is thought about the burdens that microorganisms must persist upon exchange to the aggressive and regularly cruel soil environment (Rodriguez-Navarro et al. 1991; Heijnen et al. 1992). Inoculants must be intended to give a reliable wellspring of advantageous microorganisms that make due in the soil and get to be accessible to the plant. The assembling of bioinoculants requires four noteworthy steps (a) Selection of effective strain, (b) Mass culture, (c) Carrier materials and their handling and (d) Packaging, which are to be prepared after strictly ensure the quality of a production item.

4.4.2.1 Inoculant Formulation Technology for P-Solubilizers

Formulation is an urgent perspective for producing inoculants containing a compelling bacterial strain that can decide the achievement or disappointment of

organic workers. Formulation normally comprises of setting up the dynamic fixing, i.e., microorganism (s) in a suitable carrier together with added substances that guide in the adjustment and insurance of the microbial cells amid capacity and transport at the objective site. The formulation is difficult to protect after applying in the fields from destructive ecological components, and keep up or upgrade movement of the living beings in the field (Jones and Burges 1998). Another critical thought is the cost-viability of the plan.

To encourage the performance of high cell numbers and build survival of microorganisms in soil, diverse plans utilizing carrier materials have been utilized. The issue of value inoculant production relies on upon utilization of good carrier material in biofertilizer production unit. The carrier is the conveyance vehicle of live microorganisms from the production line to the field; nonetheless, no widespread carrier or plan is accessible for the arrival of microorganisms into the soil (Trevors et al. 1992). Carrier materials might act to improve survival of inocula by giving microorganisms a defensive domain keeping in mind the end goal to escape unfavorable conditions in the soil. The explanations behind a reduction in inoculum populace in the soil after some time incorporate inadequate supplements accessible for upkeep and replication, and imperfect ecological conditions, for example, pH, ionic quality, temperature and so forth (Van Elsas and Van Overbeek 1993). Predation by bacteriovorus microorganisms, for example, protozoa, and rivalry with indigenous microbes can likewise diminish inoculum application.

To be effective, a carrier material must upgrade survival of inocula amid capacity and after performance into the soil. The carrier must show two crucial properties, i.e., it must be backing the development of the objective produce and keep up a sought populace of inoculant over an adequate time period. To accomplish these objectives, a carrier should likewise show high water-holding limit and maintenance attributes, show compound and physical consistency and be nonlethal to inoculant strains and earth safe (Stephens and Rask 2000). Extra attributes for a decent inoculant should be as per the following:

1. The inoculants should be almost sterile or effectively cleaned, and as artificially and physically uniform as could be expected under the circumstances.
2. They must have steady quality, high water-holding limit (for wet transporters) and suitable for whatever number bacterial species and strains as could be allowed.
3. The inoculant must have an effectively movable pH, and be made of a sensibly valued crude material in satisfactory supply.
4. The inoculant must be nontoxic, biodegradable and nonpolluting, and have to minimize ecological dangers, for example, the dispersal of cells to the climate or to the ground water.
5. The inoculant must have an adequate time frame of realistic usability maybe a couple of years at room temperature.

Normally, no single carrier can have every one of these qualities, yet a decent one should have however many as could be expected under the circumstances.

4.4.2.2 Types of Carriers for P-Solubilizer Inoculants

The most useful carrier for inoculants are (i) Soils: peat, coal, soils, and inorganic soil (Smith 1995). (ii) Plant waste materials: fertilizers, barnyard compost, soybean and shelled nut oil, wheat grain, press-mud, spent mushroom manure. (iii) Inert materials: vermiculite, perlite, ground rock phosphate, calcium sulfate. These arrangements can later be fused into a strong carrier or utilized as they may be.

To produce an inoculant, the objective microorganism can be brought into a sterile carrier. From an absolutely microbiological perspective, the clean carrier has huge preferences yet from a commercial point of view, it is very costly to produce sterile carrier. In any case, sterile-originated inoculants have been effectively advertised even with their higher sticker price. But the less expensive non-sterile carriers, regardless of their potential burdens, have a much bigger market in the business sector (Olsen et al. 1994). The formulation is the key issue for inoculants containing a viable bacterial strain and can decide the achievement or failure of a biological agent.

Inoculants come in four essential dispersal frames. Powder form is utilized as a seed covering before planting. The little the molecule estimate, the better the inoculant will stick to the seeds. Standard sizes differ from 0.075 to 0.25 mm, and the measure of inoculant utilized is around 200–300 g/ha. These inoculants are the most well known both in developed and developing nations (Tang and Yang 1997). Slurries depend on powder-sort inoculants suspended in a fluid (typically water). The suspension is straightforwardly connected to the furrow or on the other hand, the seeds are plunged only preceding sowing. Granular form inoculants are connected straightforwardly to the furrow together with the seeds.

4.4.3 Applications of Endophytic P-Solubilizers in Agriculture and Response of Crops to Bioinoculants

High quality of planting material is a basic requirement for the achievement of any cultivation wander. To guarantee the nature of the planting materials, a successful production and assurance framework is of principal significance. Endophytic bacterial species can be conveyed stem or established cuttings of green plants. Such a conveyance system for endophytic microbes during ahead of schedule phase of its improvement would guarantee better establishing of the planting material. A few techniques for the conveyance of endophytic microorganisms are accounted for which incorporates seed treatment, bacterization of plant spread material, soil application and even foliar application. For vegetatively spread plant species, endophytic microorganisms can be specifically conveyed into the succulent plant framework before the planting in the soil (Panhwar et al. 2013). In these plants, shoots are amiable for bacterization by endophytic microorganisms. Endophytic

microbes from tomato, apricot, apple, seabuckthorn, and *Podophyllum hexandrum* (medicinal plant) illustrative of the overwhelmingly viewed genera *Bacillus, Pseudomonas*, *Enterobacter* and *Serratia* were tried for their abilities to enhance establishing of their host plant (Mehta et al. 2014; Kumar et al. 2015; Sharma et al. 2016). After endophytic inoculation and resulting development in soil, we saw that the root structures of inoculated apple cuttings were frequently denser with numerous fine attaches contrasted with those of the noninoculated control plants. Root arrangement was moderate for noninoculated plants. Interestingly, for cuttings that were permitted to establish in the vicinity of the chose endophytes, root development was started inside of 1 week, and shoot arrangement was more declared contrasted with that of the noninoculated plants.

The use of P-solubilizers is rapidly increasing in agriculture and horticulture and offers a finest way to replace chemical fertilizers and pesticides (Zaidi et al. 2014; Ahemad 2015). Earlier, Walia et al. (2013a) had isolated and characterized different P-solubilizers from the rhizosphere soils/roots of tomato having multiple plant growth-promoting traits (PGPTs). For the testing of effective P-solubilizers, a pot culture experiment was conducted where they reported a significant increase in shoot length, root length and dry matter production of shoot and root of tomato seedlings. Among seven P-solubilizers, strain CKT1 exhibited concomitant production of PGPTs, i.e., siderophore production, indoleacetic acid production, nitrogen fixation activity, and hydrogen cyanide production. Significant increase was observed in seed germination (36.08%), shoot length (5.22%), root length (21.12%), shoot dry weight (63.50%) and root dry weight (54.08%), nitrogen (18.75%), potassium (57.69%) and phosphorus (22.22%) as compared to uninoculated control. This study, therefore, suggests that the use of single strain inoculum of CKT1 with multiple PGPTs offers a new concept to address multiple modes of action.

In an another study by Mehta et al. (2013) endophytic P-solubilizing bacterial isolate *Bacillus circulans* CB7 isolated from apple rhizosphere soil of Himachal Pradesh, India exhibited PGPTs of auxin, nitrogenase activity, ACC deaminase activity, siderophore production, and antifungal activity against *Dematophora necatrix*. In vivostudies showed remarkable increase in seed germination (22.32%), shoot length (15.91%), root length (25.10%), shoot dry weight (52.92%) and root dry weight (31.4%). Also, the nutrient uptake by plants, i.e., nitrogen (18.75%), potassium (57.69%) and phosphorus (22.22%) was increased in shoot biomass. These results exhibited strongly that isolate CB7 has the favorable PGPR traits to be developed as a biofertilizer to boost soil fertility and enhance plant growth.

The synergistic effect of the combination of three PGPRs, *Bacillus licheniformis* CECT 5106, *Pseudomonas fluorescens* CECT 5398, and *Chryseobacterium balustinum* CECT 5399 with LS 213 on the growth promotion and biocontrol on tomato and pepper against *Fusarium* wilt and *Rhizoctonia* damping off was observed by Domenech et al. (2006). They concluded that when both rhizobacterium and strain LS213 were combined together to form an inoculum, the growth parameters were significantly higher than with individual rhizobacterium, in tomato and pepper, which revealed a synergistic and most effective effect on growth

promotion. Similarly, Pandey and Maheshwari (2006) studied the interaction for plant growth promoting comprising of two species i.e. *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3 which can produce IAA and solubilize inorganic phosphate. The consortium of two strains was tested on *Cajanus cajan* in sterile soil and their results revealed an increase in seedling length, yield and weight after inoculation with these species. A similar study was also conducted by Sharma et al. (2007) who isolated two phosphate-solubilizing strains namely *Pseudomonas fluorescens* and *Bacillus megaterium*. They coinoculated them into seeds of *Cicer arietinum* and observed that the consortium of two enhanced the seedling length, radical and plumule length.

Adesemoye et al. (2008) conducted a field study to test the effect of P-solubilizers microbial inoculants on corn plant growth, yield and nutrient uptake. The field results showed that inoculants promoted grain yields (kg/ha) 7717 for AMF (Arbuscular Mycorrhiza Fungi), 7260 for PGPR + AMF, 7313 for PGPR, 5725 for the control group and also enhanced nitrogen content per gram of grain tissues. Significantly higher amounts of N, P and K were taken up by microbes thus indicated the application of inoculants lead to a reduction in buildup of N, P, and K in agricultural soils which is measure of an integrated nutrient management system. Similarly, Yazdani et al. (2009) reported that use of PSM and PGPR in addition to conventional fertilizer applications (NPK) could improve root and shoot weight, and grain number per row and finally increased grain yield of *Zea mays* L. They concluded that application of PSM and PGPR together could reduce P application by 50% without any significant reduction of grain yield. PGPR can enhance plant growth by alleviating soil stresses experimentally observed by Mehta et al. (2013a). They hypothesized that the isolated strains of *Azospirillum* sp. and *Bacillus subtilis* CB_8A may alleviate the adverse effects of drought stress on wheat and apple growth.

4.5 Conclusion and Future Prospects

In intensive agricultural practices, the application of phosphatic fertilizer requires a greater input that cannot be afforded by the farmers furthermore due to impending impacts to the biological system. Keeping this in perspective, numerous researchers have occupied their examination in finding the shrouded treasure under the soil and thus, rhizosphere competent bacteria (RCB) or endophytic P-solubilizers came into light and gained interest as inoculants or economically efficient substitute for fertilization of crops by solubilization of phosphate from inadequately accessible sources in the soil. The characteristic state of plants is by all accounts in a nearby interaction with endophytes. In the endophyte–host communications, the base commitment of the plant to the endophyte is one of giving nutrition. Endophytic microorganisms are the rich wellspring of an extensive variety of bioactive mixes, bringing about the generation of each of the five classes plant development hormones (auxins, abscisins, ethylene, gibberellins, and kinetins). The accomplishment

of this microbiological approach, in any case, relies on upon identification, preparation and delivery of multifunctional endophytic phosphate solubilizers to farm practitioners. This would be amiable when a superior learning on endophyte environment and their molecular associations is achieved. Once recognized and physiologically portrayed, phosphate-solubilizing microorganisms are liable to give advantages to crops in sustainable agriculture. Further, keeping in mind the end goal to guarantee food security in developing nations, there is a dire requirement for the eco-friendly sustainable intensification of farming production systems. In this context, efficient indigenous or genetically modified region or crop specific endophytic PSM and advancements for their definitive exchange to the fields must be produced and delivered to farmers in a relatively brief time.

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Chapter 5
Endophytic Microbes: A Resource for Producing Extracellular Enzymes

Abdul Latif Khan, Raheem Shahzad, Ahmed Al-Harrasi and In-Jung Lee

Abstract Endophytes (fungi and bacteria) have been known to live asymptomatically with plants throughout the different growth and developmental stages. Endophytic microbes provide an additional resource to the plant due to the presence of beneficial secondary metabolites, enzymes, and nutrients, which help the host to combat diverse arrays of stressful conditions of biotic and abiotic stresses. Extracellular enzymes are the product of microbial's cell growth and perform its function outside the cell in many biological or environmental processes. In fact, certain category of enzymes namely, xylanases, hemicellulases, phytases, proteases, asparaginase, cellulases, pectinases, tyrosinase, gelatinase, chitinase, amylases, etc., are some of the key enzymes produced by endophytic bacteria and fungi. Most of these enzymes have been reported from endophytes living within medicinal or crop plants, whereas they are detected through agar-based methods. The current chapter aims to identify the sources, kinds of enzymes, and the perspectives for further studies in their application in endophytic-based extracellular enzymes resources.

Keywords Extracellular enzymes · Endophytes · Bacteria · Fungi
Enzymes quantification

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5.1 Introduction

Endophytic microorganisms (bacteria or fungi) are belonging to a key class of plant symbionts, living inside the plant tissues without causing any symptoms of disease to the host. These endophytic microorganisms are associated with the plant throughout their life history, starting from seed germination to fruit development. These can be distributed in rhizosphere (roots), phylloplane (in leaves), laimosphere caulosphere (in stems), carposphere (in fruits), spermosphere (in seeds), and anthosphere (in flowers) as suggested by Clay and Holah (1999). Various workers (Lindow and Brandl 2003; Saikkonen et al. 2004; Sessitsch et al. 2012) stated the role of endophytes, bacteria, and or fungi unique in their interactions with plants. They provide an alternative resource or facilitate the distribution or production of biologically active metabolites, such as enzymes, biofunctional chemicals, phytohormones, nutrient, and minerals (Schulz et al. 2002). On the other hand, the host plant provides a protective sanctuary to reproduce and nutrients to grow inside plant tissues without compromising its own growth resources (Khan et al. 2015).

Endophytic microorganisms have also been coined for their protective role to the host during biotic and abiotic stress conditions (Arachevaleta et al. 1989; Bacon 1993; White and Torres 2010; Leitão and Enguita 2016). These stresses include salinity, drought, temperature, heavy metal, and phytopathogenic infections. In most of the previous studies, this role of counteracting stress invasion to the plant cell has been revoked or reduced through the production of essential biochemical resources (Khan et al. 2015). Among these sources, phytohormones and extracellular enzymes are few which have been regarded the most important and significant for their association with plants. Though phytohormones are one of the recent phenomena that have been known from endophytes, however, extracellular enzymes or exozymes have mostly been emphasized due to their industrial importance in food, fermentation dye synthesis, and other biotechnological applications (Traving et al. 2015).

Fungi and bacteria produce various kinds of extracellular enzymes, which are hydrolases, lyasese, oxidoreductases, and transferases (Traving et al. 2015). These extracellular enzymes target various macromolecules such as carbohydrates, lignin, organic phosphate, proteins, and sugar-based polymers to breakdown into transportable product throughout the cells and to continue heterotopic metabolism (Sinsabaugh 1994; Boer et al. 2005; Strong and Claus 2011; Wingender et al. 2012). Hallmann et al. (1997) showed that endophyte-producing enzymes could help to initiate the host symbiosis process. Besides establishment of association with host, these also initiate action of extracellular hydrolyases to counteract plant pathogenic infection (Tan and Zou 2001; Leo et al. 2016). Since, the endophytic resources offer a new source of genes, enzymes, and secondary metabolites, therefore, we aimed to investigate in the current chapter broadening our understanding related to extracellular enzymes from endophytic origin.

5.2 Extracellular Enzymes Quantification

Extracellular enzymes have been qualitatively and quantitatively measured through various ways ranging from agar plate-based methods to sophisticated advanced spectrophotometric methods. The enzyme production ability of endophytic microbes has also been coined for their ability to grow and reproduce in a specific media. However, a detailed assessment of such abilities has been least known for endophytic microbes. Overall, the endophytic microbes are isolated from plants rigorous surface sterilization methods by using tween 80 weak acids, and sterilized distilled water. Alternatively, the bulk material of plant grown in a sterilized microbial media is used where endophytic microbes were isolated. Once the pure culture is maintained, the strains are identified through either 16S rRNA sequencing or internal transcribed spacer (ITS) region of bacteria and fungi, respectively, using PCR amplification, sanger sequencing, BLASTn, and finally a detailed phylogenetic analysis. The isolated endophytes are grown in specific growth media to detect the enzymes producing either on agar plate (initial screening) or detailed spectrophotometry methods (UV/VIS or fluorescence). Currently, the advances in fluorogenic substrate such as 4-methylumbelliferone (MUB) have also been used for this purpose largely for soil or marine enzyme analysis (Hoppe 1993; Wallenstein et al. 2008; Khan et al. 2016). However, more sensitive techniques have to be adopted not only to measure time course estimation and analysis quantification of these enzymes, but also such studies may be coupled with molecular and genomic work to validate the findings and processes (III and Allison 2015) (Fig. 5.1).

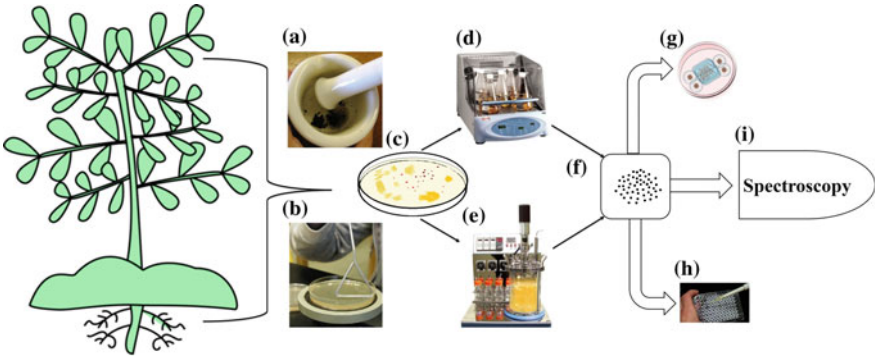

Fig. 5.1 Diagrammatic representation of the isolation, fermentation and quantification of extracellular enzymes. **a** Grinding plant, **b** isolating endophytes, **c**, **d** pure culture growth, **d** fermentation, **e**, **f**, **g**, **h** isolation of enzymes, and **i** quantification through advanced chromatographic techniques

5.3 Extracellular Enzymes from Endophytic Fungi

Endophytic fungi isolated from various plants sources have been reported for the production of various kinds of extracellular enzymes from last two decades or so (Khan et al. 2016; Esteves et al. 2014; Petrini et al. 1993). The categories of enzymes have been described in Table 5.1. Most of the endophytic fungi producing extracellular enzymes have been reported from medicinal plants (Chathurdevi et al. 2016). The endophytic fungi have been reported to be xylanase producers including *Alternaria alternate* (Wipusaree et al. 2011), *Hymenoscyphus ericae* (Burke and Cairney 1997), and *Aspergillus terreus* (Sorgatto et al. 2012). Similarly, Harnpicharnchai et al. (2009) showed that endophyte *Periconia* sp. produces β-glucosidase. De-Almeida et al. (2012) reported that endophytes of *Acremonium* species produce cellulases and hemicellulases. In another study, Suto et al. (2002) isolated and identified one hundred and fifty-five fungal strains showing their ability to produce xylanases. Silva et al. (2006) investigated the fungal strain isolated from *Annona* spp., while Luz et al. (2006) from *Passiflora edulis* to understand their potential for extracellular enzymes production.

Gazis and Chaverri (2010) isolated and identified various endophytic strains belonging to *Xylariaceae* and *Annulohypoxylon* sp. from a medicinal plant *Hevea brasiliensis*. Earlier, Wei et al. (1992) grown *A. stigyum* strain and found that this strain produces β-glucosidase with a very low level of cellulases. Some of the common endophytes such as *Alternaria* species have been isolated from eucalyptus plants such as *Eucalyptus globulus* (Lupo et al. 2001) and *Eucalyptus citriodora* (Kharwar et al. 2010). Strains of *A. alternata* are able to produce endopoly galactunorase (Isshiki et al. 1997) in the presence of pectin, and β-glucosidase in the presence of saccharose (Sáenz-de-Santamaria et al. 2016).

Among other common endophytic strains, *A. niger* was also found to produce extracellular enzymes, which is famous to produce an extensive range of extracellular glucohydrolases (β-glucosidase, pectinases, and xylanases; Ward et al. 2005). Such attributes are always helpful to fungus to colonize and propagate across different kinds of environments and plant hosts (Meijer et al. 2011). Chow and Ting (2015) reported that endophytic fungi belonging to *Colletrotrichum, Fusarium, Phoma,* and *Penicillium* species are producing l-Asparaginase in their pure culture isolated from anticancer medicinal plants.

A semiarid plant *Opuntia ficus-indica* was subjected to endophyte isolation, which resulted in the identification of 44 endophytic fungi (Bezerra et al. 2012). According to the screening assays for extracellular enzymes, *Aspergillus japonicus* and *Penicillium glandicola* have shown significant pectinolytic activity. In addition, the author showed that endophytes belonging to *Xylaria* sp. were showing significantly higher xylanase and cellulase activity. In a recent study by Khan et al. (2016), 18 different endophytic fungi isolated from the bark and leaf parts of the

Table 5.1 Enzyme production from different endophytic fungal species

Specie name	Enzyme produced	Detection method	References
Penicillium funiuclas, Trichoderma viride	Amylase, cellulose, protease, lipase	Agar plate base test	Chathurdevi et al. (2016)
Colletrotrichum, Fusarium, Phoma, Penicillium	l-Asparaginase	Pink zones on agar, Nesslerization	Chow and Ting (2015)
Aspergillus sp.,	Amylase	Agar medium	Jurynelliz et al. (2016)
Pochonia chlamydosporia	Protease	Spectrophotometer	Escudero et al. (2016)
Colletotrichum gloeosporioides	Protease, chitinase, amylase		Rabha et al. (2014)
Fusarium sp., *Chaetomium* sp., *Colletotrichum* sp., *Aspergillus flavus, Cylindrocephalum* sp., *Coniothyrium* sp., *Phoma* sp., *Aspegillus niger Colletotrichum* sp., *Mycelia sterilia* sp., *Aspergillus fumigatus Alternaria* sp., *Colletotrichum gleosporoides. Colletotrichum* sp., *Myrothecium* sp., *Fusaruim chlamydosporum. Xylaria* sp., *Fusicoccum* sp., *Mycelia sterilia* sp., *Aspergillus* sp., *Pestalotiopsis* sp., *Colletotrichum* sp., *Talaromyces emersonii, Pyllosticta* sp., *Pestalotiopsis* sp., *Discosia* sp., *Aspergillus* sp., *Mycelia streilia* sp., *Isaria* sp., *Xylaria* sp., *Phoma* sp., *Pestalotiopsis disseminate, Fusarium oxysporum, Paecilomyces variotii, Fusarium chlamydosporum, Acremonium implicatum, Nigrospora sphaerica*	Amylase, cellulase, laccase, lipase, pectinase, protease	Agar medium	Sunitha et al. (2013)

(continued)

Table 5.1 (continued)

Specie name	Enzyme produced	Detection method	References
Fusarium solani, Penicillium sp., *Mycelia sterilia* sp., *Phoma* sp., *Basidiomycetes* sp., *Colletotrichum falcatum, Phomopsis longicolla Fusarium oxysporum, Colletotrichum gleosporoides, Colletotrichum truncatum, Drechsclera* sp., *Cladosporium* sp., *Myrothecium* sp.			
Cladosporium sp., *Rhizoctonia* sp., *Aspergillus* sp., *Chaetomium* sp., *Biosporus* sp., *Fuzarium* sp., *Curvularia* sp., *Cladosporium* sp., *Colletotrichum* sp.	Amylase, protease, cellulose, lipase	Agar medium, spectrophotometer	Patil et al. (2015a, b)
Cladosporium cladosporioides, Curvularia brachyspira, C. verruciformis, Drechslera awaiiensis, Colletotrichum carssipes, Colletotrichum falcatum, Colletotrichum gloeosporioides, Lasiodiplodia theobromae, Nigrospora Sphaerica, Phyllosticta Sp. *Xylariales*	Amylase, cellulase, laccase, lipase, protease	Agar medium	Amirita et al. (2012)
Cladosporium cladosporioides, C. sphaerospermum, Acremonium terricola, Monodictys castaneae, Penicillium glandicola, Phoma tropica, Tetraploa aristata	Pectinases, cellulases, xylanases, proteases	Agar medium	Bezerra et al. (2012)
Amanita muscaria, A. muscaria, A. spissa, Boletus luridus, Cenococcum geophilum, Cortinarius glaucopus, C. purpurascens, Hydnum	Protease	Agae medium	Nygren et al. (2007)

(continued)

Table 5.1 (continued)

Specie name	Enzyme produced	Detection method	References
rufescens, Hymenoscyphus ericae, Laccaria cf., Lactarius acerrimus, L. auriolla, L.chrysorrheus, L. controversus, L. deliciosus, L. deterrimus, L. evosmus, L. pubescens, L. quieticolor, L. quietus, L. rufus, L. semisanguifluus, L. subdulcis, L. subumbonatus, L. zonarius, Piceirhiza bicolorata, Piloderma fallax, Piloderma byssinum, Russula chloroides, R. sanguinea, Suillus luteus, S. luteus, Tricholoma cf. equestre, S. variegatus, T. fulvum, T. scalpturatum			
Eurotiales, Chaelomiaceae, Incertae sadis, Aureobasiduaceae, Nectriaceae, Sporomiaceae	Celluloses, phosphatases, glucosidases	Spectrophotometer	Khan et al. (2016)
Colletotrichum sp., *Macrophomina phaseolina, Nigrospora sphaerica and Fusarium solani*	Cellulase, protease, amylase	Agar medium	Ayob and Simarani (2016)
Cochliobolus lunatus, C. australiensis, Gibberella baccata, Myrmecridium schulzeri, Penicillium commune, Phoma putaminum, Acremonium curvulum, Aspergillus niger, A. ochraceus, P. glabrum, C. lunatus, G. fujikuroi, Myrothecium verrucaria, Nodulisporium, Trichoderma piluliferum, A. chartarum, A. ochraceus, P. glabrum, Pithomyces atro-olivaceus	Cellulase, protease, xylanase, lipase	Agar medium	Bezerra et al. (2015)

(continued)

Table 5.1 (continued)

Specie name	Enzyme produced	Detection method	References
Penicillium chrysogenum, Alternaria alternate, Sterile hyphae	Amylase, pectinase, cellulase, gelatinase, xylanase and tyrosinase	Agar medium	Fouda et al. (2015)
Aspergillus terreus	l-asparaginase	Agar medium, spectrophotometer	Kalyanasundaram et al. (2015)
Phialocephala fortinii s.l. eliniomyces variabilis, Umbelopsis isabellina, Hebeloma incarnatulum, Laccaria bicolor	Protease		Mayerhofer et al. (2015)
Hormonema sp., *Pringsheimia smilacis, Ulocladium* sp., *Neofusicoccum luteum, Neofusicoccum australe*	Laccase	Agar medium, spectrophotometer	Fillat et al. (2016)
Acremonium sp., *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., *Pestalotiopsis* sp.,	Amylase, cellulase, lipase, protease	Agar medium	Maria et al. (2005)
Chaetomium sp., *Preussia* sp., *Penicillium citrinum, Thielavia arenaria, Phoma medicaginis, Aureobasidium* sp., *Preussia* sp., *Dothideomycetes* sp., *Aureobasidium pullulans, Phoma* sp., *Penicillium citrinum, Aureobasidium pullulans, Aureobasidium pullulans, Thielavia arenaria, Sordariomycetes* sp., *Fusarium proliferatum, Preussia* sp.	Glucosidase, phosphatases, cellulases	Fluorescence spectrophotometer	Khan et al. (2016)

desert wood land plant shown to produce new prospects for extracellular enzymes. The study revealed a new method of quantifying enzymes (glucosidase, phosphatases, cellulases) in outer environment of the microbial cell using fluorogenic substrates and standards.

5.4 Endophytic Bacterial Communities Producing Extracellular Enzymes

Endophytic bacterial communities are also considered an important source of extracellular enzymes. Endophytic bacterial strains have been isolated and identified from various plants such as pea (*P. sativum*), tomato (*Lycopersicum esculentum*), corn (*Zea mays*), wheat (*Triticum aesitivum*), oat (*Avena sativa*), canola (*Brassica napus*), barley (*Hordeum vulgare*), radish (*Raphanus sativus*) soybean (*Glycine max*), potato (*Solanum tuberosum*), lettuce (*Lactuca serriola*), and cucumber (*Cucumis sativa*). In addition, various bacterial strains have also been isolated from the economically important medicinal plants species. Some of the novel strains identified and characterized belong to the *Arthrobacter, Actinobacter*, *Aeromonas*, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Azospirillium, Enterobacter, Flavobacterium Pseudomonas, Acinetobacter, Azotobacter, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Rhizobium,* and *Serratia* genuses (Gray and Smith 2005).

In addition, the bacterial endophytes have been reported for the production of ACC deaminase, cellulases, protease, amylase, pectinase, esterase, lipase, protease, asparaginase, phytase, etc. (Sturz et al. 2000; Carrim et al. 2006). There are a wide array of resource studies showing that production of these enzymes by endophytic bacteria is isolated from different parts of the plant (Table 5.2). In such exploratory studies based on agar plate detection methods, Pereira et al. (2016) examined that *Lavandula dentate* harbored more than 30 endophytic bacterial strains. These endophytic microbes produced cellulases, lipases, pectinases, and proteases besides improving the growth of the host plant. In phyllosphereic part of the *Lavandula dentate,* the endophytic microbes produced higher quantities of plant cell wall-degrading enzymes, as also evidenced by Verma et al. (2001) who have reported higher number of endophytic bacteria from diazotrophs plant and their growth regulation by producing cellulase and pectinase.

In species-specific bacterial strains, *Nocardiopsis* sp. (39.2 U ml^{-1}) identified to secrete higher quantities of the α-amylase as an extracellular enzyme during different growth stages (Stamford et al. 2001). Davis et al. (1980) showed similar prospects for *Bacillus stearothermophilus,* whereas Castro et al. (1993) for *B. amyloliquefaciens*. The authors revealed a strong association of enzymes production by bacteria during different growth stages. When α-amylase was produced by *Lactobacillus plantarum*, maximum enzyme synthesis occurred during stationary phase (Giraud et al. 1993). Vijayalakshmi et al. (2016) isolated endophytic bacteria from medicinally important plants, producing α-amylase, protease, and cellulase. In one of the recent reports, Leo et al. (2016) observed the recovery of endophytic bacteria (*Alcaligenes faecalis, Burkholderia cepacia*, and *Enterobacter hormaechei*) from perennial grasses that showed the hyper-enzymatic activity of α-amylase, protease, and cellulase.

Bacterial endophyte, *Pantoea* sp. Sd-1, isolated from paddy shows a higher ligninolytic activity (Xiong et al. 2013). Castro et al. (2014) suggested that

Table 5.2 Endophytic bacterial strains producing extracellular enzymes

Species	Enzyme produced	Detection method	References
Actinomyces pyogenes, Bacillus circulans, Bacillus coagulans, Bacillus licheniformis, Bacillus megaterium, Corynebacterium renale, Pseudomonas stutzeri, Staphylococcus sp., *Bacillus* sp.	Amylase, esterase, lipase, protease	Agar medium	Carrim et al. (2006)
Pseudomonas oryzihabitans	Asparaginase	Spectrophotometer	Bhagat et al. (2016)
Bacillus sp., *Bacillus clausii, Bacillus pumilus, Bacillus licheniformis*	Amylase, protease, cellulose, lipase	Agar medium	Kannan et al. (2015)
Pseudomonas sp.	Exo-β-agarase	Spectrophotometer, NMR	Gupta et al. (2013)
Bacillus sp.	L-asparaginase	Spectrophotometer	Ebrahiminezhad et al. (2011)
Bacillus amyloliquefaciens	Phytase	Spectrophotometer	Idriss et al. (2002)
Paenibacillus polymyxa	Fibrinolytic enzymes	Agar medium, SDS Page	Lu et al. (2007)
Rhizobium, *Massilia, Kosakonia, Pseudorhodoferax, Caulobacter, Pantoea, Sphingomonas, Burkholderia, Methylobacterium*, *Bacillus*, *Curtobacterium, Microbacterium, Mucilaginibacter, Chitinophaga*	ACC deaminase, Endoglucanase, Protease	Agar medium	Chimwamurombe et al. (2016)
Acinetobacter sp., *Bacillus* sp.	ACC deaminase, Cellulase, Protease, Amylase, Pectinase	Agar medium	Joe et al. (2016)
Bacillus licheniformis, Bacillus pseudomycoides, Paenibacillus senitriformus	L-asparaginase	M9 medium	Joshi and Kulkarni (2016)
Pseudomonas hibiscicola, Macrococcus	Cellulase, xylanase, amalyase, pectinase	Agar diffusion method	Akinsanya et al. (2016)

(continued)

Table 5.2 (continued)

Species	Enzyme produced	Detection method	References
caseolyticus, Enterobacter ludwigii, Bacillus anthracis, Bacillus tequilensis, Pseudomonas entomophila, Chryseobacterium indologenes, Bacillus aerophilus			
Bacillus thuringiensis	Anthracene	Spectrophotomete	Roy et al. (2016)
Bacillus amyloliquefaciens	Exopolysaccharides	Colorimetric method	Chen et al. (2013)
Bacillus subtilis	YbdN protein	SDS-PAGE, MALD-TOF-MS	Jamal and Mudarris (2010)
Serratia marcescens, Bacillus subtilis, Bacillus methylotrophicus, Bacillus siamensis	L-asparaginase	Spectrophotometer	Nongkhlaw and Joshi (2015)
Paenibacillus polymyxa, Bacillus sp.	Cellulase, xylanase, pectinase	Agar diffusion method	Cho et al. (2007)
Paenibacillus amylolyticus	Pectin lyase	Spectrophotometer	Sakiyama et al. (2001)
Alcaligenes faecalis, Burkholderia cepacia, Enterobacter hormaechei	Cellulosic, hemicellulosic, lignin	National renewable energy laboratory methods	Leo et al. (2016)

endophytic microbes produced production of ligninases derived from Brazilian mangrove ecosystem. Yang et al. (2011) explained that *Alcaligenes faecalis* produced cellulases and xylanases with higher ability to degrade cellulosic substrate in a coculture system.

5.5 Conclusion and Perspectives

The scientific community is familiar about endophytic microbes since last two and half decades with vibrant and potent roles in agriculture, ecology, biotechnology, and industry. There are still many overlooked and unexplored aspects of these ecologically unique microorganisms which require special attention on primary metabolite production in general and enzyme in particular. This consortium of endophytes (bacteria and fungi) can thrive and capable to live together with host plants and produced synergistically hydrolytic enzymes for counteracting emerging

issues. These can be utilized in enzymology-based enzyme fermentation industries, where endophytes derived from plants living in extreme environments possess higher ability to produce higher quantities of extracellular enzymes. In addition, endophytic microbes producing enzymes can help to counteract biotic stress; however, the role of such endophytes in abiotic stresses cannot be ruled out. The significances of enzymes producing endophytes with special attention on remediating environmental pollutants such as metals, polyaromatic hydrocarbons, and polychlorinated hydrocarbons have been understood very least. Most of the researches have been performed in monoculture, while on the coculture and other aspects such as quorum sensing signaling pathway of these endophytes in growth media are yet to be elaborated. Besides the enzymes quantification methods need a rigorous review as with the advancement of technology, new techniques with higher sensitivity are much needed. Methods such as fluorescence spectrophotometer, near-infra red (NIR), and FTIR-based methods further improve enzyme analysis. The endophytic biomasses are extremely significant in determining their viability and alternative to an important resource for biofuel production. The molecular and genomic bases of these endophytic resources for enzymes production also need cross-validation.

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Chapter 6
Beneficial Effects of Bacterial Endophytes on Forest Tree Species

Akshit Puri, Kiran Preet Padda and Chris P. Chanway

Abstract Since their discovery, beneficial bacteria living inside the plant tissues (known as bacterial endophytes) have been studied widely in agricultural crop species. But their ecology and effects on tree species in a forest ecosystem could be very different yet intriguing. In this chapter, studies highlighting the isolation of bacterial endophytes, re-inoculation and detection of the endophytic population in the host tree, and benefits provided to the host tree through direct and indirect mechanisms have been reviewed. Important tree species including those belonging to the genus *Pinus*, *Populus*, and *Picea* have been reported widely to harbor bacterial endophytes belonging to the genus *Bacillus*, *Paenibacillus*, and *Pseudomonas* and possibly obtain benefits like nitrogen fixation and increased biomass production from them. Nitrogen-fixing bacterial endophytes are the most commonly studied beneficial microbes of forest tree species, and thus have been reviewed in detail in this chapter.

Keywords Endophytes · Diazotrophic bacteria · *Pinus* · *Populus* · *Picea*

6.1 Introduction

Plants are a complex micro-ecosystem that harbors a range of microbes both in their internal tissues as well as on their external surfaces. Although the importance of microbes for plant health and growth promotion has been known for a long time, internal tissues colonization was largely perceived as being related to the spread of

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disease. Even the first microorganism reported to colonize internal tissue of plant leaves was a pathogenic fungus (de Bary 1866). But now, it is widely known that microorganisms can colonize internal tissues of plants and establish beneficial symbiotic interaction with the host plant. Such microbes are known as endophytes. Literally taken, endophyte means "within plant" (Chanway 1996). Although many authors have defined the term endophyte, but in this chapter, we will use the term defined by Chanway et al. (2014). According to Chanway et al. (2014), bacterial endophytes are "bacteria that can be detected at a particular moment within the tissue of apparently healthy plant hosts without inducing disease or organogenesis." The occurrence of bacterial endophytes was first reported in internal tissues of healthy potato plant (Trevet and Hollis 1948). Since then, most studies have been focused on isolating and evaluating the benefits of bacterial endophytes in agricultural plants (reviewed by Hallmann et al. 1997; Kobayashi and Palumbo 2000; Sturz et al. 2000; Suman et al. 2016). Although there is huge literature about endophytic fungi in forest ecosystem (reviewed by Doty 2011), studies of bacterial endophytes in forest tree species are rather limited but their importance should not be underrated.

6.2 Bacterial Endophytes in Forest Tree Species

Forest trees can provide unique ecological conditions for bacterial endophytes since they have larger biomass and exist for a longer period in terrestrial ecosystems as compared to agricultural plants (Izumi 2011). Bacterial endophytes have only been reported in very limited host tree species including pine, spruce, poplar, oak, cedar, and willow. The most common bacterial endophytes isolated from forest trees belong to the genus *Acinetobacter*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Methylobacterium*, *Microbacterium*, *Pseudomonas*, *Paenibacillus*, *Rahnella*, *Sphingomonas*, and *Xanthomonas* (Izumi 2011; Pirttilä 2011). The diversity of bacterial endophytes found in forest ecosystem has been reviewed by Izumi (2011). Endophytes enhance the growth of forest tree species by various direct and indirect mechanisms. Direct mechanisms involve production of phytohormones like cytokinins (Pirttilä 2011), auxins (Taghavi et al. 2005; Madmony et al. 2005), gibberellins (Bottini et al. 2004), and nitrogen (N) fixation (Bal and Chanway 2012a, b; Anand and Chanway 2013b; Anand et al. 2013; Tang et al. 2017). Indirect mechanisms involve suppression of pathogens and improvement of the mutualistic relationship of a mycorrhizae and plant host (Anand et al. 2006). It is believed that most interactions between plants and beneficial bacteria occur in roots of host plant but shoots represent a unique ecological niche where endophytes can carry out major plant-beneficial activities. Generally, bacterial endophytes in forest trees have been isolated from shoot tips, flowers, pollens or seeds, and seedlings (Pirttilä 2011). Although shoot endophytes provide similar benefits as provided by root endophytes, they have also been reported to induce plant growth through other mechanisms like the production of adenine derivatives and vitamin B_{12} (Pirttilä

2011). Endophytic bacterial colonization sites in tree shoots and their potential growth-promoting effects have been reviewed in detail by Pirttilä (2011).

6.2.1 *Diazotrophic Bacterial Endophytes in Forest Tree Species*

N-fixing bacteria also known as "diazotrophic bacteria" are well known for their ability to fix N biologically. Apart from root nodule-forming diazotrophs living in association with leguminous plants, there are bacterial species that can fix N in association with non-leguminous plant species. The presence of diazotrophic bacteria in non-leguminous plants was first detected by Brazilian researchers in the rhizosphere of sugarcane (Döbereiner and Alvahydo 1959; Döbereiner 1961). In subsequent studies, it was determined that diazotrophic bacteria in rhizosphere contributed only small amounts of N to the sugarcane plants and diazotrophic bacteria living in internal tissues of stem and roots fix significant amounts of N from the atmosphere (Cavalcante and Döbereiner 1988; Boddey et al. 1991; Stephan et al. 1991). Cavalcante and Döbereiner (1988) isolated a diazotrophic bacteria, *Saccharobacter nitrocaptans* (renamed to *Acetobacter diazotrophicus* (Gillis et al. 1989), and then to *Gluconacetobacter diazotrophicus* (Yamada et al. 1997)), from internal tissues of sugarcane. Such diazotrophs were designated as diazotrophic bacterial endophytes (Döbereiner 1992) and were detected in many other agricultural crops like corn (Padda et al. 2017; Puri et al. 2015, 2016b), rice (Baldani et al. 2000), wheat (Sabry et al. 1997), and canola (Padda et al. 2016a, b; Puri et al. 2016a). Diazotrophic bacterial endophytes have been detected in stem tissues of forest trees like poplar (*Populus trichocarpa*) (Ulrich et al. 2008a; Scherling et al. 2009; Doty et al. 2009; Xin et al. 2009; Knoth et al. 2014), willow (*Salix sitchensis*) (Doty et al. 2009), lodgepole pine (*Pinus contorta*) (Bal et al. 2012; Bal and Chanway 2012a; Anand et al. 2013; Tang et al. 2017; Yang et al. 2016), and western red cedar (*Thuja Plicata*) (Bal and Chanway 2012b; Anand and Chanway 2013b). Diazotrophic bacterial endophytes have fixed significant amounts of N from the atmosphere (in some cases up to 79%) after establishing a symbiotic relationship with these tree species.

6.3 Plant Growth Promotion by Bacterial Endophytes in Forest Tree Species

In this section, studies highlighting the beneficial effects of bacterial endophytes in forest trees have been reviewed. We have compiled an elaborative list of bacterial endophytes that have been isolated from forest trees and have shown plant

Table 6.1 List of bacterial endophytes isolated from prominent forest tree species and their beneficial effects on host trees

Host plant	Bacterial endophytes	Benefits to host	References
Australian native pine (*Callitris preissii*)	*Nocardia callitridis* sp. nov. strain CAP 290^T	–	Kaewkla and Franco (2010)
Black cottonwood (*Populus trichocarpa*)	*Burkholderia vietnamiensis* WPB	Nitrogenase activity, production of indole-3-acetic acid (IAA)	Doty et al. (2009), Knoth et al. (2014), Xin et al. (2009)
Willow (*Salix sitchensis*)	*Herbaspirillum* sp. WW2 and *Pseudomonas* sp. H9zhy (WW6)	Nitrogen fixation	Doty et al. (2009)
Douglas-fir (*Pseudotsuga menziesii*)	*Rhodotorula graminis* WP1, *Rahnella* sp. WP5, *Burkholderia* sp. WP9, *Acinetobacter calcoaceticus* WP19, *Rhizobium tropici* bv *populus* PTD1, *Sphingomonas yanoikuyae* WW5, *Pseudomonas putida* WW6, *Sphingomonas* sp. WW7	Increases biomass, root length and shoot height	Khan et al. (2015)
Hybrid spruce (*Picea glauca* x *P. engelmannii*)	*Bacillus polymyxa* strain Pw-2R and *Pseudomonas fluorescens* strains Sm3-RN and Ss2-RN	Boosts seedling biomass and height	Chanway et al. (2000), Shishido et al. (1996a, b, 1999), Shishido and Chanway (1999, 2000)
Limber pine (*Pinus flexilis*)	Acetic Acid Bacteria (AAB)	Nitrogen fixation	Moyes et al. (2016)
Live oaks (*Quercus fusiformis*)	*Pseudomonas denitrificans* 1-15, *Pseudomonas putida* 5-48	In vitro inhibition of the pathogen, *Ceratocystis fagacearum*, reduces crown loss	Brooks et al. (1994)
Lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.)	*Bacillus polymyxa* strain Pw-2 (or Pw-2R)	Enhances seedling biomass, produces phytohormones (IAA and cytokinin)	Bent et al. (2001), Shishido et al. (1995, 1996a)

(continued)

Table 6.1 (continued)

Host plant	Bacterial endophytes	Benefits to host	References
Lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.) and western red cedar (*Thuja plicata* Donn ex D. Don)	*Paenibacillus polymyxa* P2b-2R	Nitrogen fixation, growth promotion (biomass and length)	Bal and Chanway (2012a, b), Bal et al. (2012), Anand and Chanway (2013a, b), Anand et al. (2013), Tang et al. (2017), Yang et al. (2016)
Norway spruce (*Picea abies*) seeds	*Pseudomonas* spp. and *Rahnella* spp.	–	Cankar et al. (2005)
Poplar (*Populus deltoides* x *P. nigra* DN-34)	*Enterobacter* sp. strain 638	Synthesizes plant growth-promoting compound acetoin, increases seedling biomass	Taghavi et al. (2005, 2009)
Poplar (*Populus deltoides* x (*trichocarpa* x *deltoides*))	*Pseudomonas putida* W619	Improves plant health and growth, decreases activities of anti-oxidative defense-related enzymes, reduces stomatal resistance, and degrades TCE	Taghavi et al. (2005, 2009), Weyens et al. (2009, 2010, 2012)
Poplar (*Populus trichocarpa* x *deltoides cv. Hoogvorst)*	*Pseudomonas* sp. PopHV4, PopHV6 and PopHV9	–	Germaine et al. (2004)
Poplar (*Populus trichocarpa* x *P. deltoides hybrids*)	*Rhizobium tropici* CIAT899	–	Doty et al. (2005)
Scots pine (*Pinus sylvestris L.*)	*Methylobacterium extorquens* DSM13060	Increases root and shoot dry weight, increases root and shoot potassium content	Pirttilä et al. (2000), Pohjanen et al. (2014)

growth-promoting (PGP) properties (see Table 6.1). Most studies about bacterial endophytes have been reported in tree species belonging to genera *Pinus*, *Picea*, and *Populus*.

6.3.1 *Pinus*

The genus *Pinus* is one of the largest and most important among the coniferous genera. Pines are widely distributed and mostly found in the Northern Hemisphere. They range from Alaska to Nicaragua, from Scandinavia to North Africa, and from Siberia to Sumatra (Krugman and Jenkinson 1974). The most common *Pinus* sp. found in western North America is lodgepole pine. It is a commercially important gymnosperm species that grows throughout the Rocky Mountain and Pacific Coast regions. It extends from Yukon Territory, Canada in the north to Baja California, Mexico in the south and from the Pacific Ocean in the west to South Dakota, USA in the east (Lotan and Critchfield 1990). The first evidence of endophytic colonization by plant growth-promoting bacteria in lodgepole pine was detected by Shishido et al. (1995). They isolated a bacterial endophyte (strain Pw-2) from root tissues of lodgepole pine seedlings (<3 years old) naturally regenerating at a site near Williams Lake, BC, Canada (52°N, 122°W). Preliminary characterization revealed that strain Pw-2 belongs to *Bacillus polymyxa* (now known as *Paenibacillus polymyxa*). The beneficial effects of *B. polymyxa* Pw-2 were assessed by re-inoculating it into lodgepole pine and growing in a greenhouse for 9 weeks. Inoculation with Pw-2 significantly increased shoot height, shoot dry mass, and root dry mass of lodgepole pine seedlings as compared to the uninoculated controls. A rifamycin-resistant strain, Pw-2R, was derived from Pw-2 so as to check internal root colonization of lodgepole pine after re-inoculation (Shishido et al. 1995). Pw-2R endophytically colonized the internal root tissues of lodgepole pine with a population size in the range of 10^5 cfu/g fresh tissue, 4 weeks after inoculation. In a subsequent study, Shishido et al. (1996a) ruled out the theory about the involvement of mycorrhizal fungi in growth promotion observed in Pw-2 (or Pw-2R) inoculated lodgepole pine seedlings. According to their findings, Pw-2R enhanced lodgepole pine seedling biomass significantly (up to 18%) through a mechanism that is unrelated to mycorrhizal fungi. It was also reported that strain Pw-2 is involved in elevating the levels of PGP hormones like indole-3-acetic acid (IAA) and dihydrozeatin riboside (DHZR; a form of cytokinin) produced in lodgepole pine roots (Bent et al. 2001).

In an effort to look for diazotrophic bacterial endophytes in stem and needle tissues of lodgepole pine trees (>20 years old) and seedlings (2–4 years old) growing in nutrient-poor (N-limited) forest sites of British Columbia, Canada; Bal et al. (2012) isolated an endophytic strain P2b-2R that was capable of growing on N-free medium (combined carbon medium; Rennie 1981) and consistently reduced significant amounts of acetylene in acetylene reduction assay (ARA) used for measuring N-fixing activity. Strain P2b-2R (GU132543) was identified as belonging to *P. polymyxa* (Bal et al. 2012). Since ARA is an indirect method of measuring the amount of N fixed, Bal and Chanway (2012a) used a more accurate method, ^{15}N isotope dilution method (Danso 1995), to assess the amount of N fixed. In two separate growth trials, P2b-2R inoculated lodgepole pine seedlings were able to derive 30 and 66% of N directly from the atmosphere 27 and 35 weeks

Table 6.2 Percent N derived from the atmosphere (%Ndfa) by a diazotrophic bacterial endophyte, *Paenibacillus polymyxa* P2b-2R, when inoculated into two different host trees and determined in several studies at different time periods

Host tree	Time after inoculation	%Ndfa	References
Lodgepole pine	27 weeks	30	Bal and Chanway (2012a)
	35 weeks	66	Bal and Chanway (2012a)
	12 months	40	Tang et al. (2017)
	13 months	79	Anand et al. (2013)
Western red cedar	27 weeks	23	Bal and Chanway (2012b)
	35 weeks	56	Bal and Chanway (2012b)
	13 months	36	Anand and Chanway (2013b)

after inoculation, respectively (Table 6.2). In a subsequent study, Anand et al. (2013) reported that lodgepole pine seedlings inoculated with P2b-2R were able to derive 79% of N directly from the atmosphere, 13 months after inoculation (Table 6.2). Along with fixing high amounts of N, P2b-2R inoculation also enhanced lodgepole pine shoot height by 33%, shoot dry weight by 78%, and root dry weight by 165%. They postulated that plant growth promotion was directly related to the amount of N fixed by P2b-2R. Since seedlings were grown in an N-limited environment and N fertilizer was provided only once at the onset of the experiment, so after sometime, soil N depletion would eventually restrict the growth rate of control seedlings to a point where P2b-2R inoculated (N-fixing) seedlings would outperform them. When lodgepole pine seedlings were grown in sufficient N conditions (N fertilizer provided regularly in a yearlong growth experiment), P2b-2R inoculation had no effect on the growth of lodgepole pine and the inoculated seedlings were not able to fix atmospheric N (Yang et al. 2016, 2017), thus confirming the hypothesis proposed by Bal and Chanway (2012a) that P2b-2R triggers N fixation mechanism under N-limited conditions. Full sequencing of nitrogenase reductase protein (*nif*H) of P2b-2R was also conducted by amplifying a 388-bp internal *nif*H gene fragment and performing a Southern blot analysis of total genomic DNA digested with Pst I/HindIII (Anand and Chanway 2013c). The Southern blot profile showed just one positive signal at 1.8 kb of the Pst I digest and 9 kb of the HindIII digest (Fig. 6.1), indicating that there is only one copy of *nif*H in P2b-2R. Anand et al. (2013) evidenced that P2b-2R strain can form a significant amount of endophytic colonies in the root, shoot, and needle tissues, thus indicating that perceived growth promotion and N fixation was bacteria driven. But in this study, a culture-based technique was used to assess the bacterial colonies in each part of the plant. Endospore-forming bacteria like *P. polymyxa* P2b-2R are susceptible to misidentification when such technique is used (Bent and Chanway 2002). Anand and Chanway (2013a) then applied a more precise technique to prove that P2b-2R can colonize lodgepole pine endophytically. Green fluorescent protein (GFP) tagging in conjunction with confocal laser scanning microscopy (CLSM) was used to view the sites of endophytic colonization in real time. A plasmid-borne GFP (pBSGV104) was used to tag P2b-2R and the transformed strain was named

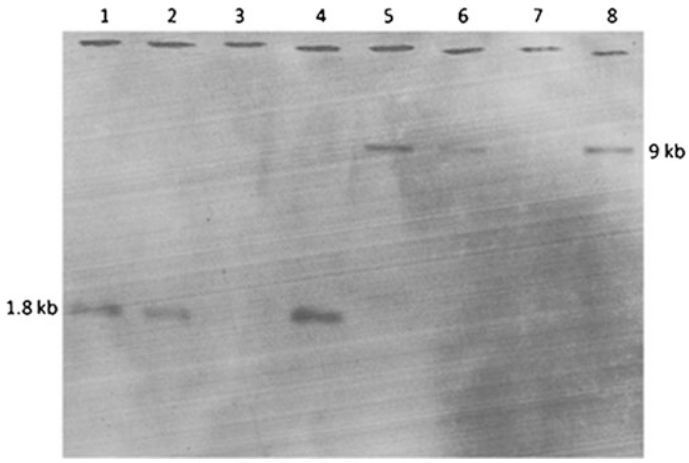

Fig. 6.1 Southern blot profile of *P. polymyxa* P2b-2R total DNA digested with Pst I (*Lanes 1*, *2*, and *4*) or HindIII (*Lanes 5*, *6*, and *8*) and probed with the *nif*H fragment (from Anand and Chanway 2013c)

P2b-2R*gfp*. With the help of CLSM, it was observed that P2b-2R*gfp* had completely engulfed the root surface of lodgepole pine, similar to what was reported by Timmusk et al. (2005). P2b-2R*gfp* effectively colonized stem cortical cell of 2–14-week-old lodgepole pine seedlings intracellularly (Figs. 6.2 and 6.3) but was not observed in vascular tissues. Thus, Anand and Chanway (2013a) provided a strong evidence that P2b-2R can effectively colonize stem tissues endophytically from as early as 2 weeks after inoculation. However, Padda et al. (2016a) reported that GFP-tagging might affect the performance of P2b-2R to promote plant growth and fix N. After some successful initial reports about colonization and plant growth promotion of agricultural crops, viz., corn, canola, and tomato by P2b-2R. Puri et al. (2015, 2016a), Padda et al. (2016a, 2017) reported that GFP-tagging could positively affect the plant growth-promoting and N-fixing capability of P2b-2R in agricultural crops like corn and canola. Recently, Puri et al. (2016b) concluded that this effect is temporary in corn and diminishes as the plant develops. However, Padda et al. (2016b) further reported that the positive effect of GFP-tagging is not temporary in canola and could be seen throughout the life cycle of the plant. Tang et al. (2017), in an effort to evaluate the positive effect of GFP-tagging of P2b-2R in its original host lodgepole pine, found that P2b-2R*gfp* outperforms the wild-type strain in the initial stages of plant development only (till 4 months after sowing) and the positive effect diminishes as the pine plant grows. It has been discovered that GFP-tagging leads to overexpression of *nif*H, *nif*D, and *nif*K genes. Therefore, the effect of reporter gene GFP on physiological activities of host–microbe cell should be taken into account in using it as a cytological marker (Unpublished data).

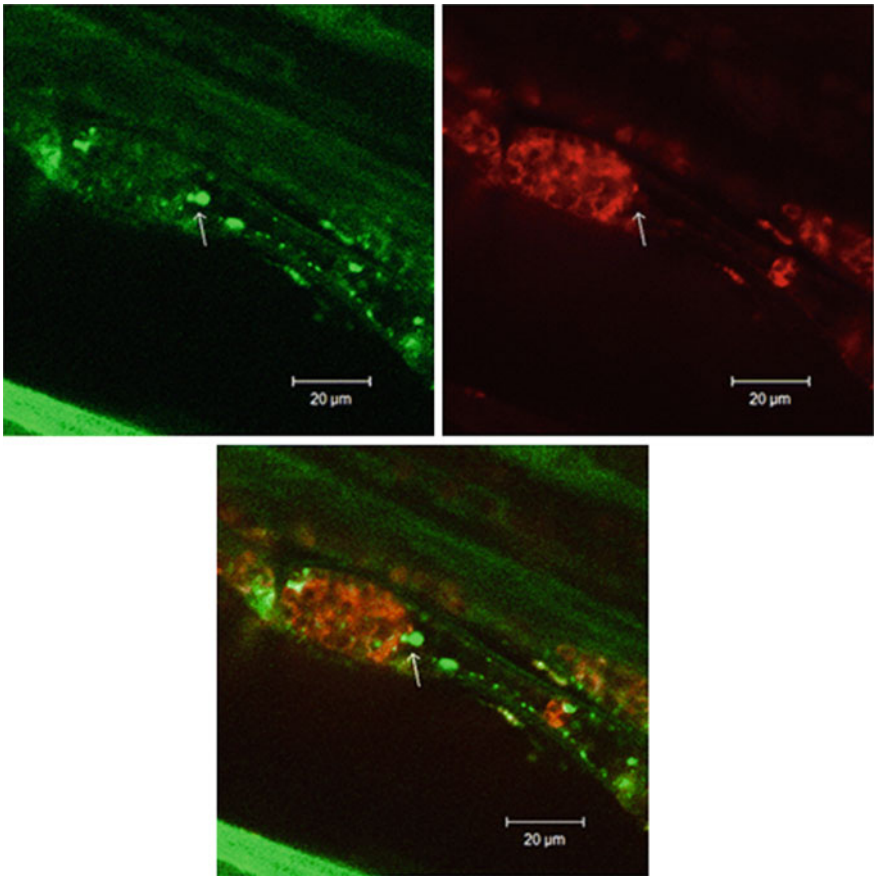

Fig. 6.2 Longitudinal section of the stem showing intracellular colonization by *P. polymyxa* P2b-2R*gfp* viewed under *green* (*top left panel*), *red* (*top right panel*), and a combination of both *green* and *red lights* (*bottom*). *Arrow* points to a bright green P2b-2R*gfp* cell that appears to contain a terminal endospore located to the right of the *red-orange* chloroplasts (Anand and Chanway 2013a)

In studies with other pine species, a novel endophytic actinobacterium strain, CAP 290^T (=DSM 45353^T = ACM 5287^T), was isolated from root tissues of a pine tree native to Australia (*Callitris preissii*) (Kaewkla and Franco 2010). Phylogenetic analysis and physiological and biochemical tests conducted on strain CAP 290^T revealed that it is a novel endophytic actinobacterium belonging to the *Nocardiaceae* family and the name *Nocardia callitridis* sp. nov. was proposed. It should be noted that beneficial effects of this endophytic strain were not evaluated in this study. In another study with Scots pine (*Pinus sylvestris* L.), a pine species

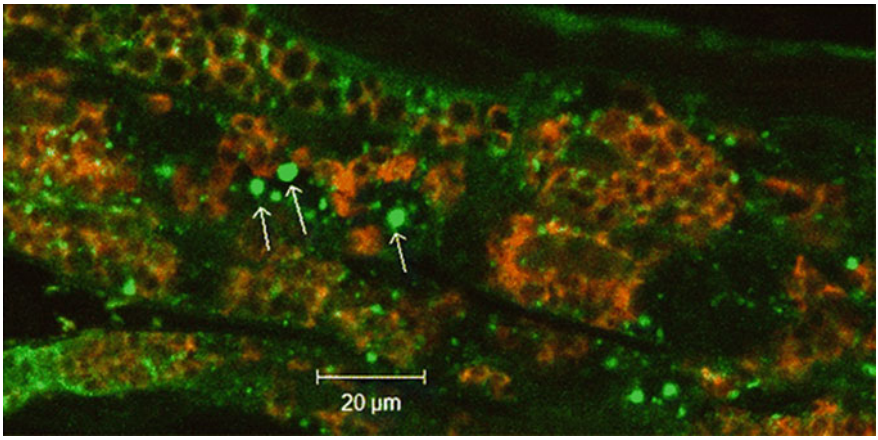

Fig. 6.3 Intracellular colonization of a 4-week-old lodgepole pine stem cortex cell by *P. polymyxa* P2b-2R*gfp*. *Arrows* point to P2b-2R*gfp* near the *red-orange* chloroplasts (Anand and Chanway 2013a)

native to Eurasia, Pirttilä et al. (2000) isolated bacterial endophytes from buds of mature, healthy Scots pine trees growing on a natural stand in northern Finland. One of the bacterial endophytic strains (isolate F) was identified as *Methylobacterium extorquens* (DSM 13060). In a subsequent study, it was reported that this bacterial endophyte produced adenine derivatives like adenine ribosides, which could be used as precursors in cytokinin biosynthesis (Pirttilä et al. 2004). In an effort to detect the endophytic colonization sites of *M. extorquens* DSM 13060 in Scots pine, Pohjanen et al. (2014) tagged DSM 13060 with GFP and viewed endophytic colonies by using CLSM. GFP-tagged DSM 13060 was observed in root epidermis and root parenchymatic and xylem tissues. Also, *M. extorquens* DSM 13060 significantly increased shoot and root dry weight of Scots pine seedlings. In addition, this bacterial endophyte in association with ectomycorrhizal (ECM) fungi (*Suillus variegatus* and/or *Pisolithus tinctorius*) was able to enhance the growth of Scots pine seedlings even more than the only ECM fungi inoculation. In a recent study with limber pine (*Pinus flexilis*), Moyes et al. (2016) found evidence of diazotrophic bacterial endophytes in foliar parts of the plant. These bacteria can provide 6.8–13.6 µg of N per square meters to limber pine stands per day or approximately 1–2 mg of N per square meters in one year (Moyes et al. 2016). In another study, Carrell and Frank (2014) found that limber pine and another conifer tree species, Engelmann spruce (*Picea engelmannii*), growing in a sub-alpine, N-limited environment are colonized by bacterial endophytes of the same phylotype. This phylotype was related to *Gluconacetobacter diazotrophicus* and other N-fixing acetic acid bacterial endophytes.

6.3.2 *Populus*

Populus is a genus of deciduous flowering plants mostly native to the northern hemisphere. It includes commercially important species like poplar, cottonwood, and aspen. The first tree species whose full genome was sequenced belong to this genus (Black cottonwood). The genome of black cottonwood (*Populus trichocarpa*) is four times larger than the genome of the first plant sequenced, *Arabidopsis thaliana* (Tuskan et al. 2006). Apart from traditional varieties of poplar and cottonwood trees, many hybrid varieties have been developed. *Populus* spp. are known for rapid growth, deep root network, and ability to grow in nutrient-poor sites (Stettler et al. 1996). They are economically important and are grown in short-rotation plantations for the production of pulp and paper, lumber, and fuel (Doty et al. 2009). The first discovery about the presence of a diazotrophic bacterial endophyte in a *Populus* sp. was reported by Doty et al. (2005). In this study, clones of hybrid cottonwood (*Populus trichocarpa* × *P. deltoides*) were evaluated for the presence of bacterial endophytes in stem tissues. *Rhizobium tropici* was the most common bacterial endophyte found in all clones of hybrid cottonwood. Although this bacterial species is well known for its nodule-forming ability and diazotrophic trait in legumes (Perret et al. 2000), this study reported its endophytic nature in a non-legume host. *Populus* spp. are also known for their phytoremediation capabilities. Endophytic bacteria belonging to genus *Pseudomonas* were isolated from xylem sap of hybrid poplar trees growing on a phytoremediation site near a motor factory in Genk, Belgium (Germaine et al. 2004). Isolated strains were tested for their ability to solubilize phosphorus, produce IAA, act as biocontrol agents, and resist heavy metal. Selected strains were tagged with GFP to visualize endophytic colonization sites. GFP-tagged strains were found to colonize inner cortex and xylem tracheid cells in the root and intercellular spaces of root xylem cells when visualized with CLSM. Although stem and leaf colonization was not observed with CLSM but through culture-based technique, it was determined that these *Pseudomonas* strains colonize root, stem/sap, and leaf tissues with population density ranging from 10^3 to 10^6.

Poplar trees harbor diverse bacterial endophytes in different parts and each bacterial community plays its own role in enhancing the growth and protecting the tree against pathogens. The diversity of endophytic bacterial communities residing inside field-grown poplar trees was evaluated by Ulrich et al. (2008b). Aerial parts (leaves and branch sections) of four hybrid poplar clones were evaluated for the presence of endophytic bacteria. Bacterial endophytes belonging to 53 different genera were isolated including *Curtobacterium*, *Plantibacter*, *Pseudomonas*, *Xanthomonas*, *Sphingomonas*, *Methylobacterium*, *Pedobacter*, and *Paenibacillus* and the most abundant genera among all clones of hybrid poplar were *Curtobacterium* and *Pseudomonas*. Several bacterial endophytes were also isolated from young poplar seedlings (black cottonwood) growing in Three Forks Park alongside the Snoqualmie River in Western Washington state, USA (Doty et al. 2009). Isolates belonging to the genus *Burkholderia*, *Rahnella*, and *Acinetobacter*

possessed *nif*H gene and were able to reduce significant amounts of acetylene in ARA. One of the strains WPB (*Burkholderia vietnamiensis*) was able to produce ethylene (concentration = 68.4 ppm) enormously higher than other strains when exposed to acetylene for 72 h. Phylogenetic analysis of *recA* gene and *nifHDK* gene cluster of WPB strain was performed in a subsequent study (Xin et al. 2009). In this study, it was also determined that WPB strain can produce 5.7 mg IAA/g dry cell after 7-day incubation with 0.1% L-tryptophan but does not produce IAA when L-tryptophan was not applied. Tryptophan generally acts as a precursor for the release of IAA (Omay et al. 1993; Hung et al. 2007; Taghavi et al. 2009), but some microbes lack the ability to synthesize tryptophan, essential for protein synthesis, and must obtain it from the plant (Radwanski and Last 1995). Thus, it can be inferred that a mutually advantageous plant–microbe interaction occurs in this case, where the plant provides tryptophan for WPB and WPB, in return, converts extra tryptophan to IAA for promoting the plant growth. Strain WPB and other diazotrophic strains isolated by Doty et al. (2005, 2009) were used in a glasshouse and a field experiment on black cottonwood and hybrid cottonwood (Knoth et al. 2014). They tested each strain individually as well as collectively by making consortiums. Diazotrophic bacterial endophytes significantly increased the biomass of black cottonwood and cottonwood hybrid in both glasshouse and field experiments and inoculation with microbial consortia made of many strains was more successful than single-strain inoculation. Inoculated cottonwood seedlings fixed up to 65% of N directly from the atmosphere in this study, clearly exhibiting the role of diazotrophic bacterial endophytes in promoting poplar tree growth by providing substantial N nutrition.

Pseudomonas spp. are one of the most common bacterial endophytes found in *Populus* trees (Ulrich et al. 2008b; Doty et al. 2009; Gottel et al. 2011; Izumi 2011). Taghavi et al. (2005, 2009) isolated several endophytes from the root and shoot tissues of hybrid poplar and used three representative strains, *Serratia proteamaculans* 568, *Enterobacter* sp. strain 638, and *Pseudomonas putida* W619, for further evaluation of their endophytic and growth-promoting properties. GFP-tagged strains colonized root surface and interior of hybrid poplar and one of the strains, *Enterobacter* sp. strain 638, significantly increased shoot biomass in greenhouse experiment (Taghavi et al. 2009). In a subsequent study, *P. putida* W619 was used to construct a trichloroethene (TCE)-degrading strain W619-TCE (Taghavi et al. 2005; Weyens et al. 2009, 2010). *P. putida* W619-TCE inoculation of hybrid polar trees reduced TCE evapotranspiration significantly and promoted plant growth (Weyens et al. 2010). In field conditions (TCE contaminated sites), TCE evapotranspiration from hybrid poplar was reduced by 90% by inoculation with this strain (Weyens et al. 2009), thus clearly establishing its phytoremediation characteristics. Weyens et al. (2012) used the GFP-tagged derivative of strain W619 (Taghavi et al. 2009) to compare the colonization ability of wild-type and GFP-tagged strain W619 along with investigating the morphological, physiological, and biochemical parameters so as to compare the PGP ability of the two strains. Although wild-type W619 was able to promote plant growth by producing IAA and cytokinins, increasing root and shoot mass, reducing stomatal resistance, decreasing

the activities of anti-oxidative defense-related enzymes like glutathione reductase and superoxide dismutase in hybrid poplar seedlings (Weyens et al. 2012), GFP-tagging of W619 had negative effects on plant growth and health since the W619 + *gfp* strain significantly lagged in the before-mentioned parameters. They also found that GFP-tagging effects the endophytic colonization ability of W619. These results are contradictory to what was observed when *Azospirillum brasilense* 8-I (Rodriguez et al. 2006) and *Paenibacillus polymyxa* P2b-2R were tagged with GFP (discussed in Sect. 6.3.1), thus casting doubts on this phenomenon of reduced microbial efficiency after GFP-tagging. In a recent study, a novel endophytic bacterium, *Pseudomonas populi* sp. nov. (KBL-4-9^T), was isolated from stem tissues of *Populus euphratica* trees (Anwar et al. 2016). The plant growth-promoting traits of this bacterial endophyte have not been fully determined yet. Since the complete genome cottonwood was sequenced (Tuskan et al. 2006), there has been an increased interest in elucidating the interaction of bacterial endophytic and rhizospheric communities with *Populus* trees at the molecular and genetic level (Schaefer et al. 2013, 2016), which will obviously help in understanding their interaction with other tree species.

6.3.3 *Picea*

Picea genus is most closely related to *Pinus* since they belong to the same family, Pinaceae. Tree species belonging to *Picea* are usually found in northern temperate and boreal regions and are commonly known as spruce trees. Commercially important species like black spruce (*Picea mariana*), engelmann spruce (*P. engelmannii*), sitka spruce (*P. sitchensis*), white spruce (*P. glauca*), Norway or alpine spruce (*P. alpestris* and *P. abies*), and Siberian spruce (*P. obovata* and *P. omorika*) are generally found in northern hemisphere (North America, North Europe, and Eurasia) (Parish and Thomson 1994). Evidence of plant growth-promoting rhizobacteria (PGPR) in spruce (hybrid spruce) was reported by O'neill et al. (1992) and Chanway and Holl (1993a, b). But the first reported spruce endophyte, *Pseudomonas* sp. Ss2, was isolated from roots of hybrid spruce (*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry) seedlings naturally regenerating near Salmon Arm, BC, Canada (51°04′N, 119°26′W, 1250 m elevation) (Shishido and Chanway 1999). Rifamycin-resistant derivative of Ss2 was generated and the resulting strain was designated as Ss2-RN. Shishido et al. (1996b) inoculated Ss2-RN into hybrid spruce (*Picea glauca* x *P. engelmannii*) seedlings and grew them in the greenhouse for 15 weeks. Inoculate hybrid spruce seedlings increased root weight by 19%, shoot weight by 10%, and seedling height by 6% in comparison to the non-treated (control) seedlings. In this study, it was also observed that bacterial inoculation had no effect on the mycorrhizal status of seedlings and growth promotion achieved by bacterial inoculation was similar in mycorrhizal and non-mycorrhizal spruce seedlings. It was also reported that strain Ss2-RN performs better when inoculated into spruce ecotype that originated from the same

geographical area as the bacteria (Shishido and Chanway 1999). In an interesting study, Shishido and Chanway (2000) used a combination of greenhouse and field trial to assess the growth-promoting effects of Ss2-RN. Hybrid spruce seedlings were first grown in greenhouse for 4 months and were then outplanted in field sites. Some seedlings were harvested after greenhouse trial to assess the growth promotion due to inoculation of Ss2-RN in the first 4 months. As expected, Ss2-RN inoculated seedlings had significantly higher root and shoot biomass than controls. Relative growth rate (RGR) of outplanted seedlings was determined 4 months after outplanting in the field. Root and shoot RGR of inoculated seedlings were 10–234% higher than controls, thus establishing the fact that bacterial endophyte Ss2-RN can perform exceptionally in field conditions. Surprisingly, Ss2-RN was not able to colonize spruce seedlings endophytically as observed in two different studies (Shishido and Chanway 2000; Chanway et al. 2000), thus indicating that this strain promotes spruce tree growth by colonizing the rhizosphere.

Shishido and Chanway (1999) had also isolated a PGPR, *Pseudomonas* Sm3, from rhizosphere of 1–3-year-old hybrid spruce seedlings naturally regenerating near Mackenzie, BC, Canada (55°11′N, 122°58′W, 780 m elevation). Sm3-RN, a rifamycin-resistant derivative of Sm3, was found to colonize rhizosphere of spruce seedlings with a population density of 10^4–10^5 cfu/g rhizosphere soil and significantly enhance root and shoot weight and seedling height (Shishido et al. 1996b). Although Sm3-RN was isolated from rhizosphere, it was able to colonize root interior of hybrid spruce seedlings grown in both greenhouse and field conditions with a population size of 10^2–10^4 cfu/g root tissue and significantly promote root and shoot RGR when grown at a field site (Shishido and Chanway 2000). Internal root tissue colonization and growth promotion of spruce seedlings by Sm3-RN in field conditions was also confirmed by Chanway et al. (2000). Apart from culture-based studies, Shishido et al. (1999) also evidenced the endophytic colonization by Sm3-RN of spruce seedlings by using immunofluorescent antibody staining (IFAS) technique. Sm3-RN strain was detected in root hairs, cortical cells, and stem vascular tissues of spruce after 4 months of inoculation. It can be concluded that Sm3-RN, a PGPR, enters the spruce seedling likely through the root openings and form detectable endophytic colonies in root and stem tissues.

A lodgepole pine endophyte, *Paenibacillus polymyxa* Pw-2R (Shishido et al. 1995), was also tested for its ability to endophytically colonize hybrid spruce and promote its growth. Strain Pw-2R was able to colonize internal root and stem tissues of hybrid spruce (*Picea glauca* x *engelmannii*) with population size of 10^4–10^5 cfu/g root tissue in controlled environment experiment 5 months after inoculation and promoted seedling biomass by 57% in field trials 17 months after inoculation (Chanway et al. 2000). Effects of Pw-2R inoculation on spruce seedlings were also assessed by Shishido et al. (1996a). Endophytic colonization of hybrid spruce by culture-based technique was also evaluated by Shishido and Chanway (2000) and it was observed that this endophyte can colonize internal root tissues with a population size of 10^2–10^4 4 months after inoculation and increase shoot RGR by up to 82%. Internal tissues colonization of spruce by Pw-2R was also evaluated by IFAS technique to have a more precise evidence since culture-based

techniques could be imprecise in detecting endophytic population (Bent and Chanway 2002). Pw-2R colonized both stem vesicular tissues and root cortical tissues in a 4-month-old spruce seedling, thus establishing its endophytic nature in spruce (Shishido et al. 1999). Earlier studies have confirmed the presence of bacterial endophytes in hybrid spruce growing in regions of BC, Canada, and their role in growth promotion of spruce seedlings both in greenhouse and field conditions. Most bacterial endophytes are isolated from either root or stem tissues of plants but Cankar et al. (2005) reported the presence of bacterial endophytes in Norway spruce (*Picea abies* L. Karst) seeds. In a previous study, this group confirmed the presence of PGPR bacteria in the rhizosphere of Norway spruce trees and characterized their beneficial effects (Geric et al. 2000). Fresh seeds from four different trees of Norway spruce growing in different locations within a 36 km^2 area in Pokljuka, Slovenia (1200–1400 m elevation) revealed the presence of bacterial endophytes in seed coat, endosperm, and embryonic tissue. Most endophytes belonged to genera *Pseudomonas* and *Rahnella*, which are well known for their plant growth-promoting and N-fixing properties (Cankar et al. 2005).

6.3.4 *Pseudotsuga, Quercus, Salix, and Thuja*

The presence of bacterial endophytes has also been reported in other coniferous trees like Douglas-fir (*Pseudotsuga menziesii*) and western red cedar (*Thuja Plicata*) and deciduous trees like oak (*Quercus* L.) and willow (*Salix* L.). Bal et al. (2012) reported the isolation of endophytic bacteria from stem and needles tissues of western red cedar seedlings (2–4 years old) and trees (>20 years old) growing at a site near Boston Bar, BC, Canada (49°50′N, 121°31′W, elevation 600 m; moist warm Interior Douglas-fir zone (IDFmw)). Endophytic bacterial strains were identified as belonging to the genera *Arthrobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus*, and *Pseudomonas*. One of the strains *Paenibacillus amylolyticus* C3b was found to produce 241 pmols C_2H_4 mL^{-1} h^{-1} of ethylene in the acetylene reduction assay and was able to grow on N-free growth medium, thereby establishing its diazotrophic ability (Bal et al. 2012). Lodgepole pine bacterial endophyte, *P. polymyxa* P2b-2R (Bal et al. 2012), was also tested for its ability to colonize and promote the growth of western red cedar seedlings. Bal and Chanway (2012b) reported that P2b-2R can colonize rhizosphere of cedar with a population size of 10^5 cfu/g root but cannot colonize the internal tissues. They also reported that P2b-2R inoculation increased the foliar N content by 33% as compared to the controls 27 weeks after inoculation. Cedar seedlings inoculated with P2b-2R derived 56% and 23% of N directly from the atmosphere 27 and 35 weeks after inoculation, respectively (Table 6.2). These results were later confirmed by Anand and Chanway (2013b). Apart from deriving significant amounts of N from the atmosphere, western red cedar seedlings accumulated 45% greater shoot biomass than control seedlings 13 months after inoculation (Anand and Chanway 2013b). Another aim of this study was to investigate the endophytic colonization of cedar

seedlings. P2b-2R endophytically colonized stem and root of cedar seedlings with a population size of 10^4–10^6 cfu/g fresh tissue and needles with 10^1–10^2 cfu/g fresh tissue.

Young willow (*Salix sitchensis*) trees growing in Three Forks Park alongside the Snoqualmie river in Western Washington state, USA were also evaluated for the presence of diazotrophic bacterial endophytes by Doty et al. (2009). Ten endophytic strains capable of growing on the N-free medium were isolated and were identified as belonging to the genera *Acinetobacter*, *Herbaspirillum*, *Pseudomonas*, *Sphingomonas*, and *Stenotrophomonas*. Two of the willow isolates, *Herbaspirillum* sp. WW2 and *Pseudomonas* sp. H9zhy (WW6), reduced acetylene to ethylene in acetylene reduction assay and it was also observed that *Pseudomonas* sp. H9zhy (WW6) possess *nif* genes necessary to encode nitrogenase enzymes (Doty et al. 2009). These willow isolates along with some cottonwood isolates (Doty et al. 2005, 2009) were tested for their ability to promote the growth of a distinct host, Douglas-fir. Khan et al. (2015) prepared an endophytic consortium by mixing these endophytic isolates. Endophytic consortium was inoculated into Douglas-fir and grown in a greenhouse environment for 15 months. Inoculated seedlings had 48% greater biomass and 13% greater root length and were 16% taller than control seedlings. Two endophytic isolates, *Acinetobacter calcoaceticus* WP19 and *Rahnella* sp. WP5, were tagged with GFP to visualize endophytic colonization sites in Douglas-fir (Khan et al. 2015). Intercellular colonization of Douglas-fir root tissues by WP19 and needle tissues by WP5 was observed 3 weeks after inoculation. These results indicate that willow and cottonwood bacterial endophytes not only colonize the internal tissues of a distinct host (Douglas-fir) but also promote its growth significantly in a greenhouse environment. Such studies increase our understanding about the bacterial endophytes that could be valuable for increasing production of seedlings in forest nurseries.

More than two decades ago, Brooks et al. (1994) evaluated the role of endophytic bacteria in suppressing oak wilt of live oaks (*Quercus fusiformis*). Mature live oaks (50–70 years old) growing in sites located near Round Rock, La Grange, and Kerrville State Park areas of central Texas, USA were sampled for bacterial endophytes. After obtaining 889 endophytic isolates from the sapwood of live oaks, bacteria were screened for in vitro inhibition of *Ceratocystis fagacearum* fungus. C. *fagacearum* causes vascular disease of oaks, commonly known as oak wilt. The traditional method of eradicating oak wilt is to remove diseased trees from the site and break the connections between the healthy and diseased tree (Gibbs and French 1980; MacDonald and Hindal 1981) or injecting a fungicide into the intravascular tissues of the oak plant (Appel and Kurdyla 1992). Brooks et al. (1994) hypothesized that biological control through endophyte inoculation could be a possible and sustainable way of controlling oak wilt. Six endophytic isolates belonging to genera *Bacillus* and *Pseudomonas* were screened after in vitro evaluation of their ability to suppress C. *fagacearum*. When injected into the stem, *Pseudomonas* spp. extensively colonized live oaks. The ability of *Pseudomonas* strains to control the oak wilt pathogen in vivo was evaluated in two growth trials. Inoculation decreased the number of trees diseased by 50% and reduced the crown loss by 17%. This study

indicates that there is potential for using bacterial endophytes to suppress deleterious pathogens in tree species.

6.4 Conclusion

In a thought-provoking commentary titled "Endophytes: they're not just fungi!", in fact, research on the existence and positive effects of bacterial endophytes on tree species has lagged far behind the amount of advanced research being conducted on endophytic fungi. But there are still many unanswered questions regarding the tree endophytes and their interactions with the host. As mycorrhizal fungi are well known for their role in the forest ecosystem, in a similar manner, bacterial endophytes can provide a range of benefits to the trees and could be the future biofertilizers of forest trees as emphasized by a report published in the Science journal.

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Chapter 7
Role of Bacterial Endophytes in Plant Disease Control

A. Muthukumar, R. Udhayakumar and R. Naveenkumar

Abstract Most of the plant diseases are generated by microorganisms dominated by fungi followed by bacteria and virus. Presently, the major method for controlling plant diseases is the application of agrochemicals. Nevertheless, this method causes toxic effect to the human beings and animals. An alternative for chemicals is the application of biology which includes application of bacterial endophytes in bio-control of wide array of plant pathogens. Endophytic bacteria belongs to the class of endosymbiotic microorganisms, ubiquitous among plants that establish in between and within the spaces of all plant parts and not causing any plant disease. They create array of relationship include mutalism, cannibalistic, commensalistic and trophobiotic in nature. Most endophytes derive from soil around the plant roots or surface of the cuticle covering the leaf epidermis; although some may be obtained from the seed. Endophytic bacteria may play a major role in developing plant growth enhancement, phytoremediation, phosphate solubilization, nitrogen fixation, modulation of plant metabolism and phytohormone signalling that lead to adaptation of environmental biotic/abiotic stress. There is an increased interest in the use of endophytes for their agricultural applications that promote plant growth under cold, drought or contaminated soil structure conditions or induce disease resistance in plants.

Keywords Endophytic bacteria · Occurrence · Colonization
Mode of action · Plant growth promotion

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7.1 Introduction

Agricultural augmentation in the twentieth century has been greatly attained through the use of farm machineries, high-yielding varieties, vigorous tillage, irrigation, fertilizers and pesticides (Foley et al. 2005). This is well illustrated by the global use of fertilizers that increased from approx. 27 to 170 million of nutrient tonnes over the past 50 years before 2010 (Bumb and Baanante 1996; Heffer 2013). However, continuous use of fertilizers over a long period leads to deleterious effects on the soil. Accordingly, environmentally safe approaches have to be implemented to maintain sustainable agricultural production to overcome threats that lead to yield loss, including unfavourable environmental conditions to plant stress, as well as biotic stress induced by plant pathogens and pests. Hence, it is necessary for using endophytic bacteria for the biocontrol of plant disease and their management (Jha et al. 2013).

Bacterial endophytes have been explained as bacteria isolated from internal plant parts remain colonized in the internal tissues, not having any harmful effects to the host (Holiday 1989; Schulz and Boyle 2006). Almost 3,00,000 plant species existed on the earth. Among these, endophytes consist of a very few (Strobel et al. 2004). Of these, complete endophytic biology was studies for only few plants. Therefore, the prospects to upbring beneficial endophyties from the diverse genera inhabit in different ecosystems.

Bacteria may live as in soils or attached to the root surface or phyllosphere, and may establish symbiotic relations with plants (Smith and Goodman 1999). Unlike phytopathogens, endophytic bacteria do not cause any symptoms on host plants, and their occurrence is not related to the morphological changes that appear in plant tissues such as formation of root-nodule by symbionts. Endophytes colonize all plant parts (inbetween the spaces of the cell walls and vascular bundles of plant roots, stems and leaves, tissues or flowers, fruits and seeds) (Compant et al. 2011; de Melo Pereira et al. 2012; Trognitz 2014). Population dynamics of endophyte bacteria may vary from 100 to 9×10^9 bacteria/g of plant tissue (Misaghi and Donnedelinger 1990; Chi et al. 2005). Generally, the highest endophytic populations is found in below ground parts when compared to above ground tissues, the apoplastic movement of endophytic bacteria from roots to rice leaves has been showed (Reinhold-Hurek and Hurek 2011). Further, roots are considered as point of invasion of the potential endophytes from soil to the host plant.

Strong union amid host plant and endophytes is mediated through the action of secondary metabolites produced by the microorganisms and the host cells (Reinhold-Hurek and Hurek 2011; Brader et al. 2014). The perusal of literature revealed the varying consequences of endophytic bacteria on plant growth. Bacterial endophytes colonize plant tissue same as that of plant pathogens, which can act as biocontrol agents (Berg et al. 2005). On the other hand, innumerable reports exhibit the endophytic bacteria have the capability to manage several phytopathogens (Sturz and Matheson 1996; Duijff 1997; Krishnamurthy and Gnanamanickam 1997), insects (Azevedo et al. 2000) and nematodes (Hallmann

et al. 1997, 1998). The major mechanism of endophytic bacteria in plant disease control is—(i) to assist nutrient availability and uptake (ii) to enhance stress tolerance and (iii) to provide disease resistance (Ryan et al. 2008; Hamilton et al. 2012).

Endophytic bacteria are correlated with the enhanced plant growth by the production of hormones that increase accessibility of nutrients, such as nitrogen, potassium and phosphorus (Glick 2012). While induced disease resistance activities are allied with the abilities to produce secondary metabolites, such as antibiotics or chitinase enzyme, which can inhibit growth of plant pathogens. Hence they act as biocontrol agents (Christina et al. 2013; Wang et al. 2014). Endophytic bacteria can also induce seedling emergence and stimulate plant growth (Chanway 1997) under stress conditions (Bent and Chanway 1998). Further, endophytic bacteria have the capacity to cope with phytopathogenic fungi with induced systemic resistance (ISR) (Pieterse et al. 2014). Due to their beneficial function such as plant growth promotion and disease control, endophytes can be used in the form of bio-formulations (seed treatment, soil application and seedling dip) in agriculture.

7.2 Nature and Occurrence

Various groups of endophytic bacteria signify their role in ecosystems and plant physiology. These bacteria colonize all plant compartments, generally the intercellular and intracellular spaces of inner tissues. Initial studies on diversity of endophytic bacteria were mostly based on characterization of isolates obtained from the plants either from rhizosphere/phyllosphere region after surface disinfection. Lodewyckx et al. (2002) characterized methods for the isolation and he found that 81 bacterial species which form endophytic associations with plants. The endophytic bacteria and plant association include a vast diversity of bacterial taxa and host plant. The early studies on composition of endophytic communities revealed that different plant hosts harbour similar community of bacterial endophytes (Mundt and Hinkle 1976). The genera of *Bacillus* and *Pseudomonas* are identified as frequently occurring bacteria in agricultural crops (Seghers et al. 2006; Souza et al. 2013). The presence of different endophytic species depends mostly on plants biotic and abiotic environmental factors. A single host plant species comprises several genera and species of endophytes but the tissue type of plant or season of isolation may determine the extent of the endophytic population (Kuklinsky-Sobral et al. 2004; Rosenblueth and Martinez-Romero 2006). An extensive research work conducted on bacterial endopyte communities revealed that although endophytic bacteria colonize entire plant, the roots usually contain higher number of species. Endophytic species mostly belong to the α, β, and γ-proteobacteria subgroups and are closely related to epiphytic species (Kuklinsky-Sobral et al. 2004). Interestingly, the γ-proteo bacteria group is the most diverse and dominant. It has been reported that most of Gram-negative endophytes act as agents of biological control (Kobayashi and Palumbo 2000), while among the Gram-positive bacteria, the

dominant endophytic species are *Bacillus* species (Gupta et al. 2002; Bacon and Hinton 2007).

Most culturable endophytic species belongs to the phylum Proteobacteria, class Firmicutes, Gram-negative and also Bacteroides are less common (Reinhold-Hurek and Hurek 2011). This suggests that 50% of endophytic bacterial communities could be identified and others are over represented based on their capability to grow on synthetic medium. To obtain clear picture of the diversity of endophytic microorganisms, recently a number of studies have been concentrated on identification of unculturable endophytes using novel metagenomic analysis approaches (Akinsanya et al. 2015). To this, direct amplification of microbial DNA from plant tissue samples and application of modern bioinformatics tools allow analysis of a bacterial community composition and its phylogenetic structure inside plant organs or tissues (Chun et al. 2007; Manter et al. 2010; Sessitsch et al. 2012) examined the structure and functions of genes of bacterial endophytes colonizing rice roots in vivo. The results showed the population was superior by members of γ-proteobacteria, comprising mostly of enterobacter-related endophytes. Whereas (Tsurumaru et al. 2015) studied that endophytic colonization on tap root of sugar beet (*Beta vulgari* L.) is a metagenome, who observed that alphaproteobacteria are dominant, followed by the actinobacteria and the betaproteobacteria. Maropola et al. (2015) analysed metagenomic study of the sorghum root and stem microbiome and revealed that both were dominated by bacterial pathogens such as *Agrobacterium, Erwinia*, *Herbaspirillum*, *Microbacterium*, *Pseudomonas, Sphingobacterium* and *Stenotrophomonas* species.

7.3 Plant Colonization with Endophytes

The apical root zone having thin-walled surface of root cells includes cell elongation and the root hair zone (zone of active penetration), and the basal root zone with small cracks are the preferable sites of bacterial attachment and subsequent entry caused by the emergence of lateral roots (zone of passive penetration) (Fig. 7.1). For active invasion, endophytic bacteria must bear the abilities of production of cellulolytic enzymes to hydrolyze exodermal cell walls of plants.

As earlier, the density of bacteria in the rhizosphere and rhizoplane is always higher than in the soil which lacks substances secreted from the roots of plants (Rosenblueth and Martinez-Romero 2006) for example, with seed germination, amount of carbon and nitrogen compounds are excreted into the surrounding environment that invites a large population of microorganisms (Okon and Labandera-Gonzales 1994). The root exudates contain that colonize different bacterial genera and they differ normally according to plant species (Bisseling et al. 2009).

Root colonization or rhizospheric beneficial microorganisms are familiar biocontrol agents and plant growth promoters. They have indirect positive effects on plants with their mechanistic behaviour that mainly includes antagonism against

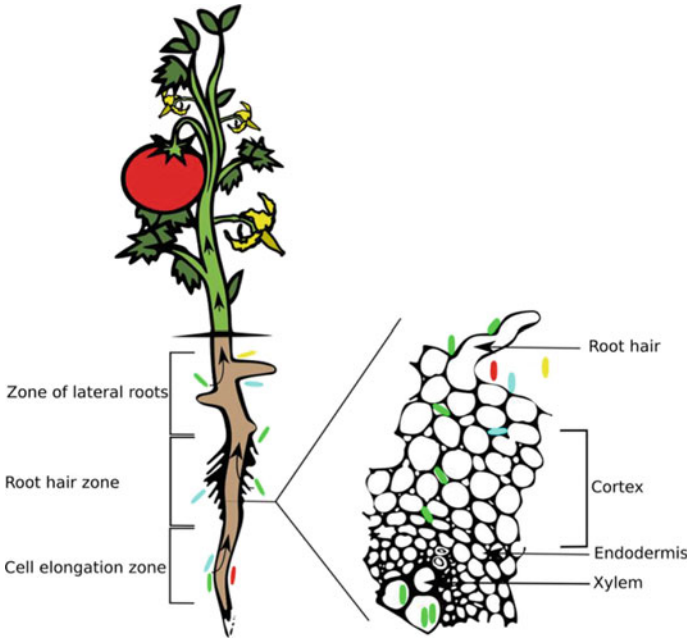

Fig. 7.1 Endophytic bacterial colonization in plants. Bacteria can enter a plant at several root zones as indicated above. Endophytes can either remain at the site of entry (indicated in *blue*) or move deeper inside or occupy the intercellular space of the cortex and xylem vessels (indicated in *green*). *Red* and *yellow* represent rhizospheric bacteria which are unable to colonize inner plant tissues

phytopathogens. Endophytic microorganisms often produce diversity of antimicrobial bioactive compound comprising extracellular chitin or lytic enzymes (glucanase and chitinase). The direct positive effects are production of phytohormones such as IAA, GA, etc. non-symbiotic nitrogen fixation, and biofortification of phosphorous and other essential nutrients include the trace elements to plants for phytostimulation and to the soil for increasing fertilization power of soil (Burdman et al. 2000). Innumerable compounds such as hydrocyanic acids (HCN), DAPG, phenazines, pyrrolnitrin, enzymes and phytohormones to protect plant from toxic effect of fungal pathogens are considered as the significant products to help endophytes to be colonized in rhizosphere (Castro-Sowinski et al. 2007; Ramette et al. 2011; Jousset et al. 2011). Besides, under iron-stress conditions in the soil and on the surface plant, endophytes produce iron-chelator molecule called siderophores used to transport iron in a competitive way and deprived for the pathogenic fungi as essential bioavailable element (Pedraza et al. 2007).

Many rhizosphere microorganisms can activate plant defence mechanisms and induce a systemic response in plants. Bacteria are able to trigger signalling pathways to produce extracellular metabolites with higher toxicity for other microorganism lead to destruction of higher pathogen, called induced systemic resistance

(ISR). Myriad of bacteria has been documented for beneficial effects, alleviation of several abiotic and biotic stresses. *Pseudomonas* and *Bacillus* sp., have been studied as potential candidate to provide ISR to plants (Chakraborty et al. 2006)

In earlier days, autofluorescent protein marker (AFP) were studied using standard microbiological enumeration associated with plant surfaces and in planta protein (AFP) (Tombolini et al. 1997; Tombolini and Jansson 1998). The colonization mechanisms of endophyte colonization have also been investigated to be utilized as β-glucuronidase (GUS) reporter system. James et al. (2002) used this technique in which *Herbaspirillum seropedicae* Z67 was inoculated on to rice seedlings via GUS stain where it acted as most severe oncoleoptiles. It was incultated that the endophytes have entered in the roots via small lesions or cracks in the root tissues at the point from where the lateral root emerged and subsequent to this become colonized in the intercellular spaces of root tissues, paerenchyma and cortical cells, and further disaminated towards xylem vessels of stems and leaves. In the exampled study, it was concluded that a compatible host plant is necessarily needed for successful colonization. An endophyte *Azoarcus* sp. strain BH72 expressed *Nif* genes in rice roots evaluated using proteomic approaches and jasmonic acid treatment to dissect rice roots responsed for colonization (which induces plant defence proteins). The strategies of adaption have been used to decipher the expression vivo expression technology (IVET) and recombination in vivo expression of bacterial gene in the rhizosphere and phyllosphere (Leveau and Lindow 2001; Preston et al. 2001; Zhang et al. 2006) The insights of these studies may provide importance of genes required by bacteria to enter, compete and be colonized in the plant and suppress phytopathogens (Table 7.1).

Table 7.1 List of endophytic bacteria isolated from major agricultural crops

Plant origin	Phylum	Endophytic bacteria	References
Black pepper	Firmicutes	*Bacillus* sp.	Aravind et al. (2009)
	Gamma proteo bacteria	*Pseudomonas* sp.	
Citrus	Firmicutes	*Bacillus cereus*, *B. lentus, B. Pumilis, B. subtilis, B. megaterium*	Araujo et al. (2001)
Cotton	Gamma proteo bacteria	*Enterobacter* sp.	Musson et al. (1995)
Canola	Firmicutes	*Bacillus* sp.	Germida et al. (1998)
	Actinobacteria	*Micrococcus* sp.	
Grapes	β-protobacteria	*Comamonas* sp.	Bell et al. (1995)
	Gamma proteo bacteria	*Pseudomonas cichori*, *Xanthomonas* sp. *Moraxella bovis*, *Enterobacter* sp.	West et al. (2010)
Kallar grass	β-protobacteria	*Azoarcus* sp.	Krause et al. (2006)
Maize	Gamma proteo bacteria	*Kilebsiella pneumoniae*	Fouts et al. (2008)

(continued)

Table 7.1 (continued)

Plant origin	Phylum	Endophytic bacteria	References
Onion	β-protobacteria	*Burkholderia phytofirmans*	Weilharter et al. (2011)
Poplar	Gamma proteo bacteria	*Serratia proteamaculans*	Taghavi et al. (2009)
Potato	β-protobacteria	*Variovorax paradoxus*	Han et al. (2011)
Rough lemon	Gamma proteo bacteria	*Pseudomonas* sp.	Gardner et al. (1982)
	Firmicutes	*Bacillus* sp.	
	Gamma proteo bacteria	*Enterobater* sp.	
Rice	Gamma proteo bacteria	*Pseudomonas* sp.	Stoltzfus et al. (1997)
	Firmicutes	*Bacillus* sp.	
Sugar beet	α-protoeobacteria	*Azospirillium* sp.	Kaneko et al. (2010)
	Actinobacteria	*Corynebacterium* sp.	
Straw berry	α-protoeobacteria	*Pseudomonas fluorescens, P. corrugate, P. tolaasii, Xanthomonas* sp.	Tanprasert and Reed (1997)
Sorghum	β-protobacteria	*Herbaspirillium seropedicae*	Pedrosa et al. (2011)
Tomato	Firmicutes	*Brebacillus brevis*	Patel et al. (2012)
	Gamma proteo bacteria	*Pseudomonas* sp. *P. syringae, P. aeruginosa*	Yang et al. (2011)
Wheat	α-protoeobacteria	*Azorhizobium* sp.	Webster et al. (1997)
	Firmicutes	*B. polymyxa*	Zinniel et al. (2002)
	Actinobacteria	*Mycobacterium* sp.	Iniguez et al. (2004)
Avacado and black grapes	Firmicutes	*Bacillus* sp.	Prasad and Dagar (2014)
Alfalfa	Firmicutes	*B. megaterium*	Ashraf et al. (2015)
Turmeric	Firmicutes	*Bacillus* sp., *B. pumilis*, *B. turingiensis*	Kumar et al. (2016)
	Gamma proteo bacteria	*P. putida*	
	Actinobacteria	*Clavibacter michiganensis*	
Switchgrass	Firmicutes	*B. subtilis, B. pumilus*	Gagne-Bourgue et al. (2013)
	Gamma proteo bacteria	*P. fluorescens*	
	Gamma proteo bacteria	*Serratia* sp.	

(continued)

Table 7.1 (continued)

Plant origin	Phylum	Endophytic bacteria	References
Cloud forest	Gamma proteo bacteria	*P. fluorescens*	Guzmán-Trampe et al. (2015)
Potato	Gamma proteo bacteria	*P. fluorescens*	Rado et al. (2015)
Devil's trumpet	Gamma proteo bacteria	*Pseudomonas* spp.	Abdallah et al. (2016)
Indian fig tree	Gamma proteo bacteria	*P. viridiflava*	Abdallah et al. (2016)
	Actinobacteria	*Streptomyces* sp.	
	Gamma proteo bacteria	*Serratia marcescens*	
Soyabean	Firmicutes	*B. megaterium*	Smita and Dipak (2015)
Tomato	Firmicutes	*Bacillus* sp.	Abbamondi et al. (2016)
	Gamma proteo bacteria	*Pseudomonas* sp.	
	α-proteobacteria	*Rhizobium* sp.	
	Proteobacteria	*Agrobacterium* sp.	
Fenugreek	Firmicutes	*Bacillus* sp.	Jasim et al. (2015)
Khejri tree	Firmicutes	*B. subtilis*	Rekha et al. (2015)
Sugarcane	Gamma proteo bacteria	*Enterobacter*	Rodrigues et al. (2016)
	Firmicutes	*Bacillus* sp.	Anjum and Chandra (2015)
Japanese honeysuckle	Firmicutes	*Bacillus* and *Paenibacillus*	Zhao et al. (2015)
Apple	Proteobacteria	*Micrococcus luteus*	Miliute et al. (2016)
	Firmicutes	*B. subtilis*	
	Gamma proteo bacteria	*P. aeruginosa*	
Sugarcane	Gamma proteo bacteria	*P. fluorescens*	Marcos et al. (2016)

7.4 Endophytic Bacterial Genomics

Till now, very few bacterial endophytes have been sequenced to know their genomic map. There are several bacterial endophytes such as *Enterobacter* sp. 638, *Stenotrophomonas maltophilia* R551-3, *Pseudomonas putida* W619, *Serratia proteamaculans* 568 and *Methylobacterium populi* BJ001 still under investigation to assess their genomic sequences (http://www.jgi.doe.gov/). *Azoarcus* sp. strain

BH72 has been studied for nitrogen fixation abilities at genomic level and complete genome was matched with related soil- and plant-associated bacteria (Hurek and Reinhold-Hurek 2003; Krause et al. 2006). Many plant-associated bacteria and pathogens contain N-acyl homoserine lactone-(AHL) for quorum-sensing system (Preston et al. 2001; Buttner and Bonas 2006). Nevertheless, (Krause et al. 2006) another factor for plant microbe interaction has been identified encoded by BH72 genome. It includes Tpe I and II protein secretion system with Type IV surface polysaccharides on pili, and flagella so as to produce chemotaxis proteins and ferric siderophore uptake systems. The valuable biological insight was provided by BH72 genome. In this perspective, it is clear that as much genome sequences of endophyte will be available, much exploration of the mechanisms involved in successful endophyte colonization.

7.5 Post Genomic View of Bacterial EndophyteS

Metagenomics analysis of endophytic bacteria associated with rice plant confirmed few traits that also exist in endophytes. Therefore, these traits evaluated through genomic analysis become potentially important for their interactions with plants. These include entire set of highly specialized bacterial secretion system, except type III due to its non-conserved nature among endophytes that are associated with rice plants. On the other hand, production of cellulolytic and pectinolytic enzymes are of major concerns along with the production of glagellins. Majority of other enzyme also involved in these interactions in terms of degradation of reactive oxygen species (ROS). Further, receptors and transporters for iron uptake also play significant role in intracellular microbial interaction with plant through quorum-sensing systems (QS). Several degradative pathways of plant metabolites and various plant growth-promoting and biocontrol traits such as ACC-deaminase activity, biological nitrogen fixation (BNF) and production of phytohormones and volatile and non-volatile antimicrobial compounds.

Applying postgenomic approaches, such as metaproteomisa, metaproteogenomics and metatranscriptomics, can link genomic potential with plant–endophyte interactions. Recently, a metaproteogenomic approach was used to study the microbial communities in the phyllosphere and rhizosphere of rice (Knief et al. 2011).

7.6 Plant Growth-Promoting Endophytes

Some of the endophytes act as biocontrol agents while others increase plant growth through the production of nutrients and minerals such as nitrogen, phosphate and other nutrients. Infact, quite a few endophytic bacterial genera such as

Pseudomonas, Bacillus, Xanthomonas and *Erwinia* are growth promontory as well as inhibit various diseases causing phytopathogen in plants. Endophytes also promote plant growth by enhance phosphate solubilisation (Verma et al. 2001; Wakelin et al. 2004) IAA production (Lee et al. 2004) siderophore production (Costa and Loper 1994) and facilitating vitamins to plants (Pirttila et al. 2004). The other physiological adjustments include osmotic, stamal regulation and morphological modifications such as alteration in root morphology enhanced immobilization of minerals nutrients along with nitrogen accumulation and its metabolism (Compant et al. 2005a, b).

Free-living and endophytic bacteria use similar mechanism to enhance plant growth and development beside, being different in their efficiency for their beneficial effect. Plant Growth-Promoting Rhizobacteria (PGPR) are able to colonize in the root vicinity thereby promoting plant growth and increase yield. Phytohormones such as IAA contributes for root abundance and hence, provide enhanced minerals and nutrient uptake to the plant. Production of diffusible and non-diffusable antifungal metabolites assists in the biocontrol soil-borne fungi. The detailed mechanism of action of endophytic bacteria is given below (Fig. 7.2).

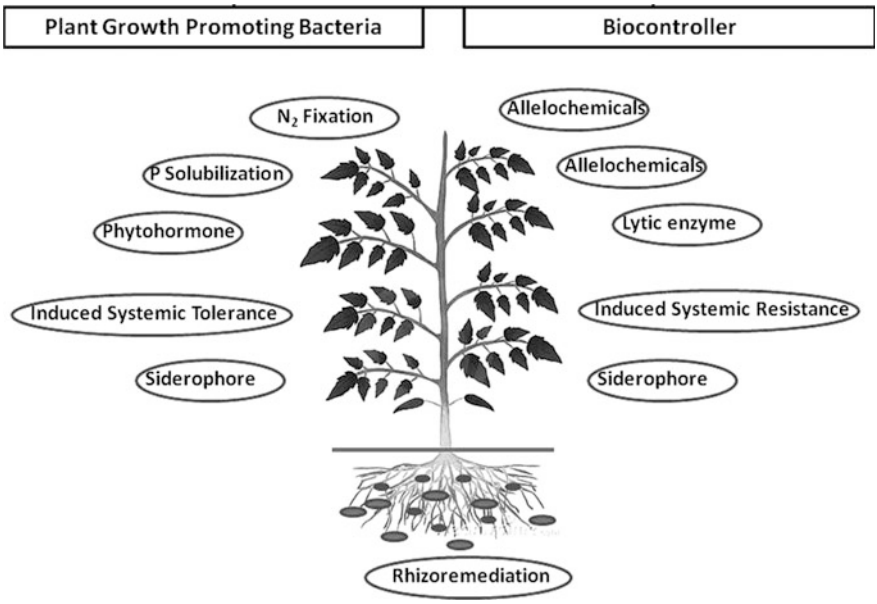

Fig. 7.2 Mechanism of action of endophytic bacteria

7.7 Bacterial Endophytes as Biocontrol Agents for Wilt

Endophytic bacteria isolated from live oak stem showed in vitro antagonism against *Colletotrichum fagacearum* causing Oak wilt (Brooks et al.1994). The endophytic bacterium *Burkholteria cepacia*, isolated from *Asparagus* exhibited antagonistic activity against banana wilt (Pan et al. 1997). Tomato seedlings treated with endophytic *P. fluorescens* as seedling dip increased resistance to wilt disease (M'Piga et al. 1997). Endophytic bacteria isolated from potato tubers showed better antagonistic activity against *F. avenaciarum, F. sambucinum* and *F. oxysporum* causing wilt of many tuber and commercial crops (Sturz et al. 1999).

Endophytic bacteria isolated from mustard and tomato plants, completely inhibited the mycelail growth of *V. dahliae* and *F. oxysporum* f.sp. *lycopersici* in vitro and in vivo, it reduces the disease incidence and also increases the plant growth parameters (more than 75% and increased the plant height and shoot dry weight) (Nejed and Johnson 2000). Endophytic *Pseudomonas* sp. (PDBCEN 8) exhibited maximum mycelia growth inhibition of *Fusarium udum* on PDA. They also recorded that endophytic *Pseudomonas* sp. (PDBCEN 7) exhibited maximum inhibition of *R. solani* on PDA. The same trend was followed in the endophytic *Pseudomonas* sp. (PDBCEN 3) against tomato *Fusarium* wilt (Rangeshwaran et al. 2002). Bhowmik et al. (2002) isolated endophytic bacteria from root and stem region of cotton seedlings and tested for its antagonistic activity against two fungal and one bacterial disease in cotton. Among these, five pseudomonads were highly antagonistic to *R. solani, S. rolfsii* and *X. axonopodis* pv. *malvacearum* (O'Sullivan and O'Gara 1992). Nagarajkumar et al. (2004) also reported that the production of siderophores, secondary metabolites and cell wall degrading enzymes by *Pseudomonas* strains may be responsible for the effective control of plant pathogens including *F. oxysporum* and *R. solani*. Ziedan (2006) revealed that peanut seeds were soaked with endophytic bacterial suspensions before sowing reduced the infection by *Aspergillus niger* and *F. oxysporum* colonization over peanut seed at 30 days after harvesting.

The PGPR strains of Pf1 and TRC 54 were effective in reducing the mycelial growth of *F. oxysporum* f.sp. *cubense* in vitro. The mycelia growth inhibition might be due to the production of enzymes and antibiotics by PGPR strains (Akila et al. 2011). Nandhini et al. (2012) who reported that entophytic bacteria were isolated from root, stem, leaves and fruits and tested for its antagonistic activity against *Fusarium* wilt disease in tomato. All the isolates belonging to four bacterial genera viz., *Bacillus, Pseudomonas, Klebsiella* and *Citrobacter*. The results revealed that only 50% of the isolates exhibited strong antagonistic activity against tomato wilt pathogen. Sundaramoorthy et al. (2012) who reported that the consortium of rhizospheric and phyllospheric bacterial strains (*P. fluorescens* (Pf1) and *B. subtilis* (EPCO16 and EPC5) strains) reduced *Fusarium* wilt incidence in chilli by 17–30% compared to control plants.

Seventy one bacterial endophytes were isolated from root and corm tissues of banana plants. Among these, six endophytic and four rhizospheric bacterial isolates effectively reduced the incidence of *Fusarium* wilt of banana. Later, combinations of these potential bacteria were evaluated for biocontrol abilities. These combination resulted complete suppression of *Fusarium* wilt thereby increased plant growth than that of control (Thangavelu and Muthukathan 2015).

7.7.1 Rots and Damping-Off

One seventy endophytic bacterial strains isolated from cotton and tested against *Rhizoctonia solani* (damping-off in cotton). Among these, 40 strains protected cotton plants from *R. solani* infection (Chen et al. 1995). Benhamou et al. (1998) revealed seed bacterization (*Serratia plymuthica*) with cucumber protected the seedlings from infection by damping-off. Cucumber seeds treated with endophytic bacterium *S. plymuthica* reduce the incidence of damping-off (Benhamou et al. 2000). Bhowmik et al. (2002) reported that seed treatment with endophytic bacterium (PR 8) reduced the incidence of damping-off disease of cotton. On the other hand, Anith et al. (2003) isolated a strain PN-026 from underground shoot portions of rooted cuttings of black pepper and tested against foot rot of pepper. The results revealed that strain PN-026 showed more efficient in reducing *Phytophthora capsici*, which causes severe infestation of foot rot disease. Kishore et al. (2005) reported that *P. aeruginosa* from groundnut rhizosphere (GRE 175) was highly inhibitory to the growth of *S. rolfisii*. Muthukumar (2008) tested nine endophytic bacterial isolates obtained from chilli plants, some of them (isolated from stem and root) exhibited higher inhibition of *P. aphanidermatum* (51.4, 41.7 and 40.0%) causing chilli damping-off. The maximum inhibition on the mycelia growth of *R. bataticola* was in chickpea by *P. fluorescens* strains PFBC-25 and 26 (Khan and Gangopadhyay 2008). Bacterial endophytes (46 strains) obtained from amaranthus and tested against *R. solani* by dual culture technique. Among these, six bacteria exhibited highest mycelia growth inhibition of *R. solani* (Uppala et al. 2009).

About 67 bacterial endophytes were isolated from cassava; they were subjected to 16S rRNA sequencing and FAME analysis. The bacterial profile revealed that 25% of all endophytic isolates belonged to the genus *Bacillus*. Among these, the isolate *B. pumilus* MAIIIM4a showed a strong inhibitory activity against *R. solani*, *P. aphanidermatum* and *S. rolfsii* (Pereira de Melo et al. 2009).

Overall 40 antagonistic bacteria obtained from rhizosphere soil (CRB-1 to CRB-20) and roots (CREB-1 to CREB-20) of chickpea plants. Among these, the isolate CRB-13 and CREB-13 showed maximum inhibition on the mycelial growth of *R. bataticola* (Veena et al. 2014).

7.7.2 Blights and Leaf Spot

Foliar application with *Pseudomonas* spp. induced disease resistance in rice against sheath blight pathogen. In spite of the absence of this bacterium on plant surfaces, its presence in the internal stem led to suppression of disease (Krishnamurthy and Gnanamanickam 1997). Endobacteria bacterium *B. subtilis*, isolated from xylem fluid of chestnuts, suppressed the growth of chestnut blight pathogen, *Cryphonectria parasitica* in vitro. The same bacteria reduced the lesion areas on stems, when applied 3 days prior to challenge inoculation (Wilhelm et al. 1998).

The endophytes, viz., *Bacillus circulance* and *Serratia marcescense* supplemented with chitin inhibited the conidial germination of early and late tikka leaf spot in groundnut (Kishore et al. 2005). Four endophytic bacteria (OS-9, OS-10, OS-11 and OS-12) were isolated from healthy leaves of *Ocimum sanctum* and tested against five plant pathogens namely *R. solani, S. rolfsii, F. solani, A. solani* and *C. lindemuthianum*. Of these, the bacterial strain OS-9 was highly inhibitory to the growth of *R. solani, A. solani, F. solani* and *C. lindemuthianum* while OS-11 alone was found antagonistic tot *A. solani* (Kalraa et al. 2010). The culture filtrate of endophytic bacteria CE-6 exhibited the highest inhibition on the mycelia growth of (61.3%) of *Cercospora* in vitro (Hima et al. 2013).

7.7.3 Powdery Mildew

The endophytic *B. subtilis* strain E1R-j exhibited high antifungal activity against wheat take all disease both in glasshouse and field conditions (Liu et al. 2009). Recently (Gao et al. 2015), who isolated 14 endophytic bacterial strains from wheat leaves and tested against *Blumeria graminis* f.sp. *tritici* causing wheat powdery mildew disease. The results revealed that *B. subtilis* strain (E1R-j) significantly reduced per cent disease index by 90.97% in pot culture under greenhouse conditions.

7.7.4 Rust

The endophytic bacteria was isolated from leaves and branches of *Coffea arabica* and *Coffea robusta* and were tested against leaf rust pathogen *Hemileia vastatrix* by detached leaf and leaf disc method. Of these, the bacterial isolates TG4-Ia (*Bacillus lentimorbus* Dutky) and TF9-Ia (*Bacillus cereus* Frank & Frank) exhibited highest growth inhibition against coffee rust pathogen (Shiomi et al. 2006). An endophytic bacteria E1R-j was isolated from wheat leaves showed strong inhibitory effect on wheat stripe rust in both greenhouse and field conditions (Li et al. 2013).

7.7.5 Downy Mildew

Sixty different endophytic bacterial isolates belonging to different genera were isolated from root and stem tissues of five medicinal plants (*Cymbopogon citratus, Azadirachta indica, Phyllanthus emblica, Boerhaavia diffusa* and *Boerhaavia repens*) and two agricultural crops (*Pisum sativum* and *Sorghum bicolor*) and one weed plant (*Parthenium hysterophorus*) and were tested against pearl millet downy mildew disease. The peral millet seeds were treated with endophytic bacteria; *P. fluorescens* ISR 34 and *Bacillus* sp. ISR 37 recorded greater control of downy mildew disease, by 68 and 63%, respectively. From the above results it is concluded that the endophytic bacterial strains not only reduces the disease incidence but also increased the plant growth by way of induced systemic resistance (Chandrashekhara et al. 2007). Endophytic bacterial isolates obtained from cucumber leaves and tested against *Pseudoperonospora cubensis* causing downy mildew disease. The results revealed that the strain CE8 recorded high level of inhibition against *P. cubensis.* In the field test, the same strain showed high level of control efficacy (42.1%) and disease reduction in cucumber. Further, the phylogenetic analysis based on 16S rDNA identified the strains as *Bacillus* sp. (Sun et al. 2013).

7.7.6 Basal Stem Rot/Ganoderma Wilt/Thanjavur Wilt

An endophytic bacterium *Pseudomonas cepacia* (B3) and *Pseudomonas aeruginosa* (P3) isolated from root tissues of oil palm exhibited strong inhibition on the growth of *G. boninense* causing ganoderma wilt (Dikin et al. 2003). Histological studies revealed that bacteria endophytes confined to the vascular bundles of the roots taken from symptomless palms (Zaiton et al. 2006). Total of 581 endophytic bacteria were isolated from root tissues of oil palm and tested against *Ganoderma lucidum* cause of wilt pathogen. Among these, three endophytic bacteria namely *Pseudomonas aeruginosa* GanoEB1, *Burkholderia cepacia* GanoEB2, and *Pseudomonas syringae* GanoEB3 were highly effective in inhibiting the mycelia growth of test pathogen. All the three isolates were under field condition. The results revealed that the only isolate *P. aeruginosa* GanoEB1 was highly effective in controlling disease incidence of 13.3–26.7% compared to control (60%) (Ramli et al. 2016).

7.7.7 Post Harvest Fungal Diseases

Finite efforts were made by using endophytic bacteria for the control of storage diseases. Endophytic bacteria were tested to control stone fruit rot pathogens *Monilinia laxa* and *Rhizopus stolonifer* (Pratella et al. 1993). One hundred and

twenty two bacterial strains isolated from different fruits including red pepper, tomato, white plum, egg plant and zucchini. Of these, 20 strains were able to control *M. laxa* in apricot and plum fruits. *R. stolonifer* was less susceptible to antagonistic bacteria than *M. laxa* and only one strain effectively controlled *R. stolonifer* (Pratella et al. 1993).

Endophytic bacteria (*B. subtilis*) isolated from stored apples have been used in the biocontrol of post harvest diseases of apple (Sholberg et al. 1995). Further, an inhibitory compound acidic peptide produced by *B. subtilis*, was responsible for the inhibition of *Botrytis cinerea* but not to *Penicillium expansum* (Bechard et al. 1998). The acidic peptide had a wide spectrum activity against Gram-negative bacteria. Similarly, 175 endophytic bacterial strains were isolated from vegetable produce and were screened for control of *B. cinerea* on pears (Bacon et al. 2001).

Two bacterial strains, *B. amyloliquefaciens* and *B. pumulis*, were effective. Incubating fruits treated with these bacteria at 200 °C for 24 h before cold storage were significantly protected against *B. cinerea* (Mari et al. 1996). The endophytic bacterium *Bacillus thuringiensis* is capable of releasing volatile substances that lead to the inhibition of *Fusarium sambucinum* in potato tubers (Sadfi et al. 2001). Two hundred and fifty eight endophytic bacteria were isolated from chilli leaves and screened against chilli fruit rot pathogen *Colletotrichum capsici* by fruit bioassay method. Of the endophytes tested, *B. megaterium* (ENB-86) recorded the highest suppression of lesion development in chilli fruits (59.66%) (Ramanujam et al. 2012).

The endophytic bacterium *B. lentimorbus* showed highest inhibition on the development of *Botrytis cinerea* causing grey mould disease fruits. This might be due to the production of antifungal substances alpha- and beta-glucosidase (Cheng et al. 2015), while fruits treated with formulation of *Bacillus cereus* CE3 showed effective control of chestnut blight and other fruit rot caused by *Endothia parasitica* (Murr) and *Fusarium solani* and also increased the shelf life of fruits (Cheng et al. 2015). Some other examples of endophytic bacteria against fungal pathogens are shown in Table 7.2.

7.7.8 Nematode Diseases

Endophytic bacteria have an additional advantage in control of phytoparasitic nematodes since the injuries produced by nematodes favour for the entry of bacteria and colonize the root surface and their introduction into the root tissue (Bookbinder et al. 1982; Khan 1993). In cotton and tomato root knot nematode infection, peanut root knot and reniform nematode infection can be effectively controlled by using *B. subtilis* (Sikora 1988). Seven endophytic bacteria, *Aerococcus viridans, B. megaterium, B. subtilis, P. chlororaphis, P. vesicularis, S. marcescens* and

Table 7.2 Biocontrol of endophytic bacteria against fungal pathogens

Endophytic bacterial isolates	Plant origin	Pathogenic fungi	Author
B. pumilus and *Pseudomonas* spp.	Oak	*Fusarium* spp.	Brooks et al. (1994)
Bacillus spp.	Cucumber	*Collectotrichum orbucullariae*	Raupach and Kloepper (1998)
Burkholderia sp.	Sugarcane	*Ustilago scitaminea* and *Fusarium* spp.	Raupach and Kloepper (1998)
Bacillus spp.	Tomato	*F. oxysporum* f.sp. *lycopersici*	Benhamou et al. (2000)
P. aeruginosa 7 NSK2	Tomato	*Botrytis cinerea*	Audenaert et al. (2002a, b)
P. fluorescens EP1	Sugarcane	*Colletotrichum falcatum*	Senthil et al. (2003)
P. fluorescens CHAO	Mousear cress	*Peronospora parasitica*	Lavicoli et al. (2003)
P. fluorescens GRP3	Rice	*Rhizoctonia* sp.	Pathak et al. (2004)
P. putida 5-48	Oak	*Ceratocystis fagacearum*	Compant et al. (2005c)
Burkholderia cepacia		*Fusarium*	Quan et al. (2006)
Bacillus and *Pseudomonas*	Wheat	*F. graminearum*	Nourozian et al. (2006)
B. subtilis and *P. fluorescens*	Peanut	*Aspergillus niger* and *Fusarium oxysporum*	Ziedan (2006)
Burkhloderia phytofirmans Ps JN	Grapevine	*Botrytis cinerea*	Compant et al. (2008)
Bacillus sp.	Cacao	*Pyhtophthora capsici*	Melnick et al. (2008)
B. pumilus SE34	Pea	*F. oxysporum* f.sp. *pisi*	Chaudhary et al. (2009)
P. fluorescens PICF7 and *P. putida*	Olive	*Verticillium dahliae*	Prieto et al. (2009)
B. subtilis	Wheat	*Gaemanomyces graminis tritici*	Liu et al. (2009)
Bacillus spp, *Pseudomonas* spp.	Peanut	*Sclerotinia sclerotiorum, S. minor, S. rolfsii* and *Fusarium solani*	Tonelli et al. (2010)

(continued)

Table 7.2 (continued)

Endophytic bacterial isolates	Plant origin	Pathogenic fungi	Author
Pseudomonas and *Burkholderia*	Banana	*F. oxysporum* f.sp. *cubense*	Fishal et al. (2010)
P. fluorescens 63-28	Pea	*Pythium ultimum* and *F. oxysporum* f. sp. *pisi*	Ardebili et al. (2011)
P. fluorescens 63-28	Tomato	*F. oxysporum* f.sp. *radicis-lycopersici*	Vanitha and Umesha (2011)
Brevibacillus brevis	Tomato	*Botrytis cinerea*	Yang et al. (2011)
B. subtilis	Loblolly pine	*F. circinatum*	Soria et al. (2012)
Bacillus spp., *Pseudomonas* spp.	Soyabean	*R. solani, F. oxysporum* and *S. rolfsii, C. truncatum, A. alternata, Macrophomina phaseolina*	Dalal and Kulkarni (2013)
B. subtilis and *B. megaterium*	Toromiro tree	*Verticillium dahliae*	Lin et al. (2013)
Bacillus sp.	Plants	*F. oxysporum* and *R. solani*	Ohike et al. (2013)
Pseudomonas spp.	Cucumber	*F. oxysporum* f.sp. *cucumerinum*	Ozaktan et al. (2015)
Pseudomonas spp.	Mousear Cress	*V. dahliae*	Iavicoli et al. (2003)

Sphingomonas paucimobilis from cotton and cucumber plants and tested against cucumber root knot nematode. Seed bacterization with endophytic bacteria completely protected cucumber seedlings from *M. incognata* infection (Hallmann et al. 1995).

Culture filtrate of *P. fluorescens* strains CHA0 or CHA0/PME3424 were tested against tomato root knot nematode. The results revealed that the inoculum levels of 10^7, 10^8, 10^9 cfu/g showed greater disease control under glasshouse conditions (Siddiqui and Shaukat 2003). An endophytic bacterium *B. subtilis* strains EPb5, 22, 31 and EPC 16 were effective against root knot nematode, burrowing nematode, root lesion nematode infection in banana (Jonathan and Umamaheswari 2006). Seed bacterization with culture filtrates of endophytic bacteria EB19, EB18, EB16 and EB3 significantly reduced the number of adult females. In another treatment, plants treated with culture filtrates of *B. subtilis, B. cereus* and *Arthrobotrys cladodes* reduced the soil population of *M. incognita* (Vetrivelkalai et al. 2009).

7.7.9 Bacterial Diseases

B. subtilis was isolated from healthy chestnut trees showed strong antagonistic activity against *Cryphonectria parasitica* cause of chestnut blight (Wilhelm et al. 1998). Five strains of *Pseudomonas* inhibited the growth of *X. axonopodis* pv. *malvacearum* and also increased cotton seed germination and seedling growth (12.8%; 22.4%) by 12.8%) (Mondal 1999). The endophytic bacterium *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* produces several antibiotics (surfactin, iturin, bacillomucine; azalomycin F, surfactin, arthrobactin; surfactin, amphomycin, arthrobactin and valinomycin) which are highly inhibitory to the growth of *X. campestris* pv. *campestris* because of black rot of crucifers (Wulff et al. 2002).

The cotton seeds treated with the endophytic bacterium (Endo PR8) reducing cotyledonary infection with black arm of cotton is caused by *X. campestris* pv. *malvacearum* (Bhowmik et al. 2002). Before planting grapevine shoots should be dipped with endophytic bacterium that produced highest fresh weight of the shoots and roots, and quick growth with more lignin deposits (Barka et al. 2002). Similarly, cotton seeds treated with bacterial endophyte (EPCO 102) showed increased plant vigour under in vivo (Rajendran et al. 2006). Endophytic bacterium (PfG32) was isolated from root region of onion plants and tested against bacterial wilt in tomatoes as seedling dip resulted in reduced incidence of wilt disease because it produces secondary metabolites (Mulya et al. 2006). Foliar spraying and seed soaking with bacterial antagonist *Delftia tsuruhatensis* (strain HR4) was isolated from root region of rice plants showed reduced the bacterial blight infection in range of 7–32% (Han et al. 2005).

Under greenhouse conditions, endophytic *B. subtilis* strain Lu144 remarkably protected mulberry plants against *Ralstonia solanacearum* causing bacterial wilt disease (Ji et al. 2008). Bacterial endophytes such as *Pantoea agglomerans, Pseudomonas* sp. and *Curtobacterium luteum* reduced the growth of *Erwinia carotovora* (Figueiredo et al. 2009). Ninety three isolates of rhizobacteria were tested against *Xanthomonas axonopodis* pv. *malvacearum.* Of these, *B. subtilis* B49 recorded highest inhibitor on the growth of pathogen in vitro and highly effective in controlling bacterial blight of cotton under greenhouse and field conditions (Salaheddin et al. 2010). Bacterial endophytes (*B. amyloliquefaciens* Bg-C31) isolated from *Bruguiera gymnorhiza* showed to be effective in controlling bacterial wilt of chilli under pot and field condition (Hu et al. 2010).

Consortium of endophytic bacterial exhibited higher disease reduction of bacterial blight by two seed-dipping treatments of 24 and 48 h incubation time. The combined application of bacterial endophytes showed better disease reduction of bacterial leaf blight in rice (Susilowati et al. 2012). Endophytic bacterium, *B. subtilis* was applied as seedling dip, soil and foliar application resulted in reduced the bacterial blight infection in rice under laboratory and field condition and it was found to increase the plant growth and yield (Nagendran et al. 2013).

P. fluorescens strain (PDY7) was highly effective in reducing the incidence of bacterial blight of rice (58.83 and 51.88%) under glass house and field condition. This is mainly due to the production of antibiotics called 2,4-diacetylphloroglucinol (DAPG) (Velusamy et al. 2013). Twenty six bacterial strains isolated from leaf, root and stem region of mangrove plant (*Rhizopora mucronata*). Among these, highest number bacterial isolates from leaf (38.5%) followed by root (34.5%) and stem (26.9%). Of these, five bacterial strains namely *Serratia, Bacillus, Pseudomonas, Micrococcus* and *Enterobacter* exhibited broad-spectrum of antagonistic activity against fungal and bacterial pathogen (Jose and Christy 2013). Among the bacterial strains tested, strain MB04 and MB08 were highly inhibitory to the growth of *X. campestris pv. oryzae* causing rice bacterial blight (Yuliar 2014).

The endophytic bacteria isolated from tomato plants tested against bacterial wilt pathogen. Of the isolates tested, only Ps1 and Ps8 can inhibit *R. solanacearum* in vitro using seed coat method. In in vivo test, 30 days old tomato seedlings were soaked with endophytic bacteria showed 8.07–9.19% disease suppression within 15–16 days incubation period (Purnawati et al. 2014). Cotton seeds treated with endophytic bacteria strains *B. subtilis* UFLA285 recorded the lowest bacteria blight incidence of 26% (de Medeiros et al. 2015). Four endophytic bacteria isolated from potato stem tissue and it was tested against the growth of *Streptomyces scabies* in agar plate method. The results revealed that all the isolates were highly inhibitory to the growth of test pathogen (Flatley et al. 2015).

7.8 Conclusion

Plant pathogenic fungi particularly *Pythium, Phytophthora, Sclerospora, Rhizoctonia, Peronosclerospora* and *Plasmopara* cause enormous crop losses. At present, fungicides are the only source to control plant diseases but they have several disadvantages that (i) many of them are toxic to human being and animals, and (ii) that develop further resistance to the pathogen. Several important questions remain unanswered concerning the practical use of endophyte 'supplements' in agriculture. However, with the correct management, they hold potential for the control of current and emerging pathogens, as well as biotic stresses, as we encounter deviation in these through climate change. This is likely to be achieved through a better understanding of signalling between the host plant and the microbiome, and, ultimately, the manipulation of root exudation profiles to recruit a more beneficial root microbiome, of which the endosphere is an integral part. The quality of these BCAs can be further increased by using fundamental knowledge to improve methods for their production and to increase their shelf life.

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Chapter 8
Endophytic Actinobacteria for Sustainable Agricultural Applications

M.F. Carvalho, Y. Ma, R.S. Oliveira and H. Freitas

Abstract Endophytic actinobacteria have the capacity to establish intimate associations with plants and colonize their inner tissues without causing apparent disease symptoms. They can protect plants by producing bioactive compounds that act as plant growth promoters or biological control agents and, in return, obtain nutrition and protection from the host plant. The application of endophytic actinobacteria in agriculture has attracted increasing attention. We address isolation and identification methods and the occurrence and diversity of endophytic actinobacteria in agricultural crops. Attention is given to the roles of endophytic actinobacteria in plant growth and development and health promotion for sustainable agriculture is discussed.

Keywords Actinobacteria · Endophytes · Sustainable agriculture
Plant growth · Promoting bacteria · Isolation · Biocontrol

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8.1 Introduction

Actinobacteria constitutes a major phylum within the domain Bacteria and includes six classes: Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, and Thermoleophilia, with the class Actinobacteria comprising 43 of the 53 families integrating the phylum (Barka et al. 2016; Gao and Gupta 2012; Goodfellow et al. 2012). Actinobacteria are Gram-positive mostly aerobic bacteria exhibiting diverse morphologies that range from unicellular organisms to filamentous forms. Due to their growth style often involving the formation of branching hyphae that can generate dense mycelia and produce spores, these microorganisms were misclassified for a long time as fungi. However, they are indeed prokaryotes having no nuclear membrane. Actinobacteria typically have a high G+C content in their genomes (>50%), and are commonly known for their remarkable capacity to produce bioactive compounds. More than half of the bioactive substances described in the literature is attributed to microorganisms belonging to this phylum (Barka et al. 2016; Berdy 2005), including antibiotics, anticancer agents, immunosuppressive agents, antiviral agents, antioxidants, enzymes, plant growth hormones, etc., that are highly important for applications in medicine, industry and agriculture (Castillo et al. 2002; Fiedler et al. 2008; Igarashi et al. 2007; Strobel and Daisy 2003). Within Actinobacteria, the genus *Streptomyces* is particularly prolific in the production of a wide range of bioactive compounds, being responsible for the production of ca. 80% of all natural products produced by actinobacteria, including agriculturally relevant compounds like insecticides and herbicides, holding a biosynthetic capacity that remains without parallel in the microbial world (Berdy 2005; Jizba et al. 1991; Tanaka and Omura 1993). These microorganisms are widely distributed, being found in both terrestrial and aquatic ecosystems, including marine environments. They are common inhabitants of soils where they spend a significant part of their life cycles as semi-dormant spores, and constitute ca. 20–30% of the rhizospheric microbial community (Bouizgarne and Ben Aouamar 2014; Coombs and Franco 2003). Actinobacteria are mostly saprophytic microorganisms that play important ecological roles in the recycling of nutrients, in the decomposition of organic matter, especially complex polymers derived from dead plants and animals, like lignin, starch, and chitin (Coombs and Franco 2003; Minotto et al. 2014; Sharma 2014), in the degradation of agricultural and urban wastes as well as in the removal of several environmental pollutants, such as petroleum, dyes, and other recalcitrant compounds (Amorim et al. 2014; Bagewadi et al. 2011; Kekuda 2016; Khedkar and Shanker 2015). Most actinobacteria are mesophilic, having optimal growth temperatures between 25 and 30 °C, and grow in soils with a neutral pH.

8.2 Actinobacterial Endophytes

Many actinobacteria have the capacity to establish intimate associations with plants and colonize their inner tissues without causing apparent disease symptoms, being defined as endophytic actinobacteria (Qin et al. 2009; Schulz and Boyle 2006; Stone et al. 2000; Strobel and Daisy 2003). Different parts of the plant can be colonized, including roots, stems, leaves, seeds, flowers, and fruits. *Frankia* was the first isolated actinobacterial endophyte, and is known for inducing the formation of nitrogen-fixing nodules in non-leguminous plants (Benson and Silvester 1993; Callaham et al. 1978; Coombs and Franco 2003). Endophytic actinobacteria play an important role in the protection of plants by producing bioactive compounds that can act as plant growth promoters, biological control agents or alleviate stress effects in plants, while in return these microorganisms can obtain nutrition and protection from the host plant (Cao et al. 2005; Conn et al. 2008; Goudjal et al. 2013; Igarashi et al. 2002; Yandigeri et al. 2012). Many studies also indicate that endophytic actinobacteria are capable of producing a wide range of pharmaceutically relevant bioactive compounds such as antimicrobial, antitumor, anti-inflammatory, antiviral agents, etc., including the production of metabolites bioactive against drug resistant pathogens (Golinska et al. 2015; Savi et al. 2015; Singh and Dubey 2015; Zhang et al. 2012). In addition, PKS and NRPS gene clusters, which are behind the synthesis of a wide variety of secondary metabolites, have also been shown to be present in many endophytic actinobacteria (Luo et al. 2013).

It is thought that almost every plant on earth hosts one or more endophytic microorganisms, where actinobacteria are included (Golinska et al. 2015; Kekuda 2016; Strobel and Daisy 2003). Endophytic microorganisms may originate both from the rhizosphere or phyllosphere and may enter plants through naturally occurring wounds or epidermal root hairs, or through the production of hydrolytic enzymes, such as cellulase and pectinase (Dudeja et al. 2012; Suman et al. 2016). Once inside the plant they can become installed at the entry location or spread through the different parts of the plant, where they may colonize the interior of the cells, intercellular spaces, or vascular systems (Suman et al. 2016). Due to their several beneficial effects in plants, endophytic actinobacteria are very promising biological resource that can be applied in environmentally friendly and sustainable agricultural approaches to control plant diseases and promote plant health and growth (Kunoh 2002). The capacity of endophytic actinobacteria to colonize seeds is particularly relevant due to the possibility of transmission of the endophytic community to the next generation (Tchinda et al. 2016).

Culture-dependent and culture-independent methods have revealed an increasing number of plants, including crops, hosting endophytic actinobacteria. Studies indicate that these microorganisms are among the predominant phyla inside the plants (Manter et al. 2010; Sessitsch et al. 2012). The potential of these microorganisms for agricultural applications is enormous, where they can be used as microbial inoculants for increasing crop yields and controlling pathogenic agents

(Conn and Franco 2004; Franco et al. 2007; Taechowisan et al. 2003). Previous studies have demonstrated that endophytic actinobacteria were capable of improving grain yields in the presence of common fungal root pathogens in a range between 5 and 60% comparing with untreated controls (Franco et al. 2007). Moreover, several actinobacterial species have been shown to be effective against various soil-borne plant pathogens such as, *Fusarium* spp. (Cao et al. 2005; Gopalakrishnan et al. 2011; Taechowisan et al. 2003), *Pythium* spp. (Hamdali et al. 2008; Verma et al. 2009), *Alternaria* spp. (Chattopadhyay and Nandi 1982; Vernekar et al. 1999), and *Rhizoctonia* spp. (Sadeghi et al. 2006; Sharma 2014), being capable of protecting different important crops. Comparing with plant growth promoting rhizobacteria, the use of endophytic microorganisms as microbial inoculants for biocontrol strategies offers considerable advantages, since competition effects are greatly reduced in the colonization of the internal tissues of the plant, thus increasing the chances of survival, growth, and effectiveness of the endophytic inoculants (Coombs et al. 2004; Rosenblueth and Martinez-Romero 2006).

8.3 Isolation of Actinobacterial Endophytes

Endophytic actinobacteria may be isolated from a wide diversity of plants. Isolation of these microorganisms is dependent on several factors, such as host plant species and age, sampling mode, sampling season, cultivation conditions, surface sterilization strategy, and selective media used (Gaiero et al. 2013; Kaewkla and Franco 2013; Zhang et al. 2006). The selected isolation procedure will determine the spectrum of endophytes recovered and should be able to yield the largest possible number of endophytes, while at the same time eliminating epiphytic microorganisms from the surface of plant tissues (Hallmann et al. 2006; Le et al. 2015; Li et al. 2012). Collected plants, or plant parts, should be processed as soon as possible within a period of 24 h. Samples should be stored at 4 °C between sampling and processing.

The critical step in the isolation of endophytic actinobacteria lies in the surface sterilization of plant tissues. This may be achieved through the use of surface sterilizing agents, with the most common ones being sodium hypochlorite (3–10%), ethanol (70–95%) and hydrogen peroxide. Other less conventional sterilizing agents, such as sodium chlorate (5%), sodium thiosulfate (2.5%), and sodium bicarbonate (10%) have also been employed for the inhibition of growth of endophytic fungi (Dochhil et al. 2013; Qin et al. 2008). The concentration of the sterilizing agents will depend on the permeability of the plant tissues. In some cases, sterilization efficiency is improved through the additional use of surfactants, such as Tween 20, Tween 80, or Triton X-100, which reduce surface tension and enable a better action of the sterilizing agent (Hallmann et al. 2006). Sterilization protocols typically include a tissue washing step, to remove soil particles and loosely adhered epiphytic microorganisms, followed by disinfection (which may or

not be preceded by a pre-treatment step with a surfactant), final rinse with sterile water, and sterility control to evaluate the sterilization efficiency. Apart from this standard protocol, some additional strategies are available to increase the isolation efficiency of endophytic actinobacteria. For instance, Qin et al. (2009) suggest the use of a thiosulfate solution after the disinfection step with sodium hypochlorite to minimize loss of endophytes caused by the presence of traces of disinfectant in treated plants tissues. Nimnoi et al. (2010) suggested soaking treated plant samples in a 10% $NaHCO_3$ solution in order to inhibit growth of endophytic fungi. Control tests of sterilization efficiency often consist in plating a sample of water derived from the last washing step or directly platting a surface-sterilized plant tissue. Microorganisms can only be assumed to be endophytes if sterility control tests are completely negative.

Ideally, the sterilization protocol should be adapted according to the plant species, age and type of plant tissue. After the surface sterilization procedure, sterilized plant tissues are inoculated in appropriate growth media, using one of two common strategies: (i) tissues are aseptically cut into small fragments (Coombs and Franco 2003; de Oliveira et al. 2010; Sardi et al. 1992) or (ii) tissues are macerated with a mortar and pestle (El-Tarabily et al. 2009; Garbeva et al. 2001; Hallmann et al. 2006; Kaur et al. 2015). In the latter case, in order to prevent inhibition of growth of endophytic actinobacteria caused by plant enzymes or toxins released during the maceration process, macerated samples may be diluted or buffered with appropriate compounds such as phosphate buffer, polyvinylpyrrolidone or EDTA (Golinska et al. 2015; Hallmann et al. 2006). More recent methods combining enzymatic hydrolysis and differential centrifugation have been alternatively used and shown very efficient in the isolation of endophytic microorganisms, especially rare endophytic actinobacteria (Jiao et al. 2006; Qin et al. 2009).

The selection of growth medium is a very important step in the isolation of actinobacterial endophytes. Nutrient poor media, such as tap water–yeast extract agar (TWYE), humic acid–vitamin B agar (HV), and yeast extract–casein hydrolysate agar (YECD), have been reported to be very effective in the isolation of these microorganisms (Coombs and Franco 2003; Qin et al. 2009). The formulation of growth media with nutrients identical to those found in plants has also been shown to be an effective strategy for the isolation of endophytic actinobacteria. Qin et al (2009) isolated rare endophytic actinobacteria comprising several genera using growth media supplemented with the aminoacids L-asparagine, proline or arginine as nitrogen sources and carbon substrates commonly found in plants, such as cellulose, fucose, or xylan. Addition of plant extracts to the growth medium is another effective strategy (Qin et al. 2011). Growth media should be supplemented with antibiotics such as nystatin, nalidixic acid, or cycloheximide (50 or 100 μg/ml) to inhibit growth of fungi and Gram-negative bacteria (Golinska et al. 2015; Lee et al. 2008; Qin et al. 2011). Examples of isolation strategies used for the recovery of endophytic actinobacteria from various plants with agricultural relevance are presented in Table 8.1.

Table 8.1 Examples of endophytic actinobacteria isolated from different crops and respective isolation strategy

Crop	Plant tissue	Sterilization method employed	Strategy of plant tissue inoculation	Isolation medium	Antimicrobial supplemented to growth medium	Number of recovered endophytic actinobacterial isolates	Identification of endophytic actinobacterial isolates	References
Maize (*Zea mays* L.)	Leaves and roots	Ethanol 70% for 30 s Sodium hypochlorite (3–5%) for 3 min Final rinse with sterile water	Roots cut into ca. 1 cm fragments and leaves divided in ca. 1 cm^2 fragments	Starch-casein-agar 2.5% water-agar	Nystatin and cycloheximide (50 μg/ml)	53	*Microbispora* spp. *Streptomyces* spp. *Streptosporangium* spp.	Araújo et al. (2000)
Wheat (*Triticum aestivum* L.)	Roots	Ethanol 99% for 60 s Sodium hypochlorite (3.125%) for 6 min Final rinse in sterile reverse osmosis-treated water	Cut into 1 cm fragments	Tap water–yeast extract agar Humic acid–vitamin B agar Flour–yeast extract-sucrose-casein hydrolysate agar Flour-calcium carbonate agar Yeast extract-casein hydrolysate agar	Benomyl	58	*Streptomyces* spp. *Micromonospora* spp. *Nocardiodes albus* *Streptosporangium*/*Microbispora*	Coombs and Franco (2003)
Tomato (*Lycopersicon esculentum*)	Roots	Ethanol 70% for 5 min Sodium hypochlorite (2.5%) for 10 min Final rinse with sterilized distilled water	Cut into 1 cm fragments	Starch casein agar S medium ISP2 agar	None	70	*Streptomyces* spp. *Micromonospora* spp. *Microbispora* spp. *Nocardia* spp.	de Oliveira et al. (2010)

(continued)

Table 8.1 (continued)

Crop	Plant tissue	Sterilization method employed	Strategy of plant tissue inoculation	Isolation medium	Antimicrobial supplemented to growth medium	Number of recovered endophytic actinobacterial isolates	Identification of endophytic actinobacterial isolates	References
Citrus rootstocks (several citrus cultivars)	Leaves and seeds	Rinsed with ethanol 70% Sodium hypochlorite (3%) for 3 min Rinsed once in ethanol 70% Rinsed twice in sterile distilled water	Leaves homogenized in a blender with 5 mL of sterile NaCl 0.85% and serially diluted Leaf and seed cut into 4–6 mm fragments	Trypticase soy agar (TSA)	Benomyl (50 μg/mL)	N.i.	*Curtobacterium flaccumfaciens*	Araújo et al. (2001)
Sweet-orange (*Citrus sinensis* Osbeck cv. Natal) Tangerine (*C. reticulata* cv. Blanco)	Branches	Ethanol 70% for 5 min Sodium hypochlorite (2%) for 5 min Ethanol 70% for 30 s Two rinses in sterile distilled water Bark of surface-disinfected branches removed with a sterilized razor blade	Bark of surface-disinfected branches removed Branches cut into 4–6 mm fragments	TSA	Benomyl (50 μg/mL)	N.i.	*Curtobacterium flaccumfaciens* *Nocardia* spp. *Streptomyces* spp.	Araújo et al. (2002)

(continued)

Table 8.1 (continued)

Crop	Plant tissue	Sterilization method employed	Strategy of plant tissue inoculation	Isolation medium	Antimicrobial supplemented to growth medium	Number of recovered endophytic actinobacterial isolates	Identification of endophytic actinobacterial isolates	References
Potato plants (*SoIanum tuberosum* cv Desiré)	Roots and stems	Sodium hypochlorite 1% supplemented with Tween-20 0.02% for 1 min Ethanol 70% for 1 min 3 times washing in thiosulfate/Ringer's solution for 1 min Incubation of grounded material with 120 mM sodium phosphate (pH 8) for 2 h	Cut into pieces of ca. 0.3 cm and macerated Incubated for 2 h at room temperature with sodium phosphate (pH 8) 120 mM	TSA (0.05x) *R2A agar*	Cycloheximide (100 µg/mL)	N.i.	*Nocardia globerula* *Corynebacterium aquaticum*	Garbeva et al. (2001)
Canola (*Brassica napus* cv. Westar)	Roots	Ethanol 95% for 1 min Acidified mercuric chloride 0.1% (w/v) for 1 min Washed 10 times with sterile tap water	Roots suspended in 1/10 PBS, macerated and serially diluted	Trypticase soy broth 0.3% (TSB) with 1.5% agar	None	65	*Arthrobacter* spp. *Curtobacter* spp. *Micrococcus* spp. *Rathayibacter* spp.	Germida et al. (1998)

(continued)

Table 8.1 (continued)

Crop	Plant tissue	Sterilization method employed	Strategy of plant tissue inoculation	Isolation medium	Antimicrobial supplemented to growth medium	Number of recovered endophytic actinobacterial isolates	Identification of endophytic actinobacterial isolates	References
Fodder leguminous plants (*Vigna unguiculata* and *Trifolium alexandrinum*)	Roots and nodules	Ethanol 70% for 5 min Sodium hypochlorite 0.9% for 20 min Washed 3 times with sterile water Sodium bicarbonate solution 10% for 10 min	Macerated tissues	Starch casein agar	None	34	*Streptomyces* spp. *Actinopolyspora* spp. *Saccharopolyspora* spp. *Micromonospora* spp. *Nocardia* spp.	Kaur et al. (2015)
Chinese cabbage (*Brassica campestris* L.)	Roots	Ethanol 90% for 1 min Sodium hypochlorite 3.1% for 6 min Washed 3 times with sterile water	Cut into 1 cm fragments	Humic acid–vitamin agar Corn meal agar	Cycloheximide (50 μg/mL) Nalidixic acid (10 μg/mL)	81	*Microbispora* spp. *Streptomyces* spp. *Micromonospora* spp. *Nocardia* spp. *Verrucosispora* spp. *Nonomuraea* spp. *Actinomadura* spp. *Thermomonospora* spp.	Lee et al. (2008)
Rice (*Oryza sativa* cv. Qilisimiao and Huajingxian)	Leaves and roots	Ethanol 70% for 5 min Sodium hypochlorite 0.87% for 15 min Sodium bicarbonate solution 10% for 15 min Rinsed three times with autoclaved distilled water	Cut into 1 cm fragments	1.2% S medium	Potassium dichromate (25 μg/mL) Nalidixic acid (15 μg/mL)	274	*Streptomyces* spp. *Streptoverticillium* spp.	Tian et al. (2004)

(continued)

Table 8.1 (continued)

Crop	Plant tissue	Sterilization method employed	Strategy of plant tissue inoculation	Isolation medium	Antimicrobial supplemented to growth medium	Number of recovered endophytic actinobacterial isolates	Identification of endophytic actinobacterial isolates	References
Cucumber seeds (*Cucumis sativus* L. cv. Cheyenne)	Roots	Exposure to propylene oxide vapors for 1 h	Macerated tissues	Humic acid–vitamin agar	Cycloheximide (50 μg/mL) Nystatin (50 μg/mL)	29	*Actinoplanes* spp. *Micromonospora* spp. *Streptomyces* spp. *Microbispora* spp. *Streptosporangium* spp.	El-Tarabily et al. (2009)
Rice (*Oryza sativa* L. cv. KDML 105)	Leaves, leaf sheathes, stems and roots	Ethanol 70% for 1 min Sodium hypochlorite 3% for 6 min Final rinse in sterile reverse osmosis-treated water	Cut into 1 cm fragments	Mannitol mung bean yeast extract mineral salt agar Tap water–yeast extract agar Humic acid–vitamin B agar TSA	Nystatin 100 IU/mL	116	*Streptomyces* spp. *Microbispora* spp. *Kineococcus* spp.	Kampapongsa and Kaewkla (2016)
Ginger (*Zingiber officinale*) Tumeric (*Curcuma domestica*)	Leaves, stems and roots	Rinsed in Tween 20 0.1% for 30 s Sodium hypochlorite 1% for 5 min Washed in sterile distilled water for 5 min Ethanol 70% for 5 min Air-dried in a laminar flow chamber	Cut into small pieces of ca. 4×4 mm^2	Humic acid–vitamin agar	Nystatin (100 μg/mL) Cycloheximide (100 μg/mL)	97	*Streptomyces* spp. *Microbispora* spp. *Nocardia* spp. *Micromonospora* spp.	Taechowisan et al. (2003)

N.i. Not indicated

8.4 Identification and Diversity of Endophytic Actinobacteria in Agricultural Plants

Plants may host a wide range of actinobacteria genera within their inner tissues. Identification of actinobacterial endophytes is often based in a polyphasic approach consisting of several morphological, biochemical and molecular studies. Morphological studies often consist in colony characterization on agar plates, which include examination of aerial and substrate mycelia colors and spore mass color, analysis of spores and hyphae morphology (usually observed in a scanning electron microscope), as well as inspection for the production and color of diffusible soluble pigments. Biochemical tests may comprise analysis of melanin production, presence of various enzymes, and utilization of a range of carbon sources, cell wall composition, whole-cell sugar distribution, cellular phospholipid composition, and menaquinone type (Barka et al. 2016; Labeda 1987; Shirling and Gottlieb 1966). Molecular analyses are based on the sequencing of the 16S rRNA gene and DNA–DNA hybridization, and are strictly necessary for the identification of new species (Barka et al. 2016).

Diversity of endophytic actinobacteria seems to be higher in woody than in herbaceous plants, with roots having the highest number and diversity, followed by stems and leaves (Kekuda 2016). The ecological environment of the plant also influences actinobacterial diversity (Sheil 1999). Endophytic actinobacteria have been isolated from various crop plants, such as maize, wheat, tomato, rice, citrus, potato, Aloe vera, etc. (Araújo et al. 2000, 2001; Coombs and Franco 2003; de Oliveira et al. 2010; Garbeva et al. 2001; Prakash et al. 2014; Thanaboripat et al. 2015; Tian et al. 2007), with the most frequently isolated genus being *Streptomyces* (Kampapongsa and Kaewkla 2016; Sardi et al. 1992; Taechowisan et al. 2003; Tian et al. 2004). Other common isolated genera are *Micromonospora*, *Microbispora*, and *Nocardia* (Table 8.1). Recent studies on the diversity of endophytic actinobacteria in various plants have also allowed the identification of more than 40 new taxa, namely of the genera *Actinoallomurus*, *Actinophytocola*, *Jishengella*, *Phytohabitans*, *Saccharopolyspora*, *Streptosporangium*, *Plantactinospora*, among others (Masand et al. 2015; Qin et al. 2011).

Studies revealed that in addition to roots, stems, and leaves, nitrogen-fixing nodules also harbor endophytic actinobacteria, and exhibited the isolation of the genera *Streptomyces*, *Agromyces*, *Curtobacterium*, *Micromonospora,* and *Microbacterium* from the nodules of different leguminous and actinorhizal plants (Carro et al. 2012; Deng et al. 2011; Trujillo et al. 2006, 2007). The two latter genera were found to be particularly predominant in plant nodules, with several new species of *Micromonospora* being isolated from these tissues (Carro et al. 2013; Garcia et al. 2010; Trujillo et al. 2006, 2007, 2015). The role of these microorganisms in plant growth promotion in not completely known, but studies

with *Micromonospora* species suggest that these microorganisms have important beneficial effects in plants (Martínez-Hidalgo 2014; Trujillo et al. 2010, 2015).

Due to the fact that culture-dependent methods are very limiting and only target less than 1% of the existing bacterial universe, culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and, more recently, next generation sequencing techniques, like metagenomics analysis, have become very important tools for the investigation of the complex microbial communities associated with plants and of the inherent endophytic population. In particular, metagenomics analysis has allowed a better understanding of the abundance, diversity, and distribution of endophytic actinobacteria in a wide variety of plants, including agriculturally important crops. Using this approach, several studies have shown that endophytic actinobacteria are well represented in different crops such as grapevine (*Vitis vinifera*), olive (*Olea europaea*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), and lettuce (*Lactuca sativa*), with the families Corynebacteriaceae, Kineosporiaceae, Microbacteriaceae, Micrococcaceae, Micromonosporaceae, Nocardioaceae, and Streptomycetaceae, being amongst the predominant taxa (Cardinale et al. 2015; Manter et al. 2010; Müller et al. 2015; Okubo et al. 2014; Pinto et al. 2014; Trujillo et al. 2015). A combination of culture-dependent and independent methods may be used for a deeper investigation of endophytic communities, with studies suggesting that these two approaches are complementary, as the microbial communities retrieved by isolation methods are often different from those obtained through molecular techniques (Garbeva et al. 2001; Qin et al. 2011, 2012).

Despite the accumulating studies suggesting that endophytic actinobacteria are beneficial to their host plants and contribute to their health, a restricted number of these microorganisms has been reported to have a pathogenic character, though when compared with other bacteria these exert a minor role in plant diseases. Examples of pathogens of agricultural plants include *Streptomyces scabies*, *S. acidiscabies*, *S. europaeiscabiei,* and *S. turgidiscabies* that cause diverse potato scab diseases (Bignell et al. 2010; Loria et al. 2006). *S. scabies* has a worldwide distribution and was the first pathogenic *Streptomyces* described in the literature, while the other species have a more recent occurrence (Barka et al. 2016; Kreuze et al. 1999; Wanner 2006). Examples of other pathogenic endophytic actinobacteria are the species *Curtobacterium flaccumfaciens* which cause disease on a variety of plants such as *Phaseolus* and *Vigna* species, sugar beet, etc. (Saddler and Messenber-Guimaraes 2012), *Leifsonia xyli* subsp. *xyli* which causes the disease ratoon stunting in sugarcane (Monteiro-Vitorello et al. 2004) and *Clavibacter michiganensis* which is pathogenic to alfalfa, maize, potato and wheat, causing considerable economic losses worldwide (Eichenlaub and Gartemann 2011; Flügel et al. 2012; Trujillo et al. 2015).

8.5 Potential of Endophytic Actinobacteria for Sustainable Agriculture

Plant growth promoting microorganisms (PGPM) have great potential to help host plants adapt to a changing environment, since they can help plants to tolerate stressors like extreme temperature, drought, and salinity, and thus better withstand the challenges of climate change in agriculture (Welbaum et al. 2004).

Although plant growth promoting bacteria are one of the well-studied groups of PGPM, only scanty information are focused on endophytic actinobacteria possessing plant growth promoting properties. Recent findings demonstrated that endophytic actinobacteria are able to enhance establishment, growth, development, and health of agricultural crops directly via production/secretion of various regulatory chemicals in the vicinity of the rhizosphere, as well as indirectly via inhibition of phytopathogens by producing specific chemicals (Fig. 8.1).

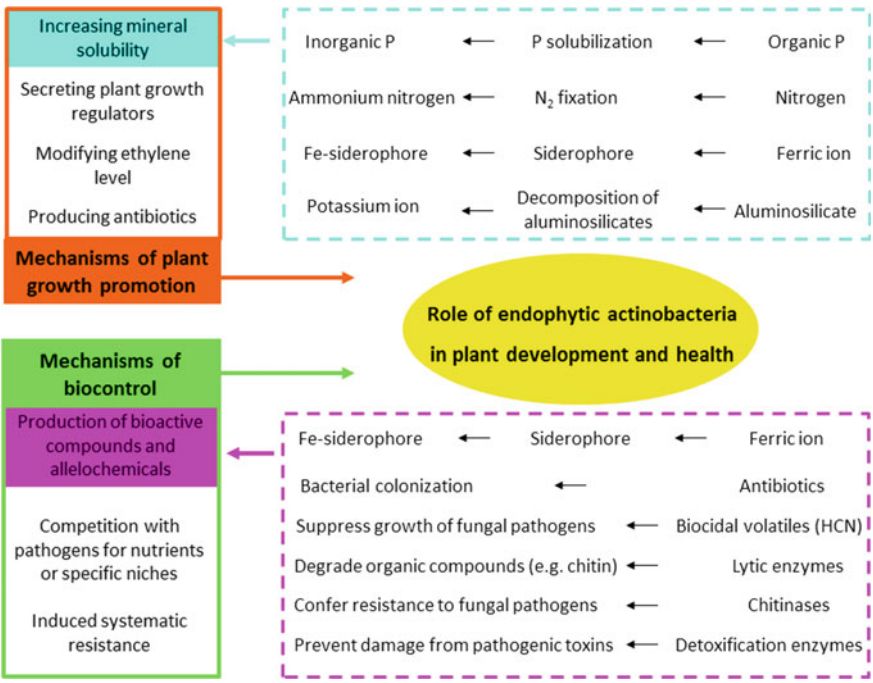

Fig. 8.1 Role of endophytic actinobacteria in plant development and health

8.5.1 *Endophytic Actinobacteria as Plant Growth Promoting Agents*

Although plants are able to adjust their activities and metabolism in the presence of stresses during their life cycle, for instance, they can synthesize various specific defensive proteins to overcome stress (Hossain et al. 2012), the exposure of plants to abiotic and biotic stresses still causes major losses in yield of agricultural crops. Certain actinobacteria have been found to be able to help their host plants by either completely or partially avoiding abiotic and biotic stresses (Gopalakrishnan et al. 2016). Especially, beneficial endophytic actinobacteria that promote plant growth under favorable and unfavorable conditions have recently received attention (Hasegawa et al. 2006). Like rhizosphere actinobacteria, beneficial endophytic actinobacteria are capable of improving plant growth via one or more plant growth promoting mechanisms, including fixation of atmospheric nitrogen, solubilization of mineral nutrients, secretion of phytohormones, and siderophores (Dudeja et al. 2012) (Fig. 8.1).

Endophytic actinobacteria are able to express nitrogenase and occupy an essential ecological niche in the living plant tissue by providing fixed nitrogen to their hosts (Soe et al. 2012). It is well known that endophytic actinobacteria possessing strong nitrogen-fixing property may confer plants the capacity to tolerate nitrogen-poor soil environment. For instance, the endophytic genera *Frankia* (Callaham et al. 1978), *Micromonospora* (Trujillo et al. 2015) and *Streptomyces* (Soe et al. 2012) were capable of enhancing plant growth under nitrogen-limited environment by fixing nitrogen. Recently, nitrogen-fixing endophytic actinobacteria have been reported to be able to increase the number of nodules, nitrogen fixation rate, as well as nitrogen uptake by plants in low nitrogen ecosystems (Le et al. 2016; Rafik et al. 2014; Trujillo et al. 2015).

Phosphorus is involved in various enzymatic reactions in living organisms, such as transport of glucose, stimulation of cell proliferation and promotion of organ development (Ahemad 2015). Although most of soil phosphorus is immobile and thus unavailable for plant uptake (Ezawa et al. 2002), some endophytic bacteria are able to solubilize precipitated phosphates through acidification, chelation, redox changes (Nautiyal et al. 2000), or to mineralize organic P through production of phosphatase (van der Hiejden et al. 2008) under environmental stress conditions, thus enhancing P bioavailability. Jog et al. (2014) found that two root endophytic actinobacteria (*Streptomyces* spp.) isolated from *Triticum aestivum* significantly improved plant growth through phosphate solubilization and secretion of phytases as well as some other plant growth promoting traits. This is probably because the phytase-P complex process facilitates plant uptake of P.

Iron is a vital element for life and is needed by almost all organisms; since it plays a significant role in physiological processes (e.g., transpiration) and enzymatic activities (Bothwell 1995). In general, most iron in soil exists in highly insoluble ferric (Fe^{3+}) form and is unavailable for plants. Siderophores produced by soil bacteria are able to solubilize iron under iron-limited conditions, therefore

improving iron availability to plant roots. In general, plants acquire iron either from bacterial siderophore-iron complex, or from the phytosiderophore-iron complex (Ma et al. 2011; Rajkumar et al. 2009). It is known that bacterial siderophores generally have higher affinity for iron than phytosiderophores and that siderophore producing bacteria can help plants accumulate more iron than the plant alone under iron-limited conditions (Ma et al. 2011). After the iron is complexed by siderophores produced by endophytic actinobacteria, plant roots are able to uptake it directly from bacterial siderophore-iron complexes (Chen et al. 1998; Rungin et al. 2012).

Endophytic actinobacteria can provide phytohormones to hosts in order to facilitate nutrients accumulation (Gopalakrishnan et al. 2016). Recently, Phetcharat and Duangpaeng (2012) investigated the role of phytohormones produced by endophytes in protecting plants against environmental stress. The authors found that the success of endophytic colonization was associated with increases in plant nutrient uptake and biomass yield. Indole-3-acetic acid (IAA) has been considered as a major auxin, which plays a vital role in stimulating plant development (Gravel et al. 2007; Shi et al. 2009), inducing plant self-defense or adaptation system (Navarro et al. 2006), and functioning as a signaling molecule (Spaepen et al. 2007). The IAA synthesized by endophytic actinobacteria is considered to have great potential to modulate the establishment and development of plant-endophyte association (Goudjal et al. 2013). Endophytic actinobacteria, such as *Streptomyces*, *Nocardia*, *Nocardiopsis*, *Spirillospora*, *Microbispora,* and *Micromonospora* were found to be involved in the production of this phytohormone, therefore benefiting plants in situ (Goudjal et al. 2013; Shutsrirung et al. 2013). El-Tarabily et al. (2009) demonstrated that some endophytic actinobacterial strains greatly enhanced growth of *Cucumis sativus* by synthesizing indole-3-pyruvic acid and IAA. However, unfavorable effects of phytohormones have also been reported by Patten and Glick (2002), who found that low concentrations of bacterial IAA induced the elongation of plant primary root, whereas high IAA concentrations caused the formation of plant lateral and adventitious roots with negative effects on primary root growth. Therefore, the endophytic actinobacteria that can modify the balance of phytohormones might be good candidates for hastening plant development.

Ethylene, a universal phytohormone, is involved in plant growth and physiological responses to both abiotic and biotic environmental stresses (Sun et al. 2006). The pathway of ethylene synthesis has been extensively reviewed (Glick et al. 2007). It is well known that plants exposed to environmental stresses such as extreme temperature, drought and salinity can induce the production of ethylene, which is able to hamper elongation of roots as well as formation of root hairs. Under such stresses, some endophytic actinobacteria might mitigate the negative impact of stress by hydrolyzing 1-aminocyclopropane-1-carboxylic acid (ACC) and subsequent diminishing plant ethylene production. It has been reported that the enzyme ACC deaminase produced by some endophytic actinobacteria may hydrolyze ACC into α-ketobutyrate and ammonia, which then serves as a nitrogen source for such microbes (Viterbo et al. 2010; Xing et al. 2012).

8.5.2 Endophytic Actinobacteria as Biocontrol Tools

Endophytes are becoming very interesting biocontrol candidates, because of their crucial role in host–plant association, such as competition with phytopathogens for colonization sites and mineral nutrients (Ma et al. 2016). Bacterial endophytes have great potential to inhibit the growth of phytopathogens, and to stimulate the growth and development of host plants (Ma et al. 2011). The understanding of the endophytic actinobacteria–host plant interaction might accelerate the application of these microbes in sustainable agriculture. Currently, endophytic actinobacteria have been isolated from various plant species, such as *Brassica rapa* (Lee et al. 2008), *Brassica oleracea* (Kanchanadevi et al. 2013), *Oryza sativa* (Mingma et al. 2015), *Lycopersicon esculentum* (Cao et al. 2004; Kanchanadevi et al. 2013), *Jatropha curcas* (Xing et al. 2012), *Glycine max* (Mingma et al. 2014), *Triticum aestivum* (Jog et al. 2014), and *Zea mays* (Costa et al. 2013); however, only few crop species have been investigated in terms of their endophytic actinobacterial diversity and their effect as biocontrol agents. Additionally, the mechanisms involved in endophytic actinobacteria–host plant interaction are still very poorly understood given the limited data currently available.

Endophytic actinobacteria have been attracting interest because of their capability to produce bioactive chemicals and/or allelochemicals, such as siderophores, antibiotics, biocidal volatiles, lytic enzymes, chitinases, and detoxification enzymes (Bérdy 2005; Clardy et al. 2006; El-Tarabily et al. 2010; Quecine et al. 2008) (Fig. 8.1). Siderophores chelate or complex soluble iron from the soil; antibiotics hinder pathogenic colonization; biocidal volatiles (e.g., hydrogen cyanide) inhibit the growth of pathogenic fungi; lytic enzymes (e.g., chitinases) degrade some organic compounds (e.g., chitin) conferring plant resistance/tolerance to pathogens; detoxifying enzymes protect against pathogen and toxins. Moreover, endophytic actinobacteria are capable of successfully competing with pathogens for specific niches and mineral nutrients in plant tissues, and of inducing systemic resistance (Doumbou et al. 2001). For instance, antibiotics produced by *Streptomyces* spp. are able to hinder the growth of a wide range of pathogenic microbes (Gopalakrishnan et al. 2016). Moreover, these compounds are considered as important agents to control soil-borne diseases with low toxic impacts on the environment and human health (Cao et al. 2004).

8.5.3 Endophytic Actinobacteria as Helpers of Agricultural Crops

The mechanisms of plant growth promotion and biological control involved in host plant–microbe association have been discussed above. In this section, we have summarized some recent publications on the beneficial functions of endophytic actinobacteria in enhancing sustainable agriculture via acting as helpers of

agricultural crops (Table 8.2). Efforts have been made in searching for biostimulator/biofertilizer for crop production, and natural biocontrol agents for crop protection. Endophytic actinobacteria, especially those belonging to the genus *Streptomyces* have become an important microbial source for application in various crops, such as *Lycopersicon esculentum*, *Triticum aestivum*, *Medicago sativa*, *Brassica rapa*, *Citrus reticulate*, *Oryza sativa,* and *Zea mays* (Table 8.2). Several studies indicate that endophytic actinobacterial species are able to enhance the performance of agricultural crops by employing a range of mechanisms, such as nitrogen fixation (Le et al. 2016), solubilization of phosphate (Jog et al. 2014), production of phytohormones (El-Tarabily et al. 2009; Goudjal et al. 2013; Meguro et al. 2006), siderophores (Hastuti et al. 2012; Rungin et al. 2012), hydrogen cyanide (Passari et al. 2015), chitinase (Singh and Gaur 2016), ammonia (Passari et al. 2015), antibiotics (Mingma et al. 2014), and other antibacterial and antifungal metabolites (Cao et al. 2004; Costa et al. 2013; Goudjal et al. 2014). However, the beneficial effects contributing to plant growth promotion and the level of biocontrol achieved by various endophytic actinobacteria are mainly performed in laboratory or identically controlled environmental conditions. In this regard, endophytic actinobacteria possessing biofertilizer and biocontrol properties for commercial use must be further tested for practical agricultural applications. Studies are, therefore, needed to evaluate the functions of these endophytic actinobacteria in terms of enhancing host plant growth, as well as inducing systemic resistance and antibiosis activity against phytopathogens in field trials.

Table 8.2 Enhanced performance of agricultural crops by endophytic actinobacteria

Endophytic actinobacteria	Isolated from	Plant beneficial trait	Effect	References
Streptomyces spp.	*Lycopersicon esculentum*	Production of antibacterial and antifungal metabolites	Growth promotion and enhanced disease resistance of tomato seedlings, but not in cucumber seedlings	Cao et al. (2004)
Streptomyces spp.	*Zea mays*	Growth inhibition of phytopathogenic fungi	Isolate 16R3B was able to reduce up to 71% damping-off incidence whereas isolate 14F1D/2 reduced disease incidence by 36%	Costa et al. (2013)
Actinoplanes campanulatus, *Micromonospora chalcea*, *Streptomyces spiralis*	N.i.	Production of IAA and indole-3-pyruvic acid	Promoted plant growth and suppressed pathogenic activities of *Pythium aphanidermatum* on seedling and mature cucumber	El-Tarabily et al. (2009)

(continued)

Table 8.2 (continued)

Endophytic actinobacteria	Isolated from	Plant beneficial trait	Effect	References
Actinoplanes campanulatus, *Micromonospora chalcea*, *Streptomyces spiralis*	N.i.	Biological control and plant growth promotion	Colonized the internal tissues of roots, stems and leaves under field conditions; promoted plant growth and yield and reduced seedling damping-off and root and crown rots of mature cucumber plants	El-Tarabily et al. (2010)
Streptomyces sp. and non-*Streptomyces*	Spontaneous plants of Algerian Sahara	Production of IAA	Promoted seed germination and root elongation	Goudjal et al. (2013)
Streptomyces sp. and non-*Streptomyces*	Native plants of the Algerian Sahara	Antifungal activity	Increased seedling fresh weight, the length of shoot and root; Reduced the severity of damping-off of tomato seedlings	Goudjal et al. (2014)
Streptomyces spp.	N.i.	Production of chitinase, phosphatase and siderophore	Increased plant height and produced higher tiller number; Inhibited the growth of *Xanthomonas oryzae* pv. *oryzae*	Hastuti et al. (2012)
Streptomyces spp.	*Triticum aestivum*	Solubilization of phosphate, production of phytase, chitinase, IAA, siderophore and malate	Improved plant growth, biomass and mineral (Fe, Mn, P) content under non-axenic conditions	Jog et al. (2014)
Streptomyces spp.	*Medicago sativa*	Growth promotion and N_2-fixation	Improved shoot weight and the number of nodules	Le et al. (2016)
Microbispora sp., *Streptomyces* sp., *Micromonospora* sp.	*Brassica rapa*	Biological control	Suppressed the occurrence of a post-inoculated strain of *Plasmodiophora brassicae*	Lee et al. (2008)

(continued)

Table 8.2 (continued)

Endophytic actinobacteria	Isolated from	Plant beneficial trait	Effect	References
Streptomyces sp. MBR-52	*Rhododendron ferrugineum*	Production of rooting-promoting plant hormones	Accelerated emergence and elongation of plant adventitious roots	Meguro et al. (2006)
Streptomyces sp. and non-*Streptomyces*	Leguminosae	Contained *LL*-isomer of diaminopimelic acid; antagonistic activity	Protected against soybean pathogen *Xanthomonas campestris* pv. *glycine*	Mingma et al. (2014)
Streptomyces sp. and *Leifsonia xyli*	Medicinal plants	Solubilization of phosphate, production of siderophores, HCN, ammonia, chitinase, IAA, antifungal activities	Improved a range of growth parameters in *Capsicum annuum* L.	Passari et al. (2015)
Streptomyces sp., *Nocardia* sp., *Nocardiopsis* sp., *Spirillospora* sp., *Microbispora* sp. and *Micromonospora* sp.	*Citrus reticulata*	Production of IAA	Promoted shoot height, fresh shoot weight and fresh root weight of seedlings	Shutsrirung et al. (2013)
Streptomyces diastaticus, *Streptomyces fradiae*, *Streptomyces olivochromogenes*, *Streptomyces collinus*, *Streptomyces ossamyceticus* and *Streptomyces griseus*	Medicinal plants	Production of chitinase; Plant growth promoting abilities and antagonistic potential	Protect chickpea against *Sclerotium rolfsii* infestation; increased the biomass and reduced plant mortality of chickpea	Singh and Gaur (2016)
Streptomyces sp.	N.i.	N.i.	Single inoculation of *Streptomyces* sp. P4 did not influence nodulation, N_2 fixation, shoot dry weight and seed weight at harvest of all soybeans	Soe et al. (2012)
Streptomyces sp. GMKU 3100	*Oryza sativa* L. cv. KDML105	Production of siderophores	Increased root and shoot biomass and lengths of rice and mungbean plants	Rungin et al. (2012)

HCN Hydrogen cyanide; *IAA* Indole-3-acetic acid; *N.i.* Not indicated

8.6 Conclusion

Endophytic actinobacteria have been isolated from several plant species and inoculated onto different target agricultural crops often resulting in enhanced plant performance. Improvements in isolation and identification methods are yielding new isolates with plant growth promoting traits and showing that endophytic actinobacteria are amongst the predominant bacterial phyla inside plants, including agriculturally important crops. The use of endophytic actinobacteria as microbial inoculants in agriculture offers considerable advantages when compared with that of rhizobacteria, since competition effects are greatly reduced in the colonization of the internal tissues of the plant, thus increasing the chances of survival, growth, and effectiveness of the endophytic inoculants. Endophytic actinobacteria are able to enhance the establishment, growth, development, and health of agricultural crops directly via production/secretion of various regulatory chemicals and indirectly via inhibition of phytopathogens. Thus, endophytic actinobacteria hold the prospect of reducing the input of chemical fertilizers and pesticides and their inoculation can be regarded as an environmentally friendly approach in agriculture. There is, therefore, great potential in using endophytic actinobacteria as biotechnological tools for sustainable agricultural applications.

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Chapter 9
Endophytic Fungi: A Remarkable Source of Biologically Active Secondary Metabolites

Pamoda B. Ratnaweera and E. Dilip de Silva

Abstract Endophytic fungi are ubiquitous in internal tissues of healthy plants and are known to biosynthesis a remarkable array of secondary metabolites with diverse chemical structures and assist host plants to overcome both abiotic and biotic stress factors in their natural environment. Screening technologies have established these natural products are an outstanding source of biologically active metabolites with promising medicinal and agricultural applications. Selection of plants from distinct environmental settings and/or with unconventional biology is expected to enhance the chances of isolating novel fungal endophytes as well as new bioactive secondary metabolites. Using selected examples from different ecological niches, this review illustrates the chemical potential of endophytic fungi for producing pharmaceutically and agriculturally valuable products. The biosynthesis of the same specific biologically active metabolites by the endopyte as well as the host plant and the factors that influence the production of secondary metabolites by the endophyte are also discussed. Finally, the current challenges in the production and commercialization of bioactive compounds of endophytic fungal origin are debated.

Keywords Bioactive · Rainforest · Mangrove · Ecological · Marine · Sedges

9.1 Endophytic Fungi

The evidence from fossil records indicates that endophyte-plant association may have evolved from the period higher plants first colonized land, thus played a long and important role in driving the evolution of life on land (Zhang et al. 2006). Fungi are a distinct group of heterotrophic eukaryotic organisms, wide spread in nature.

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A vast majority of fungi are composed of microscopic multicellular hyphae (with a few unicellular species) and show cryptic lifestyles in soil and dead matter and become noticeable only when developed fruiting bodies spores in as mushrooms or molds. Fungi are the principal decomposers of organic matter and perform important role in nutrient cycling in ecosystems (Cooke 2009). They establish parasitic relationships with both plants and animals and are known to cause widespread damage, to certain agricultural crops (Fisher et al. 2012). Endophytic fungi on the other hand, are symbionts that spend all or part of their life cycle inter and/or intracellularly colonizing the healthy tissues of a plant without causing any visible manifestation of symptoms (Tan and Zou 2001). The word "endophyte" originates from Greek, "endo" denoting within, and 'phyte' meaning plant and was first proposed in 1866 (Jalgaonwala et al. 2011; de Bary 1866). In addition to fungi, bacteria including actinobacteria are reported as the major endophytes of plants (Bandara et al. 2006).

9.2 Distribution of Endophytic Fungi

The existence of fungi inside the organs of the asymptomatic plants has been known since nineteenth century (Guerin 1898). The first description of endophytic fungi was made as far as back in the year 1904, from the seeds of *Lolium temulentum* (Freeman 1904). Since then fungi have been found from almost every plant species examined to date (Guo et al. 2008). Endophytic fungi have a long life history and their diversity among plants has been found to be one of the largest (Jalgaonwala et al. 2011).

It is noteworthy that each of the nearly 300,000 existing plant species on Earth is assumed to host at least one or even several hundred strains of endophytes (Strobel and Daisy 2003). Fungal endophytes are found in a range of host plants growing in tropical, temperate, boreal forests to extreme arctic, alphine, and xeric environments (Zhang et al. 2006). There are as many as 1.5 million different fungal species on our planet and about 1 million of them are endophytic fungal species (Strobel and Daisy 2003; Radic and Strukelj 2012; Hawksworth 2001). Among them, only about 0.1 million fungal species including endophytic fungi have been described in the past century (Radic and Strukelj 2012; Ganley et al. 2004). Accordingly, fungal endophytes are a group of mainly undescribed organisms that potentially is a rich and reliable source of genetic diversity.

Endophytic fungi are known to thrive asymptomatically in the tissues of plants above ground as well as below ground, including flowers, seeds and ovules, fruits, stems, leaves, xylem, rachis, bark, tubers, and/or roots (Zhang et al. 2006; Kusari

et al. 2012). Recent studies have shown that endophytes are not host-specific (Cohen 2006). A single species of endophytes can invade a wide range of hosts while several studies have suggested that same fungus isolated from different parts of the same host shows diverse abilities to utilize different substances (Carroll and Petrini 1983), thus host endophyte relationship may vary from host to host and endophyte in general.

9.3 The Plant-Endophyte Interaction

The relationship between the endophyte and its host may range from mutualistic symbiosis to phytopathogenesis. Sometimes the endophyte remains latent, with symptomless nature, inside the host plant until the environmental conditions are favorable for the fungus or the ontogenetic state of the host changes to the advantage of the fungus (Rodriguez and Redman 2008; Sieber 2007). Therefore, with time, endophytic fungi can also be aggressive saprophytes or opportunistic pathogens as well (Strobel and Daisy 2003; Tan and Zou 2001; Rodriguez and Redman 2008).

The mutualistic relationship between the fungal endophytes and the host plants are somewhat complex, but results in fitness benefits for both partners. The plants provide endophytes with nutrients, protection from desiccation, spatial structure, and transmission via seed dissemination to the next generation of host (Guo et al. 2008). The plant may also provide important chemical compounds that are essential for the endophytes' growth and self-defense (Metz et al. 2000; Strobel 2002). On the other hand, endophytes contribute significant benefits to their host plants by producing a plethora of bioactive substances required to adapt to abiotic and biotic stress factors (Guo et al. 2008). Resistance to abiotic stress is enhanced by increasing tolerance to drought or water stress, high temperature, low pH, high salinity and presence of heavy metals (Jalgaonwala et al. 2011). In a study done in Lassen Volcanic and Yellowstone national park, it has been shown that an endophytic *Curvularia* species isolated from a grass species collected from geothermal soils gives thermotolerance to the host, probably as a result of production of cell wall melanin that may disperse heat along fungal hyphae (Gunatilaka 2006). A study conducted with an endophytic *Penicillium minioluteum* species and soybean has shown that endophytic association has significantly ameliorated the negative effects of salinity stress damage and increased the growth and metabolism of the soybean (Khan et al. 2011).

Plants encounter biotic stress due to bacterial and fungal pathogens, and attack of insects, nematodes, and mammalian herbivore (Rodriguez et al. 2009). The bioactive secondary metabolites produced by the endophytes living in these plants are known to induce resistance to biotic stress factors (Gunatilaka 2006). Previous researches have reported that in many cases tolerance to biotic stress has been correlated with fungal natural products (Tan and Zou 2001; Zhang et al. 2006; Aly

et al. 2011). There are a number of reports describing various bioactive metabolites produced by fungal endophytes which help the plant to increase the resistance against biotic stress (Guo et al. 2008; Suryanarayanan et al. 2009). For example, production of two macrocyclic alkaloids, pyrrocidines A and B with antibiotic activity, by the endophytic fungus *Acremonium zeae* has been implicated in the protection of its host, maize, against pathogenic and mycotoxin producing fungi (He et al. 2002). In grasses and herbaceous plants, the endophytes are known to produce toxic alkaloids that prevent or poison invertebrate and vertebrate herbivores (Rodriguez et al. 2009). Accordingly in symbiotically conferred stress tolerance, endophytes act as a biological trigger to activate host defense system more rapidly and strongly (Rodriguez and Redman 2008). At the same time some endophytes are capable of enhancing the hosts' allelopathic effects on other species growing close by, being an opponent for the space and nutrients (Newcombe et al. 2009). Apart from the above benefits, many endophytes are reported to enhance uptake of phosphorus, and other important elements for plant growth, capable of fixing nitrogen and producing plant hormones such as auxin, indole acetic acid, which are essential for regulation of plant growth and development (Guo et al. 2008).

9.4 Biological Rationale in Plant Selection

Due to the vast number of plant species in the world, creative and imaginative strategies are necessary to quickly narrow down the search for bioactive endophytes. This provides the best opportunities to isolate endophytes prone to produce novel bioactive products. Plants from distinct environmental settings and/or with an unconventional biology are considered to be a promising source for isolating novel endophytes bearing new secondary metabolites (Strobel 2003). Strobel and Daisy (2003) reported several reasonable hypotheses governing the plant selection for isolating bioactive endophytes. Selection of plants from a unique environment, having unusual biology, using novel approaches for survival is one such strategy. Mangrove environments are an example for hosting such plants. A second tactic is the selection of plants that have a historic background, which have been exploited as a source of traditional medicine. Third, plants that are endemic, having an unusual longevity or that occupy a certain ancient land mass, have the prospect of lodging such endophytes. Finally, plants growing in areas of high biodiversity, such as rainforest ecosystems, are potential sources housing novel and bioactive endophytic fungi (Strobel and Daisy 2003).

9.5 Bioactive Metabolites from Endophytic Fungal Origin from Different Ecological Niches

Although the discovery of endophytic fungi dates as far back as the early 1900s, they did not receive much attention until the recent realization of their pharmaceutical and ecological significance (Gunatilaka 2006). Recent developments of screening technologies have revealed that endophytic fungi are an outstanding source of biologically active compounds with promising medicinal and agricultural applications (Aly et al. 2011).

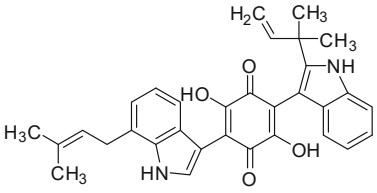
Tropical rainforest ecosystems are the richest ecosystems in the world containing more than half of the Earth's biota (Wilson 1988). The extreme biological diversity of tropical rainforests ultimately implies the chemical diversity resulting from the constant chemical innovations that exist in such ecosystems (Strobel and Daisy 2003). In tropical rainforests, the resources are limited due to the high species diversity, therefore competition among species is high, and the selection pressure is at its peak (Strobel and Daisy 2003). These factors eventually make rainforests a potentially productive source for the discovery of novel molecular structures and biologically active metabolites (Redell et al. 2000; Strobel and Daisy 2003).

Specific endophytes may have evolved within endemic plant species in areas of high plant endemicity with moist, warm, and geographically isolated climates (Strobel 2003; Strobel and Daisy 2003). This has been reported in rainforests of Venezuela, Central America, monsoonal areas of Australia, golden triangle of Thailand, Papua New Guinea, Madagascar, and upper Amazon regions (Mittermeier et al. 1999). Novel endophytic fungal taxa and series of new bioactive compounds have been discovered from each of the above areas (Mittermeier et al. 1999).

On the other hand, Strobel (2003) has stated plants growing in extremely moist conditions or plants growing in rainforests which have a more or less constant 90–100% relative humidity are prone to attack by certain extremely pathogenic fungi, thus specialized defensive mechanisms in such plants are necessary for their survival. Accordingly, such disease defences may have offered by endophytes associated with the plant (Strobel 2003). A comparative study using statistical data, revealed that tropical plant endophytes provide more active natural products and a larger number of secondary metabolites in comparison to that of temperate plant endophytes (Bill et al. 2002).

The metabolite demethylasterriquinone B-1, L-783,281 **(1)**, isolated from an endophytic *Pseudomassari* sp. collected from an African rainforest tree has acted as an antidiabetic agent (Strobel et al. 2004; Zhang et al. 1999). Unlike insulin, this non-peptide secondary metabolite (L-783,281) does not get ineffective in the digestive tract and thus can be a lead for an orally ingested drug for diabetes. Similarly, Ambuic acid **(2)** is an antifungal agent isolated from a common rainforest

endophyte *Pestalotiopsis microspora* (Li et al. 2001). Pestaloside **(3)**, an aromatic β-glucoside, and two pyrones namely pestalopyrone and hydroxypestalopyrone are other secondary metabolites isolated from *P. microspora* with antifungal and phytotoxic activities (Lee et al. 1995). Antibacterial helvolic acid **(4)** is a nor-triterpenoid isolated from *Xylaria* sp. from an endemic endangered rainforest orchid *Anoectochilus setaceus* in Sri Lanka (Ratnaweera et al. 2014). Helvolic acid has reported for antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA, MIC 4 μg mL^{-1}) and *Bacillus subtilis* (MIC: 2 μg mL^{-1}).



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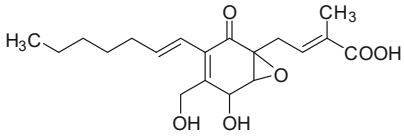

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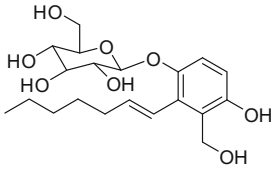

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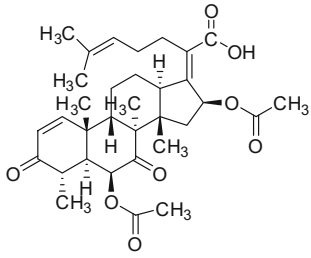
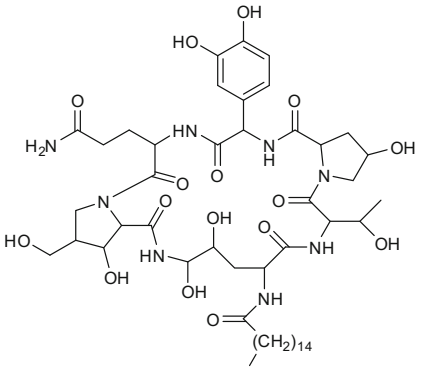


(4)

Several important bioactive natural products found in other terrestrial plants are as follows. Cryptocandin **(5)**, a peptide antifungal agent was isolated and characterized from the endophytic fungus *Cryptosporiopsis quercina* inhabiting in the medicinal plant *Tripterygium wilfordii* (Strobel et al. 1999). This compound has shown excellent antifungal activity against several human fungal pathogens, *Candida albicans*, *Trichophyton* spp. and number of plant pathogenic fungi, including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Currently several companies have tested and developed Cryptocandin to use against a number of fungi causing skin and nail diseases (Strobel 2003).

Enfumafungin **(6)**, is a hemiacetal triterpene glycoside, isolated from *Hormonema* sp. comprising in mesophyll tissue of leaves of *Juniperus communis* L (Aly et al. 2011). Enfumafungin is a specific inhibitor of fungal cell wall glucan synthesis. The compound has shown in vitro antifungal activity with 0.07 μM, EC_{50} value against *C. albicans* (Aly et al. 2011). Extensive structural modifications of the Enfumafungin resulted in the development of an orally available semi-synthetic inhibitor derived from this fungal secondary metabolite. This inhibitor, with EC_{50}, 0.6 ng mL^{-1} against *C. albicans* and 1.7 ng mL^{-1} against *Aspergillus fumigatus*, has entered phase I clinical trials as the first oral glucan synthase inhibitor for fungal infections therapy (Motyl et al. 2010).



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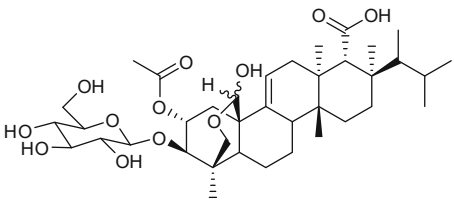
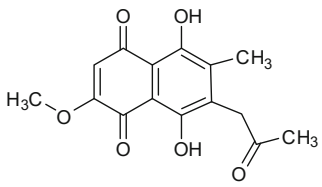


(6)

Highly antibacterial naphthaquinone, javanicin **(7)**, has been isolated from the endophytic fungus *Choridium* spp. from root tissues of *Azadirachta indica*. The sensitivity to javanicin with MIC value of 2 μg mL^{-1} showed antibacterial activity against *Pseudomonas aeruginose* and *P. fluorescens* (Kharwar et al. 2009). Phomol **(8)** is a novel polyketide lactone with antibacterial, antifungal, and anti-inflammatory activities, isolated from an endophytic *Phomopsis* sp. in Argentinian medicinal plant, *Erythrina crista*-galli (Weber et al. 2004). The diterpenoids guanacastepenes A-O, have been encountered in an unidentified endophytic fungal strain CR115, occurring in *Daphnopsis americana*. Guanacastepenes A **(9)** and I **(10)** exhibited antibacterial activity against drug resistant strains of *Staphylococcus aureus* and *Enterococcus faecalis* (Brandy et al. 2001). Recent discovery of two new metabolites, antibacterial active eupenicinicols A and B **(11, 12)**, from an endophytic fungus, *Eupenicillium* sp. harbored in the roots of a Chinese medicinal plant, *Xanthium sibiricum* showed the unfailing potential of endophytes as probable antimicrobial agents (Li et al. 2014).



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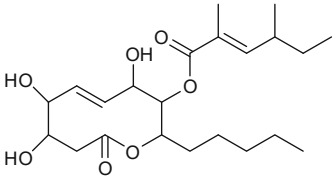

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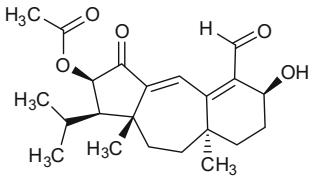

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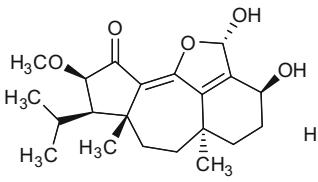

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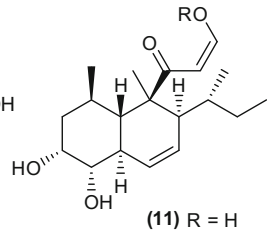
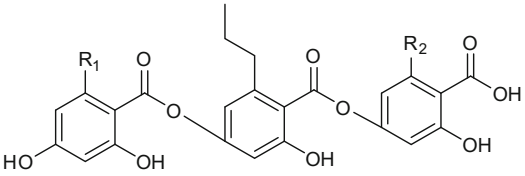


(11) R = H
(12) R = CH_3

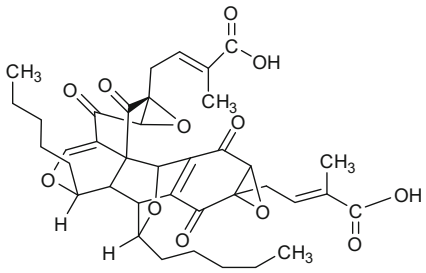
Besides the above-mentioned endophytic fungal antibiotic metabolites, there is a plethora of endophytes, with no certain compound isolated, but have been reported to show strong antibiotic activity for tested microorganisms. Methanol extract of a new endophytic fungus *Colletotrichum gloeosporioides* from the medicinal plant *Vitex negundo* with antimicrobial activity against methicillin-penicillin-and/or vancomycin-resistant clinical strains of *S. aureus*, is an example for the former statement (Arivudainambi et al. 2011).

Cytonic acid A and B **(13, 14)** are two novel human cytomegalovirus protease inhibitors isolated from endophytic fungus *Cytonaema* sp. (Guo et al. 2000). The absence of appropriate antiviral screening systems in most programs is the main limitation in this type of compound discovery. Cytochalasins are alkaloids, common in endophytic *Xylaria*, *Phoma,* and *Hypoxylon* spp. exhibiting antitumor activities (Wagenaar et al. 2000). Torreyanic acid **(15)**, is a selectively cytotoxic unusual dimeric quinone isolated from *Pestalotiopsis microspora* endophytic to the endangered tree *Torrya taxifolia* (Lee et al. 1996). Torreyanic acid, in general has demonstrated 5–10 times more potency to several cancer cell lines that are sensitive to protein kinase C agonists and caused cell death by apoptosis (Lee et al. 1996). A recent study has reported a new epitetrathiodioxopiperizine, secoemestrin D **(16)** from an endophytic fungal strain *Emericella* sp., occurred in mesophyll of *Astrgalus lentiginosus.* Secoemestrin D exhibited significant cytotoxic activity with IC_{50} values ranging from 0.06 to 0.24 μM and moderate selectivity to human glioma and metastatic breast adenocarcinoma cell lines (Xu et al. 2013).



(13) R_1 : $(CH_2)_4CH_3$ R_2 : $(CH_2)_2CH_3$

(14) R_1 : $(CH_2)_2CH_3$ R_2 : $(CH_2)_4CH_3$



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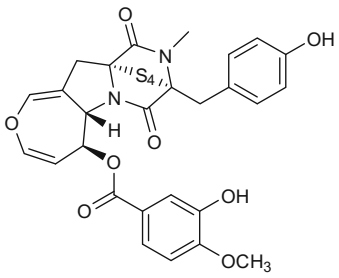
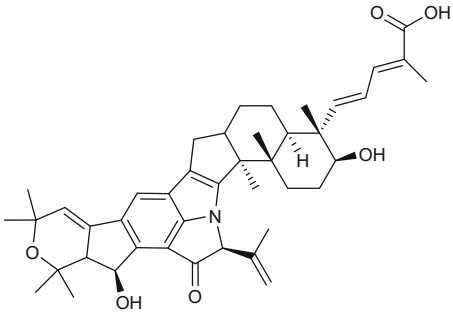


(16)

Apart from the antibiotic activities, endophytic fungi have been a potential source of various other interesting behaviors. Nodulisporic acid A **(17)** is an insecticidal fungal metabolite isolated from endophytic *Nodulisporium* sp. from the Hawaiian plant *Bontia daphnoides*. This compound has shown systemic efficacy against fleas by modulating an invertebrate-specific glutamate-gated ion channel and has resulted in identifying a potent and effective oral agent for control of fleas and ticks in mammals (Ondeyka et al. 1997; Aly et al. 2011). Subglutinols A and B **(18, 19)** are immunosuppressive compounds produced by endophytic fungus *Fusarium subglutinans*, from *Tripterygium wilfordii*. Both compounds showed IC_{50} value of 0.1 μM in the mixed lymphocyte reaction assay (Lee et al. 1995). Pestacin **(20)** and isopestacin **(21)** are two antioxidants secreted by an endophytic *P. microspora* isolated from *Timonius morobensis* growing on the north coast of Papua New Guinea (Strobel et al. 2002; Harper et al. 2003). A new alkaloid named 16α-hydroxy-5 N-acetylardeemin **(22)**, demonstrating acetylcholineesterase inhibitory activity (EC_{50}: 58.3 μM), has been isolated from a fermented broth of endophytic *A. terreus* from stems of *Artemisia annua* collected from the Zijin Mountain in China (Ge et al. 2010).



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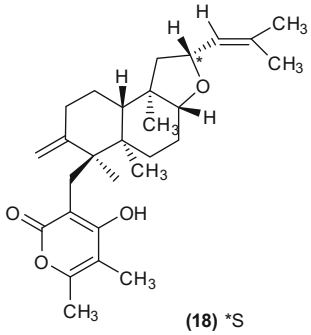
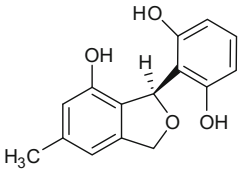


(18) *S
(19) *R



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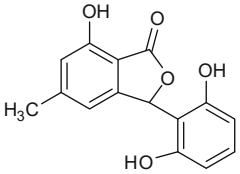

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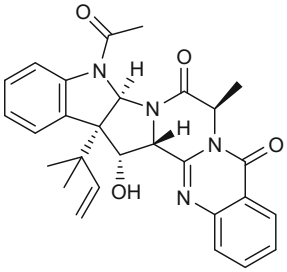


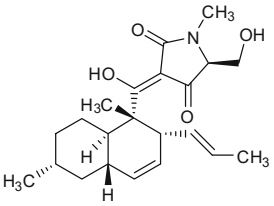
(22)

Shipunov et al. (2008) have mentioned that in the host's invaded range, endophytes increase the competitiveness of the host by producing metabolites inhibitory to evolutionarily native plants. An endophytic fungus *Fusarium* sp. of the invasive cactus *Opuntia dillenii* contained antimicrobial secondary metabolite equisetin **(23)** (Ratnaweera et al. 2015a). The production of such biologically active substances may enhance the competitive ability of the host against microorganisms and perhaps increase its adaptability to withstand the biotic and harsh abiotic stress factors that assist in the successful establishment of *O. dillenii* to the detriment of native plants in the area.

Various workers have reported grasses and sedges are reservoirs for a number of endophytic fungi and result in enhancement of the ecological fitness and tolerance to biotic and abiotic environmental stresses (Gunatilaka 2006; Mukhtar et al. 2010). In grasses and herbaceous plants, the endophytes are known to produce toxic alkaloids that prevent or poison invertebrate and vertebrate herbivores (Rodriguez et al. 2009). A Korean study has shown endophytic fungal isolates of the roots of *Monochoria vaginalis*, a weed of rice paddy significantly promote the growth of the plant mainly due to higher secretions of Gibberellins (Ahmad et al. 2010). Among the bioactive secondary metabolites, solanioic acid **(24)** isolated from *Rhizoctonia solani* from *Cyperus rotundus* showed antibacterial activity (Ratnaweera et al.

2015b). Solanioic acid has a highly functionalized and rearranged steroidal carbon skeleton and is a potent antibiotic, active at 1 μg/mL against MRSA. The endophytic *Aspergillus* strain CY725 and *Rhizoctonia* sp. strain Cy064 isolated from the leaves of *Cynodon dactylon*, have afforded antimicrobial helvolic acid, rhizoctonic acid, monomethylsulochrin and ergosterol (Li et al. 2005; Ma et al. 2004). *Paspalum conjugatum* harbored an endophytic *Microthyriaceae* sp. which contained a known mycotoxin sterigmatocystin which exhibited antiparasitic activity against *Trypanosoma cruzi*, with an IC_{50} value of 0.13 μmol L^{-1} and a novel polyketide integrasone B (Almeida et al. 2014).

Mangrove forests are considered as biodiversity 'hotspots' for marine-derived fungi (Shearer et al. 2007). This is mainly because, the permanently and intermittently submerged mangrove trunks and aerating roots are host to terrestrial, marine and an overlap of terrestrial and marine fungi (Sarma and Hyde 2001; Shearer et al. 2007). According to Schmit and Shearer (2003), 106 fungi have been reported from mangrove habitats in the Atlantic Ocean, while 173 and 128 are documented from Pacific and Indian Ocean mangroves, respectively. Among the mangrove-derived fungal community, the fungal endophytes play an important role protecting their host against various aggressions (Cheng et al. 2009). According to reports more than 200 species of endophytic fungi have been isolated and identified from mangrove plants and dominant among them are species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Fusarium*, *Paecilamyces*, *Penicillium*, *Pestalotiopsis*, *Phoma*, *Phomopsis*, *Phyllosticta,* and *Trichoderma* (Liu et al. 2007). Mangrove-derived endophytic fungi are believed to contribute to their hosts' ability to adapt to endure the extreme habitat conditions (Debbab et al. 2013). In addition, these mangrove endophytic fungi are proven to be a promising source of structurally unique natural products, and drug leads with remarkable bioactivities (Tan et al. 2008). Cytosporone B **(25)** is such a novel natural product, isolated from an endophytic *Dothiorella* sp. from mangrove plant *Avicennia marina* at an estuary in China, with broad antifungal activities against *A. niger*, *Trichoderma* sp. and *Fusarium* sp. and high activity against human epidermal carcinoma and several other cell lines (Xu et al. 2005). Recent report of two new antibacterial α-pyrone derivatives, infectopyrones A **(26)** and B **(27)** from the mangrove endophytic fungus, *Stemphylium* sp. isolated from a *Brguiera* sp. also demonstrates the potential of mangrove endophytes to produce bioactive chemical scaffolds (Zhou et al. 2014). Two new compounds pinazaphilones B and (±)-penifupyrone with significant α-glucosidase inhibitory activity have been discovered from a mangrove endophytic *Penicillium* sp. isolated from the fresh branches of the mangrove plant *Cerbera manghas* (Liu et al. 2015).



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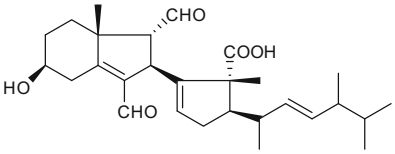

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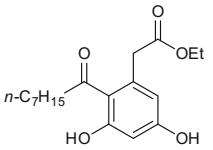

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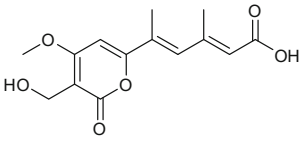

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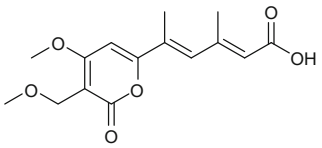
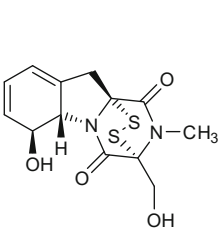


(27)

Mangrove associates are species mainly distributed in terrestrial or aquatic habitat but also occur in the mangrove ecosystem (Parani et al. 1998). According to Tomlinson criteria, mangrove associates are also distinguished from true mangroves by lacking aerial roots, vivipary, and no physiological mechanism for salt exclusion (Wang et al. 2011). However, mangrove associates growing in the mangrove habitat also have to face the same extreme ecological conditions as the true mangroves. Therefore, these mangrove associates also have the potential of producing bioactive natural products as the true mangroves. This is evident by the recent report of Ratnaweera et al. (2016), who described the isolation of antimicrobial gliotoxin **(28)** and Bisdethiobis (methylthio) gliotoxin **(29)** from an extract of the endophytic fungus *Hypocrea virens* from the plant *Premna serratifolia* from a mangrove habitat.

Inland fresh water bodies also are productive ecosystems in the world which house diverse microorganisms. Aquatic plants highly adapted to its environmental and ecological conditions also harbor endophytic fungi having bioactive metabolites. A recent investigation of endophytic fungi of *Nymphaea nouchali* led to the isolation of the known secondary metabolites chaetoglobosin A and C **(30, 31)** from *Chaetomium globosum*, with chaetoglobisn A showing good antibacterial activities (Dissanayaka et al. 2016).



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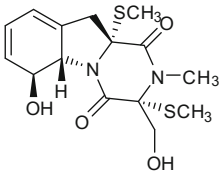

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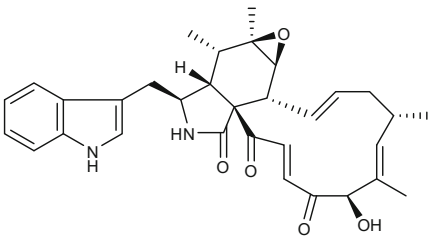

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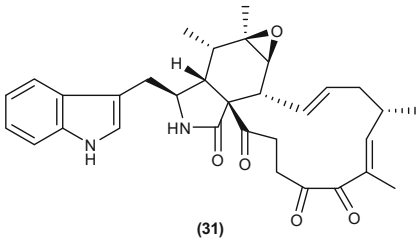
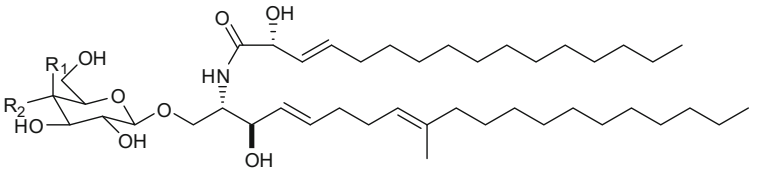
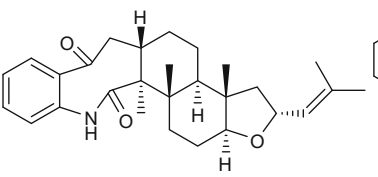


(31)

Among the vast diversity of marine-derived fungi are endophytic fungi from macro-algae, sea grasses and other marine plants. Most of these fungi belong to class Ascomycota and their distribution is governed by plant metabolites, temperature, salinity and pH (Ji and Wang 2016). These fungi are proven to be prolific producers of huge array of bioactive natural products. Up to date more than 300 natural products have been identified from endophytic fungi of marine macro-algae. From the published natural products 43% were reported novel compounds with various biological activities such as antioxidant, anticancer, antiplaspodial, and antimicrobial (Flewelling et al. 2015). Among the novel antimicrobial metabolites are Asperamide A, B (**32**, **33**), Asporyzin A-C (**34–36**) and Asperversin A (**37**), from endophytic *Aspergillus* spp., isolated from *Colpomenia sinuosa*, *Heterosiphonia japonica,* and *Sargassum thunbergii,* respectively (Zhang et al. 2007; Qiao et al. 2010; Miao et al. 2012). Myrocin A (**38**) and asperwentin A-C (**39–41**) are some of the anticancer compounds isolated from *Apiospora montagnei* from *Polsiphonia violacea* and *Aspergillus wentii* from *Sargassum fusiforme* (Klemke et al. 2004; Miao et al. 2014). Three 2-pyridone alkaloids, the known N-hydroxy-2-pyridone PF1140 (**42**), and two new 2-pyridones **43** and **44** have been isolated from a *Penicillium* species associated with the New Zealand marine brown algae *Xiphophora gladiata* (de Silva et al. 2009). PF1140 was active against *B. subtilis* and *C. albicans* and to that of murine leukemia P388 cells. Both **43** and **44** were inactive pointing to the importance of the presence of the N–OH functionality meant for bioactivity.



(32) R_1: OH, R_2: H
(33) R_1: H, R_2: OH



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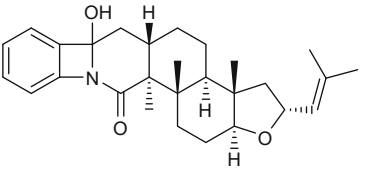

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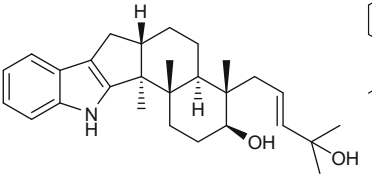

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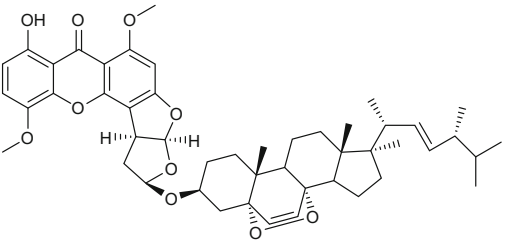

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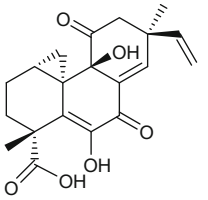

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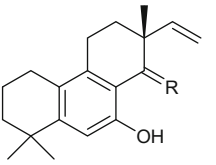
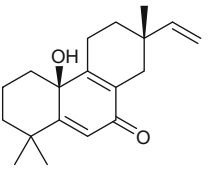
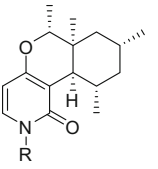


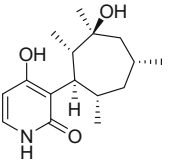
(39) R=H_2
(40) R=O



(41)



(42) R=OH
(43) R=H



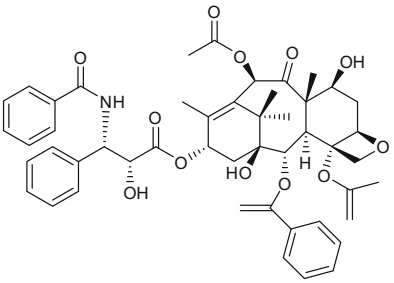
(44)

9.6 Production of Similar Metabolites by Endophytic Fungi and Host Plants

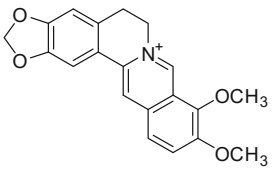
The long co-evolution of endophytes with their host plants has resulted a genetic recombination. It has opened the path for some endophytes to produce the same bioactive compounds originally characteristic of the host plant (Tan and Zou 2001). Taxol **(45)**, Berberine **(46)**, Sanguinarine **(47)** Camptothecin (CPT) **(48)** producing endophytic fungi are examples for this phenomenon. Taxol (paclitaxel) **(45)**, the first billion dollar anticancer drug was discovered initially from *Taxus brevifolia* and later from 11 other *Taxus* species in the world (Stierie et al. 1993). Therefore, as an alternative source, taxol producing endophytic fungi have been investigated from these yew plants, and *Taxomyces andreanae*, was the initially discovered taxol producing endophytic fungus from host plant *Taxus brevifolia* (Strobel 2003).

Berberine **(46)**, with diverse pharmacological properties is an isoquinoline alkaloid isolated from several medicinal plants including *Berberis aristata*, *Hydrastis canadensis*, *Coptis chinensis*, *Coptis rhizome*, *Coptis japonica*, *Phellondendron amurense,* and *Coscinium fenestratum* (Timothy et al. 1997; Tillhon et al. 2012). This natural product is currently undergoing 10 clinical trials (Tillhon et al. 2012). Berberine has also been reported from the endophytic fungus, *Fusarium solani*, from the roots of *Coscinium fenestratum* a critically endangered plant species (Diana and Agastian 2013). Since *C. fenestratum* also been reported to produce berberine, it supports the theory that, with the long co-evolution with the host, an endophyte can adapt to the special microenvironments through genetic modification which includes uptake of some plant DNA into their own genomes (Germaine et al. 2004; Diana and Agastian 2013).

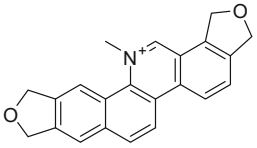
Sanguinarine **(47)** is an antimicrobial benzylisoquinoline alkaloid reported from several plants belonging to the family Papaveraceae including *Macleaya cordata* (Nicoletti and Fiorentino 2015). This compound has also been isolated from the endophytic fungal strain of *Fusarium proliferatum* inhabiting the leaves of *Macleaya cordata* (Wang et al. 2014). CPT **(48)** is another anticancer agent first isolated from the extracts of *Camptotheca acuminata*, and later from several other plants (Wall et al. 1966; Asano et al. 2004). The production of CPT in *Ophiorrhiza mungos* was first reported by Tafur et al. (1976). Later, Salim et al. (2011) isolated the CPT producing endophytic fungus *Glomerella cingulata* from *O. mungos* providing an alternative strategy to reduce the need to harvest slow-growing and possibly rare plants consequently helping to preserve the world's ever diminishing biodiversity. In addition, it is easier and more economical to produce a valued phytochemical by exploiting a microbial source than using a plant, which eventually leads to increase availability and low market price (Radic and Strukelj 2012).



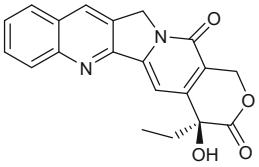
(45)



(46)



(47)



(48)

9.7 Factors Influencing the Production of Secondary Metabolites of Endophytic Fungi

In the natural setting, the climatic conditions, soil, season, location, age and tissue of the host plant, all affect the endophytes' biology, and consequently considerable variations in the production of secondary metabolites (Strobel and Daisy 2003). Therefore, the chemical substances isolated from two endophytic fungi of the same species may differ from each other. At the same time, the differences also in the isolation methods and in vitro cultivation conditions can impact the kind and range of secondary metabolites (Gunatilaka 2006). It has been reported that the size of the plant tissue fragments used for the isolation, time since harvesting of the tissue, composition of the culture media and culture conditions such as aeration, temperature, pH, incubation period, agitation, shape of the culturing flask (with respect to liquid media), all affect the production of secondary metabolites in laboratory (Aly et al. 2011; Kusari et al. 2012). Even, the production of six new secondary metabolites by the plant associated fungus *Paraphaeosphaeria quadriseptata*, only when the water used to make the media changed from tap water to distilled water is a good example to prove this fact (Paranagama et al. 2007).

9.8 Current Challenges

To achieve a competent endophyte with promising bioactivity is a challenging task (Scherlach and Hertweck 2009; Kusari et al. 2012). In the traditional way, this requires screening of a plethora of endophytes from a host. Most of the isolating endophytes are incompetent, i.e., they do not possess desired potential to produce bioactivity. However, whole-genome sequencing strategies have revealed that the incompetent endophytes express only a subset of biosynthetic genes under laboratory culture conditions (Scherlach and Hertweck 2009; Winter et al. 2011). Therefore there is possibility of utilizing the large reservoir of cryptic natural metabolites by experimenting with different in vitro culture conditions and understanding the chemical ecological interactions of endophytes (Kusari et al. 2012).

Another major challenge is the low yield of the active desirable compound/s obtained from the cultures (Yu et al. 2010; Aly et al. 2011). This is a major drawback for bioactive compounds from entering the commercial industry. For an example, the yield of the anticancer drug paclitaxel obtained from several endophytic fungal cultures are 846.1, 187.6 and 163.4 μg L^{-1}, which is too low for commercial production (Gangadevi and Muthumary 2008; Liu et al. 2009). Therefore, so far fungal endophytes have not been an industrial source of paclitaxel (Aly et al. 2011) or any other pharmaceutical. However, genetic engineering technology and research identifying the regulatory gene/s in the biosynthesis pathway of the active compound can lead to increase production of the compounds (Yu et al. 2010; Radic and Strukelj 2012).

The mammalian toxicity of any prospective drug developed has also become a major concern in the field (Yu et al. 2010). Most of these isolated bioactive metabolites precluded clinical use due to the toxicities to animals and humans (Waring and Beaver 1996). However, Strobel (2003) stated that plants as an eukaryotic system, have naturally served as a selection system for microbes having bioactive molecules with reduced toxicity toward higher organisms.

Compounds showing moderate biological activity most often cannot be used as potential chemotherapeutic agents (Yu et al. 2010). Totally or partly unknown biosynthesis, regulation, and synthesis, of these natural products in endophytes are other issues in the field (Yu et al. 2010). The rapidly diminishing rainforests, which is a huge reservoir for novel fungal endophytes and their bioactive products, is one of the major problems facing the future in this area (Strobel 2003). Therefore, countries need to establish information repositories of their biodiversity and at the same time should take conservation measures to protect the biodiversity.

Despite speculation by many authors no endophytic fungal-derived metabolite has so far become commercially useful (Kusari and Spiteller 2011). However, interest in the biosynthetic abilities of the endophytic fungi by the scientific community has not diminished but in fact is on the rise.

9.9 Conclusion

Exploration of competent endophyte for only subset of biosynthetic genes expressed under laboratory culture conditions is not enough to utilize the large reservoir of natural metabolites produced endophytes. Therefore, incorporation of genetic manipulation technology so as to advance the research to identify the regulatory gene/s of several biosynthesis pathways of metabolite production can lead to increase production of the compounds to be used in human welfare. As such, innovative approaches are bound to result in the productive utilization of this important and remarkable resource of much potential in the coming years.

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Chapter 10
Endophytes: Potential Source of Therapeutically Important Secondary Metabolites of Plant Origin

Shahid Iqbal Mohammed, Mohini Panditrao Patil, Ravindra Himmatrao Patil and Vijay Laxminarayan Maheshwari

Abstract Use of plants and plant-derived metabolites for human health and well-being is as old as human civilization. The plant kingdom contains an estimated 400,000–500,000 different species and each plant produces a number of secondary metabolites which enables them to withstand various biotic and abiotic stresses. The plant secondary metabolites such as alkaloids, steroids, flavonoids, terpenoids, etc., are known to have a number of biological activities. Moreover, because of their natural origin, the plant-derived metabolites are increasingly preferred for therapeutic applications all over the world. However, the overharvesting of plants for biologically active secondary metabolites is rapidly diminishing the valuable treasure of medicinal plants. Endophytes are the microbial symbionts which live in the internal tissues of plants and mimic the chemistry of the host plant. Because of their huge diversity and ability to produce a range of metabolites similar to host plant, they have attracted significant attention of scientific community all over the world. The plant- and endophyte-derived metabolites which have attracted sufficient research in last decade include compounds with antioxidant, antihypercholesterolemic, antidiabetic, and anticancer activities. The present article reviews the current state of research on biologically active metabolites from plant and endophytic fungi. The work carried out in our lab on bioprospecting of endophytic fungi for molecules with antihypercholesterolemic potential is also included.

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10.1 Introduction

Secondary metabolites are naturally derived substances from plants, animals, and microorganisms (Baker et al. 2000). Plant secondary metabolites are known to provide protection from pathogen invasion to their host plant (Yang et al. 1994). As per world health organization (WHO) estimates, 80% of the people in the developing countries use traditional plant derived drugs for their primary health need. Moreover, plant secondary metabolites have featured significantly in the treatment of many diseases as well as used as base substances for the development of efficient drugs. For example, Digitoxigenin, a steroid glycoside produced by *Digitalis purpura,* is the oldest of the commonly used compounds in the treatment of heart diseases. After the discovery of Penicillin, the wonder drug, followed by advancement in the fermentation technology for cultivation of microbial cells, the potential of microbes as a good source of secondary metabolites was recognized by world scientific community. Microorganisms produce a range of secondary metabolites, many of them novel/new, apparently as a part of survival/adaptation strategy to overcome constant metabolic stress and challenging environmental conditions that they live and encounter continuously (Schutz 2001). On the other hand, despite the abundant biological diversity of plants on earth, discovery of bioactive secondary metabolites has focused on microorganisms which were mainly isolated from soil. Therefore, selection of the ecological niche for isolation of the desired microbes is an important stage in new drug discovery rather than using a totally random approach.

Throughout history, humans have used plants and plant-derived medicines to treat all kinds of illnesses. However, extensive use of plant derived medicines has questioned the survival of many medicinally valuable and rare plant species. So it is a need of hour to find alternate sources and strategies for the production of bioactive metabolites of plant origin. Approximately 0.4–0.5 million plant species exist on the earth and, every plant inhabits/hosts one or other endophytic microorganism in its internal tissues. Thus, this huge and relatively unexplored biodiversity can be tapped for the search of novel microbiota significant for chemical and functional diversity.

De Bary (1866) first coined and introduced the term “endophytes”. They are the microbes that colonize inside the living internal tissues of the host plant without causing any negative effect on them (Bacon and white 2000) and establish symbiotic relationship with their host. Among the endophytic microorganisms, the endophytic fungi are highly diverse and are known to produce biologically active and structurally diverse natural products of pharmaceutical importance. The bioactive compounds isolated from endophytic fungi have been found to produce a range of compounds/metabolites having diverse biological properties including antimicrobial, antioxidant, antidiabetic, anticancer, antihypercholesterolemic, etc. (Ruma et al. 2013). Use of endophytic fungi as a source of therapeutically important

compounds/metabolites has several advantages. For example, (i) When a plant based drug is produced from the source like endophytic fungi, it will not only reduce our dependence on plant sources for these metabolites but also help in preserving the continuously decreasing plant biodiversity on earth (Strobel et al. 2004). (ii) Environmental conditions which affect the quality and quantity of desired phytochemicals *in planta* can be avoided if isolated endophytes are cultured successfully in vitro by providing optimum physicochemical parameters. (iii) Production of plant derived drugs using microbial sources can be easily scaled up for industrial process. It is also possible to obtain improved versions of existing drugs by simply altering the cultural conditions. (iv) Isolation of drugs from microbial sources is relatively easier and cost-effective as compared to extraction and purification of drugs from the whole plant or plant part(s) including tissue culture. (v) Since the plant and fungal endophytes, both are eukaryotic systems, the metabolites produced by the endophytes are less likely to show mammalian toxicity when used for human applications and, (vi) High-value metabolites/compounds of medicinal importance are produced in easier and economical way using the endophytic microorganisms (Strobel et al. 2004).

It has been proved that both plant and endophytic fungi isolated from the same plant produced similar compounds with same bioactivity (Kusari et al. 2012). The possible reason for this could be the genetic recombination between the host and endophytes or vice versa that occurred during the course of evolution (Tan and Zou 2001).

In the ensuing paragraphs, an attempt has been made to briefly review the studies on plants and endophytic fungi which could produce antioxidant, antihypercholesterolemic, antidiabetic, and anticancer metabolites of therapeutic importance.

10.2 Antioxidant Activity of Plants/Plant Extracts

Reactive oxygen species (ROS) generated in the biological system are the major cause of the degenerative conditions such as aging, cancer inflammation, atherosclerosis, etc. (Sandesh et al. 2014). Naturally occurring antioxidants/enzymes, superoxide dismutase, catalase, glutathione, etc., can stabilize the adverse effects of ROS and, thus help maintain the redox balance in the body. However, when generation of ROS is more than what can be processed by the endogenous antioxidant system, it results in oxidative damages leading to several other disorders such as arthritis, atherosclerosis, cancer, diabetes, and many others (Castaneda et al. 2003). Sufficient amounts of exogenous antioxidants are required to reduce the effects of ROS to the human body. In response to the growing consumer demand for food supplements that are free of synthetic antioxidants with carcinogenic potential (Baardseth 1989), there has been tremendous increase in the search for naturally occurring antioxidants during the past decades (Gould 1995). Of the vast amount of literature, a concise list of studies on the antioxidant activities of plant/plant parts using DPPH free radical scavenging assay, mainly in last 10 years is shown in Table 10.1.

Table 10.1 List of plants with DPPH radical scavenging activity

Name of plant	Extract	Results	References
Alnus incana	Catkins methanolic extract	Showed IC_{50} value 18.9 µg/ml	Stevie et al. (2010)
Tansy vulgare	Arial part methanolic extract	Showed EC_{50} value 37 µg/ml	Juan-Badaturuge et al. (2009)
Tussilago farfara	Flower bud ethanolic extract	Dose dependent increase in scavenging activity	Li et al. (2012)
Gemmae betulae	Bud ethanolic extract	Normalized phospholipid level	Mashentseva et al. (2011)
Sorbus aucuparia	Fruit aqueous extract	Dose dependent increase in radical scavenging activity	Zlobin et al. (2012)
Carica papaya	Seed ethyl acetate fraction	Increase in radical scavenging activity	Zhou et al. (2011)
Manihor esculenta	Leaves aqueous extract	Increase in radical scavenging activity	Tsumbu et al. (2011)
Arnebia benthamii	Plant ethyl acetate extract	700 µg/ml extract showed 87.99% inhibition	Ganie et al. (2014)
Bauhinia vahlii	Plant methanolic and aqueous extract	Methanolic extract showed strong antioxidant potential	Sowndhararajan and Kang (2013)
Hygrophila auriculata	Root extract	Showed significant antioxidant activity	Shanmugasundaram and Venkataraman (2006)
Phyllanthus niruri	Methanolic and aqueous extract of leaves and fruits	Inhibited reactive oxygen species	Chatterjee et al. (2006)
Pistacia lentiscus	Ethanolic extraction from ethyl acetate fraction	Showed radical scavenging activity (90%) equivalent to standard BHA (89%)	Atmani et al. (2009)
Polyalhia cerasoides	Plant ethanolic extract	Dose dependent inhibition of DPPH scavenging activity with IC_{50} 25 µg/ml	Ramkumar et al. (2009)
Teucrium polium	Plant aerial part petroleum ether, chloroform, methanol and water extract	Highest antioxidant activity was observed in methanolic extract showed IC_{50} value 20.1 µg/ml nearly similar to standard (18.3 µg/ml) BHT	Sharififar et al. (2009)
Bidens pilosa	Leaves and flower essential oil	Radical scavenging activity with IC_{50} value 21 and 36 µg/ml for leaves and flower essential oil respectively	Deba et al. (2008)
Rheum ribes	Stem and root methanolic extract	87.07 and 60.60% inhibition with 100 µg/ml for stem and root extract respectively	Ozturk et al. (2007)

10.2.1 Antioxidant Compounds from Endophytes

Polysaccharides from plants and microorganisms have been extensively reported as potent natural antioxidants (Chen et al. 2009). Liu and coworkers (2009), for the first time, reported the capacity of endophytic microorganisms to produce polysaccharides with antioxidant activity. Patil et al. (2015) isolated endophytic *Aspergillus flavus* from Indian medicinal plant, *Aegle marmelos*, which produced bioflavonoid, rutin with excellent antibacterial and antioxidant activities. Graphislactone A that showed potent radical scavenging activities was obtained from endophyte *Cephalosporium* spp. isolated from *Trachelospermum jasminoides* (Strobel and Daisy 2003). Other endophytic fungi such as *Aspergillus niger* and *Alternaria alternata,* isolated from *Tabebula argentea* have been shown to produce Lapachol with excellent antioxidant activities (Sadananda et al. 2011). An endophytic strain of *Colletotrichum gloeosporioides* from a fruit of plant *Forsythia suspensa* was found to produce plant derived metabolite, phillyrin in liquid cultures (Zhang et al. 2012). A representative group of antioxidant compounds obtained from endophytic fungi that were previously produced by their host plants is shown in Fig. 10.1.

A number of endophytic strains from *Vitis vinifera, Vitis quinquangularis*, and *Polygonum cuspidatum* belonging to the genera *Alternaria, Aspergillus, Botryosphaeria, Cephalosporium, Geotrichum, Mucor,* and *Penicillium* were shown to produce resveratrol, which is a stilbene phytoalexin with excellent antioxidant properties (Shi et al. 2012). Another phyllosphere species of *Alternaria*, together with root strains of *Fusarium solani, F. oxysporum* and *F. proliferatum* from *Cajanus cajan*, have been found to produce cajanin stilbene acid, a related

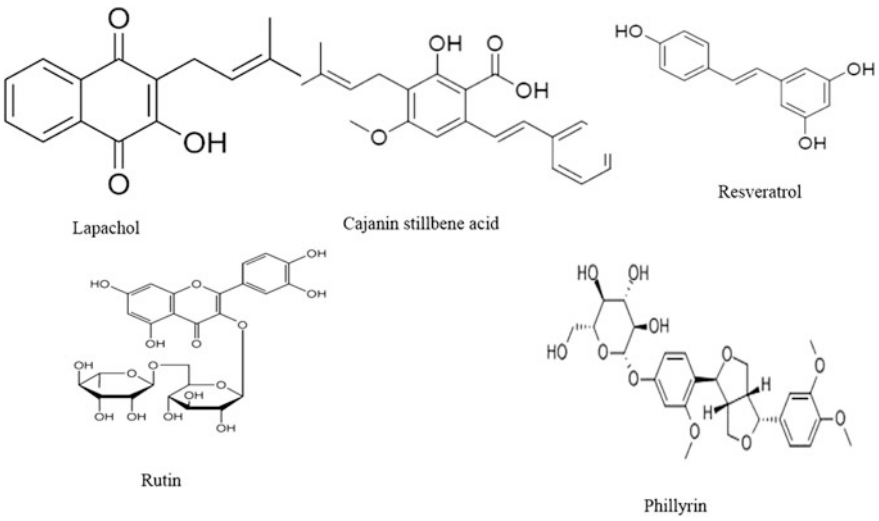

Fig. 10.1 Antioxidant compounds from endophytic fungi originally produced by their host plant

antioxidant compound originally characterized from its host plant (Zhao et al. 2012). The Tanshinones are diterpenoid quinone compounds that have been obtained from endophytic strains of the *Salvia miltiorrhiza*, particularly *Emericella foeniculicola* TR21 (Ma et al. 2011), and *Trichoderma atroviride* D16 (Ming et al. 2012).

10.3 Studies on Antihypercholesterolemic Activity of Plants

Cardiovascular diseases (CVDs) are the major cause of death in the developed as well as in the developing countries. Among CVDs, hypercholesterolemia is the most important contributing factor responsible for development of CVDs and atherosclerosis. Epidemiological studies have proved that the elevated levels of blood cholesterol increase risk of CVDs several times. Krishnakumari and Priya (2006) evaluated hypolipidemic activity of *Achyranthes aspera* against sesame oil fed lipidemic rats. Rats treated with powdered seed aqueous extract of the plant showed significant reduction in lipid profile parameters and increase in level of high-density lipoprotein to normal level. Similarly, antihypercholesterolemic effect of *Piper beetle* ethanolic extract and its purified eugenol constituent against triton WR-1339 induced hypercholesterolemia in rats was demonstrated by Venkadeswaran et al. (2014). Hypercholesterolemic rats treated with 500 mg/kg body weight *Piper beetle* extract or 5 mg/kg body weight of the purified constituent, eugenol orally for seven days showed significant improvement in parameters of lipid profile. The improvement by the plant extract was found to be at par with that of standard lipid lowering drug, lovastatin at 10 mg/kg body weight. Subash and Augustine (2012) evaluated hypolipidemic effects of methanol fraction of *Aconitum heterophyllum* in diet-induced obese rats. Treatment of obese rats with extract decreased level of total cholesterol, triglycerides, apolipoprotein B and increased the level of high-density lipoprotein and apolipoprotein A level as compared to control rats. In our laboratory, hypolipidemic effect of bark ethanolic extract of *Terminalia arjuna* against high fat induced hypercholesterolemic rats treated with 40 mg/kg body weight of plant extract showed statistically significant reduction in total cholesterol, triglycerides and low density lipoprotein, with a concomitant increase in level of high-density lipoprotein, lipoprotein lipase, and enhanced bile acid synthesis as compared to control rats (Patil et al. 2011).

A list of selected antihyperlipidemic studies of plant/plant extracts, along with methods/model employed for study and high light of the obtained results in past decade is shown in Table 10.2.

Table 10.2 Plant/plant extracts with antihypercholesterolemic activity

Name of plant	Extract/plant part	Model/method	Results	References
Alchornea cordifolia	Leaves n-butanolic fraction	Streptozotocin-induced diabetic wistar rats	Significant decrease in total cholesterol	Mohammed et al. (2012)
Aloe vera	Processed *Aloe vera* gel	Non-insulin dependent diabetes mellitus mouse (feeding mouse with a high fat diet)	Hypoglycemic and hypolipidemic activity	Kwanghee et al. (2009)
Cajanus cajan	Methanolic leaf extract	Alloxan-induced hyperlipidemia in diabetic rabbits	Significant decrease in LDL/HDL ratio	Akinloye and Solanke (2011)
Curcuma longa	Rhizomes methanol extract	Alloxan-induced hyperlipidemia in diabetic rabbits	Decrease in plasma triglyceride	Nwozo et al. (2009)
Eclipta prostrata	Leaves aqueous extract	Atherogenic diet induced hyperlipidemic rats	Significant decrease on total cholesterol and triglycerides	Dhandapani (2007)
Emila praetermissa	Leaves aqueous extract	Wistar rats	Hypolipidemic activity	Nwodo et al. (2014)
Eugenia jambolana	Composite extract of seeds	Streptozotocin-induced diabetic male albino rat	Serum lipid profile came significantly close to normal level in a dose dependent manner	Mallick et al. (2006)
Moringa oleifera	Leaves petroleum ether extract	Fat diet induced obesity in rats	Antiobesity and hypolipidemic	Bais et al. (2014)
Pithecellobium dulce	Leaves aqueous extract	Triton Wr-1339 induced hyperlipidemic rats	Significant decrease in the levels of serum cholesterol, phospholipids, triglyceride, LDL, VLDL and significant increase in the level of serum HDL	Sundarrajan et al. (2010)
Stachytarphela augustifolia	Plant methanolic extract	Streptozotocin-induced diabetic wistar rats	Significant decrease in total cholesterol, low density lipoprotein and lipid peroxides as function of treatment	Garba et al. (2013)
Tephrosia purpurea	Leaves ethanolic extract	Streptozotocin-induced diabetic rats	600 mg/kg body weight normalized the lipids and lipoproteins profile	Pavana et al. (2007)
Urtica dioica	Leaves ethanolic and aqueous extract	Alloxan-induced diabetic or hyperlipidemic rats	Rats treated with ethanolic and aqueous extract showed significantly reduction in levels of triglyceride and cholesterol	Mahjoub et al. (2012)

10.3.1 Antihypercholesterolemic Compounds from Endophytes

HMG–CoA reductase (HMGR) is the key enzyme in the cholesterol biosynthesis pathway and it is the attractive target of several antihypercholesterolemic drugs. Statins, the fungal secondary metabolites, are widely used as competitive inhibitors of HMG–CoA reductase all over the world. Lovastatin, a highly potent inhibitor of HMG–CoA reductase is commercially produced using a micro-fungus, *Aspergillus terreus* (Patil et al. 2011). Endophytic fungus, *Aspergillus niger* was isolated from *Taxus baccata* which was able to produce lovastatin when cultivated in solid-state fermentation (Raghunath et al. 2012). In another study, rosuvastatin, a potent inhibitor of HMG-CoA reductase, used for treating dyslipidemias, was produced from *Penicillium citrinum* and *P. brevicompactum* (Scott et al. 2004). Bhargavi et al. (2014) studied lovastatin production using soil and endophytic fungi, demonstrated that the soil isolate, *Aspergillus terreus* NCBI (KM017963) produced lovastatin whereas none of the endophytic fungi tested showed lovastatin production when cultured in solid-state fermentation. Endophytic fungi have been recognized as an important source for these antihypercholesterolemic compound/metabolites and structures of a few compounds obtained from them are shown in Fig. 10.2.

Another metabolite, chartarlactams A-P, phenylspirodrimanes produced by Sponge- associated endophytic fungus *Stachybotrys chartarum* exhibited potent antihyperlipidemic activity in HepG2 cells assessed by Oil Red O staining (Yong et al. 2013). On the other hand, endophytic fungus *Mycosphaerella* sp. PF13 was

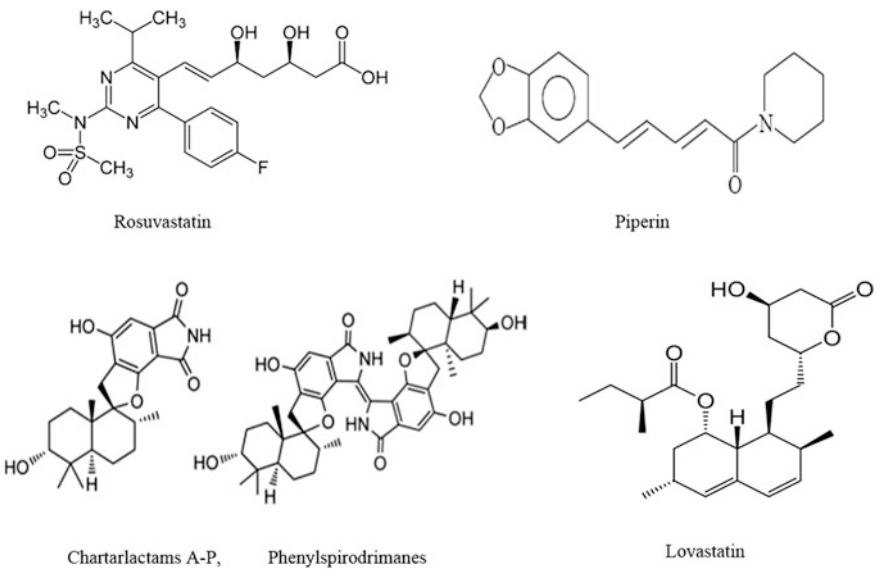

Fig. 10.2 Antihypercholesterolemic compounds produced by endophytic fungi

found to have the potential to produce piperine (Chithra et al. 2014a). Piperine has also been reported to possess a wide range of applications because of its antimycobacterial, antihyperlipidemic, antiandrogenic, immunoregulatory, and antitumor properties (Chithra et al. 2014b).

Obesity is also a contributing factor for variety of serious diseases like hypertension, hyperlipidemia, atherosclerosis, and type II diabetes (Birari and Bhutani 2007). One of the key enzymes involved in lipid metabolism is pancreatic lipase (PL) which acts on triglycerides and converts them into glycerol and free fatty acids. The free fatty acids further increase the level of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) in the blood and ultimately contribute to development of CVDs. Inhibition of PL would decrease the level of LDL and VLDL and hyperlipidemia is prevented (Sreerama et al. 2012; Onakpoya et al. 2015). Therefore, PL can be recognized as attractive therapeutic target for management of hypercholesterolemia and diet-induced obesity. Endophytic fungi have been identified as promising good source of effective PL inhibitors. Recently, Gupta et al. (2015) isolated 70 endophytic fungi and screened them qualitatively using in vitro screening methods. The PL inhibitory effect of endophytic *Penicillium* spp. in their study was comparable with the standard PL inhibitor, Orlistat. Other studies have also shown the potential PL inhibitory activity of polyphenol-rich extracts in vitro (Birari and Bhutani 2007; McDougall et al. 2009). In our lab, we have isolated, screened, and identified a number of endophytic fungi which could produce metabolites with antihypercholesterolemic potential. One of our isolates, obtained from *Terminalia* arjuna and identified as *Diaporthe arengae,* showed strong in vitro as well as in vivo antihypercholesterolemic activity. Administration of the extract of *Diaporthe arengae* at 100 mg kg^{-1} body weight dose in rats showed significant decrease in the levels of serum total cholesterol (TC), triglycerides (TG), very low density lipoproteins (VLDL), and low density lipoprotein (LDL) cholesterol (unpublished data).

10.4 Antidiabetic Activity of Plants/Plant Extracts

Diabetes mellitus (DM) is characterized by hyperglycemia resulting from either lack of insulin, or insulin resistance at the cellular level (Haire-Joshu 1996). It has been estimated that 366 million people may be affected by diabetes worldwide by the year 2030. In India, about 40.9 million people are affected by hyperglycemia and this number may rise up to 60.9 million by the year 2025 (Maahs et al. 2010). Present diabetes management strategies primarily employ insulin and other synthetic antidiabetic agents like sulfonylureas, biguanides, glinides, etc. Despite considerable success in diabetes management by these strategies, there is a need for newer strategies to overcome the limitations of existing compounds (Ghazanfar et al. 2014). It should be noted that diabetes management with minimal side effects is a challenge before the scientific community (Saxena and Vikram 2004). There

has been an enhanced focus on exploring indigenous medicinal plants with antidiabetic potential so that a low cost, safer, and effective alternative strategy to insulin and other synthetic compounds for diabetes management can be realized. Hypoglycemic effects of several plants used to treat diabetes are already known and the underlying mechanisms of the observed affects are also being worked out (Patel et al. 2012). Recently, Mohammed et al. (2016), in a comprehensive study, evaluated antidiabetic activity of *Coccinia grandis* against streptozotocin-induced diabetic rats. Treatment of diabetic rats with *Coccinia grandis* leaf ethanolic extract (500 mg/kg) for 21 days showed significant reduction in blood glucose level, increase in body weight and serum insulin in a dose dependent manner. Diabetes induced hypercholesteremia and hypertriglyceridemia, which can lead to secondary complications, were also found to be significantly improved as a result of treatment. Marked recovery was also recorded in liver and kidney function tests of diabetic rats. The study demonstrated a strong antioxidant activity of the ethanolic leaf extract of the plant. It should be pointed out here that oxidative stress is a natural consequence of chronically elevated blood glucose level and thus, compliments diabetes (Rahimi et al. 2005). Baldea et al. (2010) evaluated antidiabetic activity of crude ethanolic extract of 17 Boreal forest medicinal plants by in vitro on Caco-2 human enterocylic cell lines and in vivo on normal rats by oral administration of 250 mg/kg body weight of extract. In in vitro experiments, of the 17 plants, 13 showed approximately 40% decreased glycaemia and another 2 plants showed reduction in intestinal glucose absorption in rats. Similarly, antidiabetic activity of *Allium cepa*, *Allium sativum*, and *Zingiber officinale* aqueous extract was evaluated against alloxan-induced diabetic rats. All three plant extracts showed decrease in blood glucose level in treated diabetic rats as compared to control rats (Eyo et al. 2011). A comprehensive list of studies of the last 5–6 years on antidiabetic activities of plant/plant extracts demonstrating the interest and scope in the field is given in Table 10.3.

10.4.1 Antidiabetic Compounds from Fungal Endophytes

Dhankar et al. (2013) demonstrated the antidiabetic and hypolipidemic activity of endophytic fungi, *Aspergillus* sp. and *Phoma* sp. isolated from *Salvadora oleoides* (Salvadoraceae). The study showed that 2,6-di-tert-butyl-p-cresol and Phenol, 2,6-bis [1,1-dimethylethyl]-4-methyl, isolated from the broth extract of endophytic fungi, significantly reduce blood glucose level in glucose loaded, fasting and alloxan-induced diabetic Wistar albino rats. Bioactivity-guided fractionation of the culture filtrate of an endophytic fungus, *Dendryphion nanum,* isolated from *Ficus religiosa* yielded a compound herbarine 1 and its analogue herbaridine A2 showing promising antidiabetic activities. Both of them were found to be naphthaquinones (Mishra et al. 2013).

Berberine from several medicinal plants is a compound known for its cardioprotective, antidiabetic, antibiotic, and antitumor roles (Sun et al. 2009). Recently, it

Table 10.3 List of plant/plant extracts with antidiabetic potential

Name of plant	Extract/plant part	Model/method	Results	References
Acalypha indica	Aerial part chloroform and ethanolic extract	In vitro alpha amylase inhibition assay	Showed IC_{50} value 173.53 µg/ml for chloroform extract and 180.80 µg/ml for ethanolic extract	Dineshkumar et al. (2010)
Annona muricata	Plant aqueous extract	Streptozotocin-induced diabetic rats	Plant extract was not effective in normal rats but showed 75% reduction in blood glucose level at 100 mg/kg after 28 days of treatment	Florence et al. (2014)
Anthocleista djalonensis	Ethanolic root extract	Alloxan-induced diabetic rats	Reduced blood glucose level	Okokon et al. (2012)
Aralia taibaiensis	Plant total saponins extract	Streptozotocin-induced diabetic rats	Reduced fasting blood glucose and glycosylated hemoglobin	Weng et al. (2014)
Azadirachta indica	Ethanolic leaf extract	Streptozotocin-induced diabetic rats	Improved pancreatic lesion in diabetic rats	Akinola et al. (2010)
Berberis aristata	Bark methanolic extract	Inhibition of DPP4 enzyme	Showed IC_{50} value 14.4 µg/ml	Chakrabarti et al. (2011)
Blighia sapida	Root aqueous extract	Normoglycaemic rats	Decreased blood glucose level	Saidu et al. (2012)
Cola acuminata	Stem methanolic extract	Alloxan-induced diabetic rats	Decreased blood glucose level	Adediwura et al. (2011)
Cucurbita pepo	Seed mixture powder	Alloxan-induced diabetic rats	Increase in activity of aspartate aminotransferases and alanine aminotransferases as a function of treatment	Makni et al. (2011)
Daniellia oliveri	Aqueous leaf extract	Alloxan-induced diabetic rats	Blood glucose level decreased in both normal and diabetic rats	Manosroi et al. (2011)
Gymnema sylvestre	Callus methanolic extract	Alloxan-induced diabetic rats	Significant increase and regeneration of pancreatic β-cells	Ahmed et al. (2010)
Hunteria umbellate	Alkaloid rich fraction	Alloxan-induced diabetic rats	Decreased post absorptive glucose concentration	Adewale et al. (2013)

(continued)

Table 10.3 (continued)

Name of plant	Extract/plant part	Model/method	Results	References
Ipomoea batatas	Plant aqueous extract	Streptozotocin-induced diabetic rats	Decreased blood glucose level in normal and STZ-induced diabetic rats	Olowu et al. (2011)
Malmea depressa	Root butanolic extract	In vitro alpha glucosidase inhibition	Showed IC_{50} value 109 μg/ml	Kumar et al. (2011)
Mangifera indica	Leaves methanolic extract	Inhibition assay of DPP4 enzyme	Showed IC_{50} value 182.7 μg/ml	Yogisha and Raveesha (2010)
Pine densiflora	Bark extracted pycnogenol	In vitro alpha glucosidase inhibition	Showed IC_{50} value 5 μg/ml	Kumar et al. (2011)
Polyalhia longifolia	Leaves ethanolic extract	In vitro alpha amylase and in vivo in diabetic rats	Showed IC_{50} value 154 μg/ml and decrease in blood glucose level	Sivashanmugam and Chatterjee (2013)
Sphenocentrum jollyanum	Root ethanolic extract	Alloxan-induced diabetic rabbits	Decreased blood glucose level both in normal and diabetic rabbits	Mbaka et al. (2014)
Stevia rebaudiana	Leaves extracted polyphenols and fibers	Streptozotocin-induced diabetic rats	Reduction in blood glucose and increase in insulin concentration as compared to diabetic control rats	Shivanna et al. (2013)

has been produced by a strain of *F. solani* isolated from roots of the medicinal liana, *Coscinium fenestratum* (Vinodhini and Agastian 2013). An endophytic fungus, *Pseudomassaria* sp. isolated from African rainforest was found to produce a metabolite [L-783] which showed significant antidiabetic activity. Aspergillusol, a compound isolated from the marine derived endophytic fungus *Aspergillus aculeatus* isolated from the leaves of *Cassia siamea Lam.* also proved to be good source of α-glucosidase inhibitors in previous studies (Abdul et al. 2013; Ingavat et al. 2009).

A number of bioactive compounds with antidiabetic potential are characteristics of their host plant that have been isolated from endophytic fungi of different medicinal plants (Fig. 10.3). Methyl eugenol production by *Alternaria* sp. isolated from Rose (Kaul et al. 2008); phillyrin-producing endophytic fungi *Colletotrichum gloeosporioides (Forsythia suspensa)* (Zhang et al. 2012); sterigmatocystin, arugosin C, and epiisoshamixanthone from *Emericella* sp. (inhabiting *Astragalus lentiginosus*) have shown potential antidiabetic activities (Xu et al. 2013). Another example includes Helvolic acid from *Xylaria* sp. *(Anoectochilus setaceus)* (Ratnaweera et al. 2014), diphenyl ether producing *Verticillium* sp. *(Rehmannia glutinosa)* (Ola et al. 2014); Dihydroanthracenone metabolites from *Diaporthe melonis (Annona squamosa)* (Peng et al. 2013), piperine production by *Colletotrichum gloeosporioides (Piper nigrum L)* (Chithra et al. 2014), a chromone alkaloid such as rohitukin produced by endophytic fungi isolated from *Dysoxylum binectariferum Hook. f* and *Amoora rohituka (Roxb)* have been reported (Kumara et al. 2014).

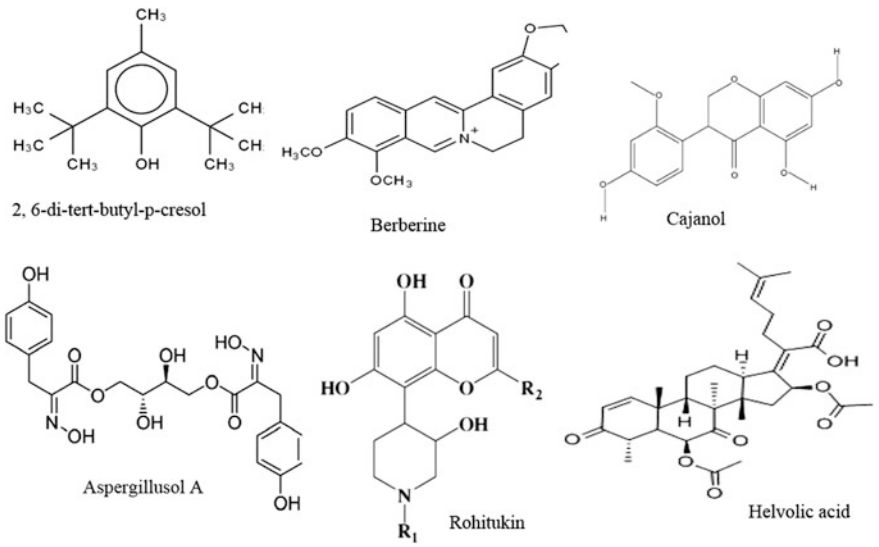

Fig. 10.3 Antidiabetic compounds of plant origin produced by endophytes from host plant

10.5 Anticancer Activity of Plants/Plant Extracts

Cancer is currently one of the leading causes of death worldwide and it is estimated that there are more than 1.6 million deaths occurred due to invasive cancer in 2013 (Siegel et al. 2013). The economic burden of cancer has necessitated the search for new, safe, affordable, and effective anticancer drugs. Purified asiatic acid fraction from *Centella asiatica* when tested in vitro on human melanoma SK-MEL-2 cells showed induction of apoptosis and decline in their viability in a time and dose dependent manner (Park et al. 2005). Similarly, induction of apoptosis in Hela cervical cancer cell line was recorded with Goniothalamin isolated from *Goniothalamus macrophyllus* (Aied et al. 2013). Ethanolic extract of *Atractylis lancea* showed inhibition of HEP-G2 liver cancer cell lines in a concentration and time dependent manner (Wei et al. 2013). Apoptosis of cancer cell in human intestinal epithelium was observed in presence of sesquiterpenes, Salograviolide-A isolated from *Centaurea ainetensis* (El-Najjar 2008). Kundusen et al. (2011) demonstrated decrease in tumor volume and increase in weight of Swiss albino mice as a function of treatment with methanolic leaf extract of *Citrus maxima*. Similarly, ethanolic extract of plant, *Derris scandens* resulted in death of human colon cancer cell line HT-29 in in vitro (Arunee et al. 2014). Both the aqueous and methanolic extract of plant *Ficus religiosa* showed cytotoxicity to HT-29 and MDA-MB-435S cancer cells in in vitro (Uddin and Grice 2011). Decrease in growth of breast cancer cell lines as a function of treatment with aqueous leaf extract of *Taraxacum officinale* (Sigstedt et al. 2008), suppression of proliferation of prostrate, breast, ovary, colon, lung, and bladder cancer cell lines by isolated flavonoids from *Silybum marianum* (Agarwal et al. 2006) and death of human T-47D cancer cell lines by chloroform extract of plant, *Physalis minima* (Ooi et al. 2010) are few other example from many such studies carried out using different plant/extracts and cancer cell lines.

10.5.1 Anticancer Compounds from Endophytes

Endophytic fungi have been reported to produce a myriad of anticancer substances (Table 10.4). The bioactive potential of the endophytic fungi was first recognized when world's first multibillion dollar anticancer drug, Paclitaxel (Taxol), was obtained from an endophytic fungus. Taxol is known to be a potent chemotherapeutic agent, used for a variety of cancers including ovarian and breast cancers (Weaver 2014). Wani et al. (1971) first isolated and chemically characterized the Taxol from the bark of yew plant (*Taxus brevifolia*). However, the amount of taxol produced by the slow growing African yew is very small and a number of plants need to be sacrifice to obtain a few milligrams of compound. The cost of the taxol obtained from the plant source was a major constrain for its clinical use (Gangadevi et al. 2009). Stierle et al. (2001) first reported an alternative source for Taxol

Table 10.4 Anticancer compounds from endophytes and respective host plant

Host plant	Endophytic fungi	Bioactive compound	References
Taxus brevifolia	*Taxomyces andreanae*	Diterpenoid	Strobel (1997)
Torreya taxifolia	*Pestalotiopsis microspora*	Torreyanic acid	Lee (1996)
Catharanthus roseus	*Mycelia sterilia*	Vincristine	Yang (1994)
Plumeria acutifolia	*Colletotrichum gloeosporioides*	Taxol	Nithya (2009)
Spondias mombin	*Phomopsis* sp.	Phomopsidin & Phomopsichalasin	Kobayashi (1995)
Rhizophora annamalayana	*Fusarium oxysporum*	Taxol	Elavarasi (2012)
Tinospora cordifolia	*Fusarium culmorum SVJM072*	Taxol	Sonaimuthu (2010)
Annova squamosa	*Penicillium* sp.	Meleargine and Chrysogine	Yunianto (2014)
Tripterygium wilfordii	*Rhinocladiella* sp.	22-oxa-(12)-cytochalasins	Wagenaar (2000)
Nothapodytes foetida	*Entrophospora infrequens*	Camptothecin	Puri (2005)
Podophyllum hexandrum	*Trametes hirsute*	Podophyllotoxin	Puri (2006)
Sequoia semperivirens	*Aspergillus parasiticus*	Sequoiatones C-F	Stierle (2001)
Nothapodytes foetida	*Phycomycetes*	Campthothecin	Puri (2005)
Adenophora axilliflora	*Chetomium* sp. *IFB-EO15*	Chaetominine	Rui (2006)

production using endophytic fungus *Taxomyces andreanae*. Another important drug for cancer is the alkaloid "Camptothecin"—a potent anticancer compound found in the plant *Camptotheca acuminata* Decaisne (Nyssaceae) (Wall et al. 1966). Camptothecin and 10-hydroxycamptothecin are widely used as precursors in the synthesis of topotecan, and irinotecan, which are clinically used as potent anticancer drugs (Uma et al. 2008). An endophytic fungus *Fusarium solani* isolated from the same plant produced Camptothecin and 10-hydroxycamptothecin when cultivated in submerged cultures (Kusari et al. 2009). Subsequently, several workers reported endophytic fungi which could produce Camptothecin and other similar compounds (Puri 2005; Rehman et al. 2008).

Guo et al. (1998) reported that *Alternaria* sp. isolated from the phloem of *Catharanthus roseus* had the ability to produce well-known alkaloid, vinblastine (Fig. 10.4). Later, another endophytic fungus *Fusarium oxysporum* from the phloem of *C. roseus is* shown to produce vincristine (Zhang et al. 2000). In another

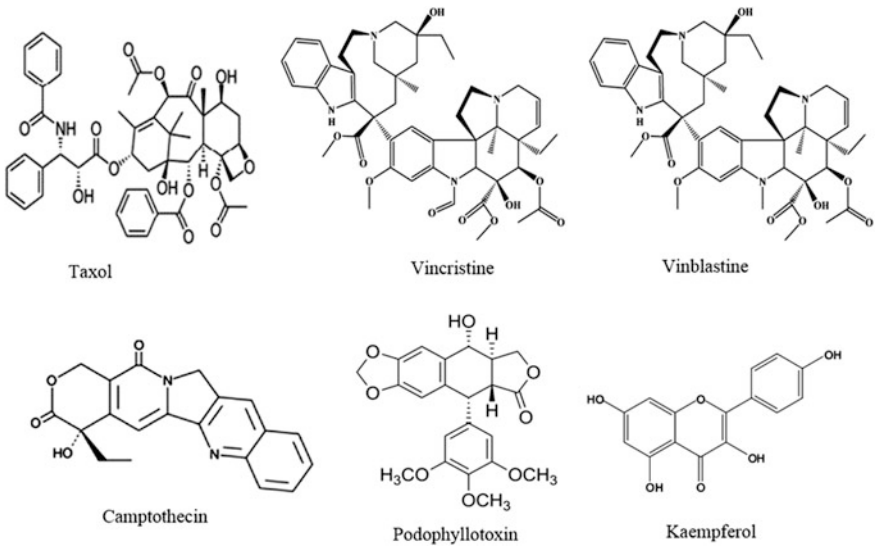

Fig. 10.4 Structure of anticancer compounds produced by endophytes

study, an unidentified vincristine producing endophytic fungus was isolated from the leaves of *C. roseus* (Yang et al. 2004). A new compound "Ergoflavin" with excellent anticancer activity was extracted from the culture filtrate of an endophytic fungus isolated from an Indian medicinal plant *Mimusops elengi* (Deshmukh et al. 2009). Similarly, Cytotoxic quinone dimer, Torreyanic acid is another important anticancer agent produced from *P. microspore* and isolated from *T. taxifolia (Florida torreya)*. Recent studies showed that *Hypocrea lixii*, a novel endophytic fungi, isolated from *Cajanus cajan*, produced anticancer agent cajanol (Zhao et al. 2013). Another endophytic fungus, *M. fragilis* exhibited production of bioactive metabolites, viz., podophyllotoxin and kaempferol (Huang et al. 2014) besides, guanacastane diterpenoids reported from the plant endophytic fungus *Cercospora* sp. (Feng et al. 2014).

A pentacyclic triterpenoid, ursolic acid, a known compound for its anticancer and cardioprotective properties (Liu 1995), was found to be produced by endophytic strain of *Annulohypoxylon stygium* (Cheng et al. 2014). A sterol ergosta-8(9), 22-diene- 3,5,6,7-tetrol (3β, 5α, 6β, 7α, 22E) (Compound 1) along with other three known sterols, namely 3β, 5α, 6β-trihydroxyergosta-7, 22-diene (2) 3β-hydroxy-5α, 8α-epidioxyergosta-6,22-diene (3) and ergosterol (4) from the unidentified endophytic fungi obtained from *Castaniopsis fissa* (Hou et al. 2004).

Endophytic fungi seem to be the store house of bioactive compounds with anticancer potential characteristics of their host plant. A new compound naphtho-ã-pyrone, 5-hydroxy-6,8-dimethoxy-2-benzyl-4H-naphtho[2,3-b]-pyran-4-one (1), together with three known compounds 5,7-dihydroxy-2-methylbenzopyran-4-one (2), 3,5-dihydroxy-2,7dimethylbenzopyran-4-one (3) and cyclo (Tyr-Tyr) (4) has

been isolated from the mangrove endophytic fungus *Phomopsis* sp. (Huang et al. 2010). Primary bioassays showed that compound 1 exhibited cytotoxicity against Hep-2 and HepG2 cells with IC_{50} values of 10 and 8 μg/ml, respectively.

10.6 Conclusion

The injudicious use of plant biodiversity for human use, increased commercial and scientific interests is continuously increasing the pressure on the population of higher plants. India, despite being one of the global biodiversity hotspot, uncontrolled use of medicinal plants is leading to many valuable medicinal species at risk of extinction. Globally, about 100,000 species of angiosperms are used for medicinal purpose. However, because of overharvesting, irrational use and loss of habitat about 15,000 species are near to extinction. Therefore, the sustainable use and conservation of medicinal plant biodiversity is the need of time.

Endophytic microorganisms, by virtue of their ability to produce metabolites of plant origin, can be of great significance to save fast diminishing plant biodiversity. Microbial endophytes have been intensively studied in last decade because of their potential to produce diverse biologically active metabolites of therapeutic importance. Their coevolution with host plant is thought to enable them to mimic host chemistry. Isolation of potential endophyte followed by their successful laboratory cultivation and scale-up are the important steps for exploring the endophytes for biotechnological purpose. Of late, thousands of endophytic microorganisms have been isolated, identified and their products such as steroids, alkaloids, peptides, terpenoids, tannins, polyketones, flavonoids, and phenolics have been characterized. However, producing the metabolites of plant origin using endophytes has several limiting factors. The laboratory cultivation conditions are completely different from that of *in planta* conditions. Moreover, the complex host–endophyte relationship controls expression of genes coding for secondary metabolite production. It is therefore concluded that, in spite of huge biotechnological potential, endophytic microorganisms are relatively less explored microbial community that can be tapped as rich source of bioactive metabolites of plant origin.

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Chapter 11
Harnessing Endophytic Microbial Volatile Organic Compound (MVOC) for Sustainable Agroecosystem

Dinesh Chandra, Pallavi and A.K. Sharma

Abstract Endophytic bacteria and fungi emit a wealth of volatiles, representing a promising group of microorganisms, as they are a largely untapped reservoir of metabolic diversity. These volatile organic compounds (VOCs) occur as mixtures of low molecular mass hydrocarbons, alcohols, heterocyclic compound, aldehydes, ketones, and other small molecules. They have characteristic aromas and are produced during primary and secondary metabolism of microbes. Their ability to diffuse makes them excellent chemical signaling molecules in nonaqueous habitats and facilitates the ability of microbes to engage in chemical conversations. The methods for the collection and detection of MVOC are steam distillation, liquid–liquid extraction, simultaneous distillation extraction, purge and trap, supercritical fluid extraction, and solid phase microextraction (SPME). Among them, SPME is the most commonly used technique as it integrates the extraction, concentration, and introduction in one step thus resulting in reducing preparation time. A growing body of evidence indicates that MVOCs are eco-friendly and can be exploited as a cost-effective and sustainable strategy in agricultural practice as agents triggering plant immunity and promoting plant growth. Also, MVOC-mediated conversion of solid cellulosic biomass to liquid biofuels may provide a renewable energy source for transportation fuels.

Keywords Endophyte · MVOC · Plant growth · Biofuel · SPME

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11.1 Introduction

The term endophyte was first coined by De Bary in 1884 for microorganisms including bacterial, yeast, archaeal, fungal, and protistic taxa colonizing internal plant tissues (De Bary 1884). Hallmann et al. (1997) defined endophytes as, considering any microorganism as an endophyte if it can be isolated or extracted from inside surface disinfected plant tissue and it does not seemingly harm the plant. This definition has been widely accepted for cultivable species in most laboratories in the world over the last 20 years. However, due to the suspected lack of adequate elimination of nucleic acids after sterilization of plant surfaces, this definition seemed to be less suited for non-cultured species upon the interpolation of molecular detection techniques in endophyte research. Coombs and Franco (2003) defined microbial endophytes as ubiquitous colonizers of the interior tissues of host plants and can constitute a range of different relationships such as symbiotic, mutualistic, and commensalistic where they do not usually have any substantial morphological changes and disease symptoms.

11.2 Microbial Volatile Organic Compound (MVOC)

Several prokaryotic and eukaryotic microorganisms generate a plethora of complex and dynamic array of gaseous secondary metabolites, usually known as volatile organic compound (VOCs) of low molecular weight lipophilic (<300 Da) compounds, high vapor pressure (0.01 kPa or higher at 20 °C), low boiling point, belonging to different chemical classes that vaporize and diffuse easily through air and water-filled pores and thus play essential biological/ecological roles in aboveground as well as belowground habitats (Penuelas et al. 2014; Tyc et al. 2014; Wenke and Piechulla 2013). Recent studies showed that soil microbiotas can use these volatile compounds as ideal info-chemical as growth stimulants, growth inhibitors, and inhibitors of quorum-sensing, i.e., quorum quenching (Effmert et al. 2012; Kim et al. 2013). In living organism, these compound are formed as part of normal metabolism and derived from different biosynthetic pathways and act as signal molecules for inter- and intra-organismic communication between plants, antagonists, and mutualistic symbionts both below (soil) and above ground (atmosphere) (Kanchiswamy et al. 2015). At the plant–microbe community level, substantial progress has been made in studying the multifaceted role of MVOCs produced by bacteria, fungi, and phytopathogens in agroecosystems. Researcher considers MVOCs as potential semiochemicals that function as attractants and repellents to insects and other invertebrates, as biocontrol agents to control various phytopathogens, as biofertilizers for plant growth promotion, as a potential source of biofuel, and are also used to prevent postharvest plant diseases.

11.3 Diversity of Microbial Volatiles

The available literature and databases allow estimating the known structural diversity of volatiles derived from plant flowers ca. 1,700 volatiles from 991 species (Dunkel et al. 2009), 1,093 volatiles from 491 microbes, including 135 fungi and 356 bacteria (Lemfack et al. 2014). Yet considering that 2,98,000 of plant species (Mora et al. 2011), 10^7–10^9 bacterial species (Schloss and Handelsman 2004), and 1.5 million fungal species (Hawksworth 2001) might exist on earth, the number of volatiles will be added more to databases as new species are being characterized and discovered. The 1,093 volatilome from the MVOC database (Lemfack et al. 2014) grouped into 13 chemical classes (Table 11.1).

11.4 Microbial Volatile Organic Compound (MVOCs) Collection and Detection

For bioprospecting the microbial volatile compound, the first and foremost requirement is their detection in the source, identification and afterwards their collection. However, analysis of these volatile compounds poses challenges as they tend to occur in mixtures, possess different chemical properties, and are generally

Table 11.1 Per cent contribution of major and minor group of microbial volatiles (Lemfack et al. 2014)

	% contribution of volatile	Total diversity
Major group of volatile		
Terpenes	11	Major groups of volatile compound representing 64% of total diversity
Ketones	13	
Organic acid	10	
Alcohol	16	
Aromatic compounds	14	
Minor groups of volatile		
Alkanes	5	Minor groups of volatile compound constitute 36% of total diversity
Alkenes	3	
Nitrogen compound	5	
Sulfur compound	7	
Aldehydes	7	
Esters	7	
Furans	2	
Ethers	<1	

present in low concentrations. Thus, for accurate interpretation, it is crucial to obtain as many volatile components as possible from the samples, therefore, the desired sampling technique must be able to extract different polar and structural biological VOCs.

Over time, many strategies have been applied in the field of MVOC detection and concentration. Steam distillation (Vanhaelen et al. 1978; Kaminski et al. 1972), liquid–liquid extraction (Wu et al. 2005), and simultaneous distillation extraction are some of the conventional methods that were employed by the researchers. They required long extraction times, large amounts of solvents, and multiple steps. These methods also result in degradation of unstable volatiles such as alkene, ester, and some unsaturated VOCs. However, because of their simplicity, they are still extensively applied for the fragrance-and-aroma characterization. Another method for collection of microbial volatiles is purging and trapping. In studies of odor formation in moist cereal grain during granary storage, the volatiles were collected by the withdrawal of intergranular air through adsorbent cartridges (Abramson et al. 1980, 1983). Norrman (1977) developed a fast method to study volatile production by direct injection of a headspace sample into a gas chromatograph with a packed column.

However, currently the widely used method is headspace solid-phase microextraction method (HS-SPME) because it integrates the extraction, concentration, and introduction in one step thus resulting in reduced preparation time and simultaneously increasing sensitivity over other extractions (Tait et al. 2014). The HS-SPME procedure includes introduction of fused silica fiber coated with a polymeric organic material into the headspace above the sample. The volatile organic analytes are extracted and concentrated in the fiber coating and then transferred to the analytical instrument which is mostly gas chromatograph for thermal desorption and analysis. The technique has great importance in microbiological studies and food technology (Zhang and Pawliszyn 1993; Nilsson et al. 1995). The fiber chosen for extraction may have a marked effect on the detected VOC profile. Different fiber coatings are commercially available for SPME, like polydimethylsiloxane, carbowax-divinylbenzene (Jia et al. 2010), and polyacrylate (Buchholz et al. 1994) that have exclusively been applied to the analysis of phenols. Only for a few phenols comparative results are available, indicating higher sensitivity with the polyacrylate than with the polydimethylsiloxane fiber coating for these more polar compounds.

After extraction, analysis of samples is performed by coupled (GC) and mass spectrometry (MS) (Madrera et al. 2005). Other methods of volatile analysis include comprehensive two-dimensional gas chromatography (Welke et al. 2014), ion trap mass spectrometry (Noguerol et al. 2009), or time-of-flight mass spectrometry (Bordiga et al. 2014). Among innovative procedures, near-infrared (NIR) spectroscopy is becoming popular in the field of volatile studies as a rapid, accurate, simple to operate, as it requires no sample pretreatments prior to analysis (Buratti et al. 2011). Recently, Ye et al. (2016) have used this method in detecting volatiles in apple wines. Near-infrared region of the electromagnetic spectrum (700–2500 nm) provides more sophisticated structural information based on the variation behaviors of combinations of bonds (Bauer et al. 2008; Reid et al. 2006). Proton transfer reaction-mass

spectrometry (PTR-MS) is another method that can be used to measure the concentration volatile emission by ionizing organic molecules in the gas phase through their reaction with H_3O^+, forming mostly MH^+ molecules (where M is the neutral organic molecule), which can then be detected by a standard quadrupole/multiplier mass analyzer (Ezra et al. 2004).

11.5 MVOCs as Signals Mediating Intra- and Interspecies Communications

Recently, a new communication path, sense of smell has been well established in many organisms. This sense of smell or volatile-mediated communication played significant function in both above and belowground ecosystems. Living organisms use these volatile as ideal semiochemical for chemical conservations among intra- and interspecies. For example, vertebrates and invertebrates are capable to detect minute quantities of volatiles even over very long distances. The plants use volatiles to broadcast with their pollinators as well as with plants of the same species or other plants and microorganisms as well use these sweet scents for communication among microbes and plants so they can interact with each other (Heil and Walters 2009; Effmert et al. 2012).

11.5.1 MVOC in Bacterial–Plant Interactions

A number of bacteria preferentially live in the soil in close association with plant roots utilizing root exudates as their food source. These exudates are ordinarily rich in sugars, amino acid, organic acids, and other compounds, many of them promote plant growth. These bacteria are called as rhizobacteria, whereas the root environment they colonize is called the rhizosphere (Bhattacharyya and Jha 2012; Mendes et al. 2013). Some of these microbes get genetically modified to acquire traits like endophytic competence to be able to colonize the interior of the plant. For long, scientists have speculated that all rhizobacteria can be expected to be endophytic at least at one point in their entire life cycle (Sturz et al. 2000; Hardoim et al. 2008, 2012).

VOCs produced by rhizobacteria are involved in their interaction with plant pathogenic microbes and host plants and exhibit antimicrobial and plant growth regulating activities. The bacterial VOCs such as 2-heptanol, 2-pentanone, 2-pentadecanone, 2-undecanone, 2-tridecanone, 4-heptanone, and sodorifen produced by *Serratia odorifera* are able to interfere with plants. Forty-two soilborne bacterial strains were screened and evaluated for their volatile-mediated effect on 6-day-old seedlings of *Arabidopsis thaliana*. A total 36 volatile compounds of bacterial origin were selected, many of them exerting negative consequences on

plants growth while only three compound indole, 1-hexanol, and pentadecane stimulated plant growth. Cocultivation of *A. thaliana* with *S. odorifera* in divided Petri dishes, which only let volatiles to diffuse from one side of the plate to the other, resulted in dramatic growth inhibition of plants (Kai et al. 2010; Blom et al. 2011; Weise et al. 2014). Groenhagen et al. (2013) in their study compared the effect of volatiles produced by three *Burkholderia* strains isolated from clinical environment, pea rhizosphere, and maize roots. Exposure of *Arabidopsis thaliana* plants to these volatiles resulted in significant increase in biomass, as well as growth inhibition of *Rhizoctonia solani* and *Alternaria alternata*. Also, volatile profiles of these strains were found to be similar, and dimethyl disulfide was the most abundant compound and sulfur compounds, ketones, aromatic compounds were other significant components. This indicates that like their rhizospheric counterparts endophytic strains are also capable of producing the similar volatiles and this can be further explored in many other important species.

Application of dimethyl disulfide (DMDS) produced by a *Bacillus cereus* strain significantly protected tobacco and corn plants against gray mold *Botrytis cinerea* and southern corn leaf blight *Cochliobolus heterostrophus*, respectively. It also reduced the expression of *Nicotiana attenuata* sulfur assimilation genes, methionine biosynthesis, and recycling (Huang et al. 2012). Acetoin and 2,3-butanediol (2,3-BD) were frequently released from strains of *Bacillus subtilis* and *B. amyloliquefaciens* and were found to raise the total leaf surface area and induced systemic resistance (ISR) of *Arabidopsis thaliana* (Ryu et al. 2003; Rudrappa et al. 2010). The study of D'Alessandro et al. (2014) revealed that production of 2,3-BD by *E. aerogenes* rendered corn plants more resistant against the northern corn leaf blight fungus *Setosphaeria turcica*. A large number of volatile produced by lemon rhizobacteria is benzaldehyde, tridecanal, acetophenone, tetradecanal, and 6,10,14-trimethyl 2 pentadecanone have differential effects on *Arabidopsis* roots is correlated to the type and quantity of compounds produced by the bacteria (Gutierrez-Luna et al. 2010). Similarly, 3-exanone produced by *Burkholderia ambifaria* significantly enhanced *Arabidopsis* biomass, as did acetophenone and DMDS produced by lemon rhizobacteria and *Bacillus cereus*, respectively (Groenhagen et al. 2013).

11.5.2 MVOC in Bacterial–Bacterial Interactions

Very scanty information is available about the nature of volatiles in bacterial–bacterial communication, what is known till yet is briefly described here. The communication may be stimulatory and inhibitory depending upon the one species exerts an effect on other species. The stimulating effect of volatile compound produced by *Collimonas pratensis* and *Serratia plymuthica* observed on the growth of *Pseudomonas fluorescens*. The unique volatiles produced by the both bacterial strains were benzonitrile, methyl thiocyanate, S-methyl thioacetate, and DMDS. A blend of volatiles emitted by four bacteria namely, *Paenibacillus* sp., *Pedobacter*

sp., *Collimonas pratensis* and *Serratia plymuthica* did not affect *P. fluorescens* growth. Moreover, the specific volatiles produced by *C. pratensis* were methyl salicylate, methyl 2-methylbutanoate, methyl 3-methylbutanoate,2-methyl propanal, 3-methyl 2-pentanoene, 3-methyl 2-heptanone, 3-hexanone, 4-methyl 3-penten-2-one, ethenyl acetate, myrcene, and terpinene. Similarly, the specific volatiles produced by *S. plymuthica* were chlorobenzene, dimethylsulfone, ethyl butanoate, 2-pentadecanone, 2-octanone, 1H-pyrrole, and 5-dodecanone (Garbeva et al. 2014).

Many workers demonstrated the inhibitory effect of two *Pseudoalteromonas* strains on the growth of *Burkholderia cepacia* complex (Bcc) strains through the synthesis of Methyl-2,3,3,4-tetrahydroxytetrahydrofuran, indole and its derivatives, quinolones and (S)-3-hydroxytridecan-4-one volatile organic compound (Papaleo et al. 2013; Orlandini et al. 2014; Kanchiswamy et al. 2015). Similarly, Dandurishvili et al. (2011) examined that some strains of *P. fluorescens* and *S. plymuthica* inhibited the growth of *Agrobacterium tumefaciens* and *A. vitis* strains in vitro. The tomato treated with *S. plymuthica* produced DMDS that strongly suppressed *Agrobacterium* growth and might be involved in suppression of oncogenicity in plants.

11.5.3 MVOCs in Bacterial–Fungal Interactions

A number of rhizobacterial species such as *P. fluorescens*, *P. trivialis*, *Stenotrophomonas maltophilia*, *S. rhizophila*, *Serratia plymuthica,* and *S. odorifera* are known to synthesize and emit complex blends of volatile organic compound that inhibit growth of many phytopathogenic as well as non-phytopathogenic fungi (Kai et al. 2010). Pyrrolnitrin (PRN) is a chlorinated phenylpyrrol antibiotic from *Burkholderia pyrrocinia*,*Pseudomonas* sp., *Enterobacter* sp., *Myxococcus* sp., and *Serratia* sp. (Garbeva et al. 2004). This compound has shown broad spectrum activity against a wide range of fungi belonging to the ascomycota, basidiomycota, and deuteromycota, including several economically important phytopathogens such as *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Rhizoctonia solani,* and *Verticillium dahliae*. The stem rot of *Euphorbia pulcherrima* is caused by *Rhizoctonia solani* and also suppressed by *B. cepacia* strain 5.5B mediated PRN (Hwang et al. 2002).

MVOCs emitted by *S. rhizophila* P69, *S. maltophilia* R3089, *S. plymuthica* 3Re4-18, *S. plymuthica* HRO-C48, *S. odorifera* 4Rx13, *P. trivialis* 3Re2-7, and *Bacillus subtilis* B2g had exerted a strong negative impact on the mycelial growth of soilborne phytopathogenic fungus *R. solani* (Kai et al. 2007). Also, the volatile O-anisaldehyde emitted by *Bacillus atrophaeus* CAB-1 exerts the highest inhibition on the mycelial growth of the fungal pathogen *Botrytis cinerea* (Zhang et al. 2013). Growth inhibition of phytopathogen is also dependent on the varying concentration of volatile produced by different microbes. The high concentration of volatile-mediated growth inhibition of *R. solani* and *Alternaria alternata* was observed with 2-undecanone, DMDS, dimethyl trisulfide, S-methyl

methanethiosulphonate, 4-octanone, and 1-phenylpropan-1-one produced by *Burkholderia ambifaria.* The mycelial growth of *Fusarium culmorum*, *F. oxysporum*, *Colletotrichm gloesporioides,* and *Sclerotum rolffsi* was significantly inhibited by fifteen *Burkholderia tropica* strains (Groenhagen et al. 2013).

11.6 Endophytic Microbial Volatiles as Promising Source of Next Generation Biofuel Production

Recently, a number of endophytic bacteria and fungi have been discovered that make hydrocarbons while utilizing cellulosic polymers found in plant-based agricultural wastes. The action of different hydrolytic enzymes converts the agricultural waste substrate into volatile compounds that are either identical to or are closely linked to those specific categories of molecules that are found in diesel such as mono-terpenoids, alkanes, cyclohexanes, cyclopentanes, and alkyl alcohols, ketones, benzenes, and polyaromatic hydrocarbons. For example, the *Phomopsis* sp. produces VOCs such as sabinene, pinene, 3-methyl-1-butanol, 1-propanol, 2-methyl and 2-propanone, and benzeneethanol that are being explored as the components for the next generation aircraft fuel (Grigoriev et al. 2011; Strobel et al. 2011). Similarly, *Ascocoryne sarcoides*, *Ascocoryne cylichium,* and *Ascocoryne solitaria* produced a broad range of volatiles including alkanes, alkenes, alcohols, ester, ketones, acids, benzene derivatives, terpenes, and esquiterpenes. Majority of these VOCs are similar to diesel because of their cyclic and branched structure (Rude and Schirmer 2009; Griffin et al. 2010; Mallette et al. 2014).

An endophyte, *Nodulisporium* sp. was isolated from *Myroxylon balsamum* produced VOCs with fuel potential. Under microaerophilic growth environments, the organism produced 1,8-cineole, propylcyclohexane, acetone, 2-pentanone, 3-hexanone, 4-methyl and 5-hepten, 2-one, 4-methyl, 3-hexanone, -methyl-1-butanol, 1,4-cyclohexadiene, 2,4-dimethyl, 1-4 pentadiene and cyclohexene, 2-hexanone, 1-methyl-, 1-methyl-4-(1-methylethenyl)-, along with some alcohols and terpenoids of interest as potential fuels. In an aerated large fermentor, *Nodulisporium* sp produced a number of products such as 3-methyl-1-butanol, 2-methyl-1-propanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, benzene derivatives, alkyl alcohols, ketones, esters, a few terpenoids, 1-nonanol along with phenylethyl alcohol as ingredient of diesel (Mends et al. 2012). The 1,8-cineole, 1-methyl-1,4-cyclohexadiene, and (+)-amethylene-a-fenchocamphorone also have the potential to be used as a fuel additive, produced by *Hypoxylon* sp. (CI-4A) (Tomsheck et al. 2010). In addition to alkanes and long-chain hydrocarbon, many fungal species produce other potential biofuel targets, such as ethylene, ethane, propane, and propylene (Ladygina et al. 2006).

However, little information is available about the bacterial volatiles as fuel potential as compared to traditional bioethanol and plant oil-derived biodiesel. The identified relevant volatile compounds include various short-to-medium chain alkanes, alkenes, alcohols, and isoprenoids, which hold large potential to substitute

or supplement petroleum-derived fuels. For example, undecene, butanol, and isopentanol are consistently observed in *Pseudomonas* sp. 1-Undecene is particularly fascinating due to its superior physical properties, which may receive a direct application as a fuel (Rui et al. 2014).

11.7 Potential of MVOCs for Applications in Agriculture

The list of VOC produced by many endophytic microorganisms and their effect on interacting organisms is summarized in Table 11.2. Several rhizobacterial species enhanced and regulate the growth of plants, impart resistance against abiotic and biotic stresses through volatile-mediated compounds and exhibit their potential in biocontrol. The *B. amyloliquefaciens*-borne volatile 2,3-butanediol mediates the growth promotion and ISR against *E. carotovora* in *A. thaliana* (Ryu et al. 2004; Farag et al. 2006;). Several strains of *Bacillus* isolated from rhizosphere of lemon plants facilitate the growth and root architecture of *A. thaliana* by VOC production (Gutierrez-Luna et al. 2010). It has been also shown that *Serratia plymuthica* and *Stenotrophomonas maltophilia* borne volatile had negative consequences on the growth of model plant Arabidopsis thaliana (Wenke and Piechulla 2013). Sensor kinase GacS mediated synthesis of 2,3-butanediol in *P. chlororaphis* enhanced the growth of tobacco and also imparted drought tolerance in *A. thaliana*. Among three stereoisomers of 2,3-butanediol, only 2R,3R-butanediol was effective in plant growth promotion suggesting the presence of specific receptors for this isomer in plants (Han et al. 2006; Cho et al. 2008). Moreover, VOC produced by several genera such as *Burkholderia*, *Chromobacterium*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas* may have negative consequences on the plant growth and health (Bailly and Weisskopf 2012).

Endophytic *Bacillus* and *Psuedomonas* strains are capable of species-specific production of six volatile compounds such as dimethyltrisulfide, n-decanal, nonanal, benzothiazole, cyclohexanol, and 2-ethyl-1-hexanol which completely inhibit mycelial growth and sclerotial germination of *Sclerotinia sclerotiorum* (Bitas et al. 2013). A more extensive survey involving 1,018 bacterial isolates showed that VOC from 328 isolates, which belong to families Alcaligenaceae, Bacillales, Micrococcaceae, Rhizobiaceae, and Xanthomonadaceae, inhibited spore germination and mycelial growth of two nematicidal fungi. Seven VOC including acetamide, benzaldehyde, benzothiazole, 1-butanamine, 1-decene, methanamine, and phenylacetaldehyde appear to play roles in fungistasis. Benzothiazole is the only VOC that was found in both surveys, suggesting that different species produce different VOC for fungistasis, antifungal activity of many compounds are target specific, or a combination of both (Zou et al. 2007).

VOC emitted from the fruiting body of *Pleurotus ostreatus* (oyster mushroom) such as 1-octanol, 3-octanol, 3-octanone, 1-octen-3-ol, benzoic acid, and benzaldehyde inhibited the growth of several bacterial species (Beltran-Garcia et al.

Table 11.2 List of endophytic bacterial and fungal VOCs and their effects on plants, bacteria, and fungi

Bacterial and fungal strains	Host plant	Identified volatile compound	Effective on	Effect on interacting organisms	References
Bacillus atrophaeus XW2	*Populus tremula*	Volatile mixture	Fungi and plants	Volatiles produced by XW2 inhibited hyphal growth of *Colletotrichum gloeosporioides* by 60.2% and were antagonistic against the germination of *C. gloeosporioides* spores	Huang et al. (2015)
Bacillus megaterium strain *BP17*	*Piper nigrum*	Pyrazine, 2-ethyl-3-methyl-; Pyrazine, 2,5-dimethyl-; Pyrazine, ethyl-; and Pyrazine, methyl	Fungi and plants	BmBP17 released antimicrobial volatiles against several pathogens, viz., *Phytophthora capsici, Pythium myriotylum, Athelia rolfsii, Gibberella moniliformis, Colletotrichum gloeosporioides, Rhizoctonia solani, Magnaporthe oryzae, Ralstonia solanacearum*, and *Xanthomonas axonopodis* pv. *Punicae*and which can be exploited for crop protection	Munjal et al. (2016)
Bacillus sp. B55	*Nicotiana attenuate*	Dimethyl disulphide (DMDS)	Plants	Growth promotion	Meldau et al. (2013)
Burkholderia ambifaria	*Zea mays*	Dimethyl di- and trisulfide, 4-octanone, S-methyl methanethiosulphonate, 1-phenylpropan-1-one, and 2-undecanone, while dimethyl trisulfide, 1-methylthio-3-pentanone, and o-aminoacetophenone	Plants	Biomass increase in the model plant *Arabidopsis thaliana* as well as growth inhibition of two phytopathogenic fungi (*Rhizoctonia solani* and *Alternaria alternata*)	Groenhagen et al. (2013)
Enterobacter aerogenes	*Zea mays*	2,3-butanediol	Fungi and plants	The production of 2,3-BD by *E. aerogenes* rendered maize plants more resistant against the Northern corn leaf blight fungus *Setosphaeria turcica*	D'Alessandro et al. (2014)

(continued)

Table 11.2 (continued)

Bacterial and fungal strains	Host plant	Identified volatile compound	Effective on	Effect on interacting organisms	References
Pseudomonas putida BP25	*Piper nigrum*	Heneicosane, Tetratetracontan, Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3 (2methylpropyl), Tetracosyl heptafluorobutyrate, 1-3-Eicosene(E)-1-Heneicosanol, Octadecyl trifluoroacetate, 1-Pentadecene, 1-Undecene, Disulfide dimethyl, Pyrazine, methyl-Pyrazine, 2,5-dimethyl Isoamyl alcohol, Pyrazine, Methyl, Dimethyl trisulfide	Fungi, plant parasitic nematode and plants	The bacterium inhibited broad range of pathogens such as *Phytophthora capsici, Pythium myriotylum, Giberella moniliformis, Rhizoctonia solani, Athelia rolfsii, Colletotrichum gloeosporioides and plant parasitic nematode, Radopholus similis* by its antimicrobial volatile substances that can be exploited in crop protection	Sheoran et al. (2015)
Pseudomonas sp. strain P2	*Olea europaea*	Dimethyl disulfide and dimethyl trisulfide	Fungi and plants	VOCs-producing P2 strain could be a promising agent in the protection of tuber crops against fungal diseases	Elkahoui et al. (2015)
Psedomonas fluorescens strain ALEB 7B	*Atractylodes lancea*	Dimethyl disulphide (DMDS), 2-Piperidinone	Fungus	Antagonizing *Athelia rolfsii*	Zhou et al. (2014)
Ascocoryne sarcoides NRRL 50072 (earlier it was known as *Gliocladium roseum*)	*Eucryphia cordifolia*	Pentyl, hexyl, heptyl, octyl, sec-octyl and decyl alcohols, undecane, 2,6-dimethyl, decane, 3,3,5-trimethyl, cyclohexene, 4-methyl, decane, 3,3,6-trimethyl, and undecane, 4,4-dimethyl	–	–	Griffin et al. (2010)
Botrytis sp. BTF21	*Musa* spp.	Butane 2-methyl, β-butyrolactone, 2-butenedinitrile	Fungi	Inhibit the growth of the pathogenic *Fusarium oxysporum* f. sp. *cubense* race 4 (FocR4) via production of volatile	Ting et al. (2011)

(continued)

Table 11.2 (continued)

Bacterial and fungal strains	Host plant	Identified volatile compound	Effective on	Effect on interacting organisms	References
Hypoxylon sp.	*Persea indica*	1,8-cineole, 1-methyl-1,4-Cyclohexadiene, (+)-α methylene α-fenchocamphoron	Fungi and plants	*Hypoxylon* sp. displayed maximal VOC-antimicrobial activity against *Botrytis cinerea, Phytophthora cinnamomi, Cercospora beticola*, and *Sclerotinia sclerotiorum* suggesting that the VOCs may play some role in the biology of the fungus and its survival in its host plant	Tomsheck et al. (2010)
Muscodor vitigenus, M. equiseti and *M. heveae* sp. nov.	*Hevea Brasiliensis*	3-methylbutan-1-ol, 3-methylbutyl acetate and azulene and many other volatile compounds	Fungi and plants	The VOCs produced by the *Muscodor* strains have the potential for biological control of bacteria, yeast and filamentous fungi. The VOCs of *M. heveae* were active against the pathogenic fungi *Phellinus noxius and Rigidoporus microporus* that cause root disease in the rubber tree	Siri-udom et al. (2016)
Muscodor albus	*Cinnamomum zeylanicum*	Isoamyl acetate, 2-Methyl butanol, isobutyric acid	Fungi and bacteria	Collectively they acted synergistically to kill a broad range of plant- and human-pathogenic fungi and bacteria also volatile mixture were effectively used to control postharvest plant diseases	Mercier and Jimenez (2004), Strobel et al. (2011)

(continued)

Table 11.2 (continued)

Bacterial and fungal strains	Host plant	Identified volatile compound	Effective on	Effect on interacting organisms	References
Muscodor crispans	*Ananas ananassoides*	Mixture of volatile compounds	Fungi and bacteria	Effective against a wide range of plant pathogens, including the fungi *Pythium ultimum*, *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum*, and *Mycosphaerella fijiensis* (the black sigatoka pathogen of bananas), and the serious bacterial pathogen of citrus, *Xanthomonas axonopodis* pv. *citri*. In addition, the VOCs of *M. crispans* killed several human pathogens, including *Yersinia pestis*, *Mycobacterium tuberculosis*, and *Staphylococcus aureus*	Mitchell et al. (2010)
Muscodor kashayum sp. nov.	*Aegle marmelos*	3-cyclohexen-1-ol,1-(1,5-dimethyl-4-hexenyl)-4-methyl, 1,6 dioxacyclododecane-7,12-dione, 2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl) phenol, 2,4-di-tert-butylthiophenol and 4-octadecylmorpholine	Fungi and bacteria	Growth of 75% of test fungi/yeasts and 72% of the test bacteria were completely inhibited by VOC produced by *Muscodor kashayum* sp. nov.	Meshram, et al. (2013)
Muscodor sutura	*Prestonia trifidi*	Thujopsene, chamigrene, isocaryophyllene, and butanoic acid	Fungi	Volatile compounds in the mixture possess wide spectrum antifungal activity and no observable antibacterial activity	Kudalkar et al. (2012)
Muscodor tigerii sp. nov.	*Cinnamomum camphora*	4-Octadecylmorpholine, 1-Tetradecanamine, N,N-dimethyl and 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Fungi and bacteria	The in vitro VOC stress assay completely suppressed the growth of *Alternaria alternata* and *Cercospora beticola* while the growth of other fungal species was inhibited in a	Saxena et al. (2015)

(continued)

Table 11.2 (continued)

Bacterial and fungal strains	Host plant	Identified volatile compound	Effective on	Effect on interacting organisms	References
				range of 10–70%. The growth of *Candida albicans* in the presence of VOC was reduced by 50–65% while in bacteria 50–80% reduction in growth was observed. Thus, *M. tigerii* stands as a potential candidate to be further developed into a biocontrol agent	
Myrothecium inunduatum	*Acalypha indica*	3-octanone, 3-octanol, and 7-octen-4-ol, terpenes, organic acids, ketones, and alcohols	Fungi	VOC showed inhibitory activity against a number of plant pathogenic fungi including *Pythium ultimum* and *Sclerotinia sclerotiorum*	Banerjee et al. (2010)
Nigrograna mackinnonii E5202H	*Guazuma ulmifolia*	Terpenes and main component of the polyenes such as (3E,5E,7E)-nona-1,3,5,7-tetraene (NTE)	–	–	Shaw et al. (2015)
Phialocephala fortinii	*Pinus sylvestris*	β-caryophyllene, ethanol, acet-aldehyde, toluene	–	–	Back et al. (2010)
Phoma sp.	*Larrea tridentate*	Unique mixture of VOCs, including a series of sesquiterpenoids, some alcohols, and several reduced naphthalene derivatives	Fungi	The volatiles of *Phoma* sp. possess antifungal and fuel properties	Strobel et al. (2011)
Phomopsis sp.	*Odontoglossum* sp.	Sabinene, isoamylalcohol, 2-methyl propanol, 2-propanone	Fungi	Possess antifungal properties against a wide range of plant pathogenic fungi including: *Pythium, Phytophthora, Sclerotinia, Rhizoctonia, Fusarium, Botrytis, Verticillium,* and *Colletotrichum*	Singh et al. (2011)

(continued)

Table 11.2 (continued)

Bacterial and fungal strains	Host plant	Identified volatile compound	Effective on	Effect on interacting organisms	References
Trichoderma gamsii YIM PH30019	*Panax notoginseng*	Dimethyl disulfide, dibenzofuran, methanethiol, ketones	Fungi and plants	*T. gamsii* YIM PH30019 displayed antagonistic activities against the pathogenic fungi (*Fusarium solani, Fusarium oxysporum, Phoma herbarum, and Rhizoctonia solani*) of *Panax notoginseng* via production of VOCs	Chen et al. (2015a)
Trichoderma koningiopsis YIM PH30002	*Panax notoginseng*	Alkanes, monoterpenes and arenes, heterocycles, and aldehydes	Fungi and plants	*Trichoderma koningiopsis* YIM PH30002 exhibited significant inhibition of the growth of four host root-rot phytopathogens, *Phoma herbarum, Fusarium flocciferum, Scytalidium lignicola*, and *Epicoccum nigrum* by producing volatile organic compounds	Chen et al. (2015b)
Quambalaria cyanescens strain IMI298177	*Ipomoea carnea*	Volatile mixture	Fungi	The sesquiterpenoid (+) globulol with antimycotic property has been reported from *Q. cyanescens*	Padhi and Tayung (2013)

1997). Certain endophytes produce antimicrobial VOC which may directly contribute to defense against pathogens. *Muscodor albus*, a fungal endophyte originally isolated from cinnamon tree, emits a blend of VOC that inhibits or kills a broad range of bacteria, fungi, and oomycetes (Strobel et al. 2001). The GC-MS analysis of its VOC revealed that many of the identified compounds such as 1-butanol and 3-methyl-acetate have antimicrobial activity (Strobel 2006; Porras-Alfaro et al. 2011).

Some biocontrol fungi appear to employ VOC to control pathogenic fungi (Hynes et al. 2007). For instance, many strains of *Trichoderma* have been proven to effectively prioritize a wide range of soilborne fungal pathogens by employing mechanisms such as mycoparasitism, nutrient competition, and secretion of inhibitory compounds and hydrolytic enzymes (Lorito et al. 2010; Harman 2011). *Trichoderma viride* and *T. aureoviride* emitted VOC that inhibit the growth and protein production of *Serpula lacrymans*, a wood-rotting basidiomycete. However, *T. pseudokoningii* had no effect on any of the *Serpula* isolates tested, suggesting the species-specific nature of antifungal VOC production (Humphris et al. 2002). Also, VOC produced by *Trichoderma* spp. are useful in growth inhibition of *F. oxysporum* f. sp. *ciceris*, a soilborne fungal pathogen that causes chickpea wilt (Dubey et al. 2007). *F. oxysporum* strain MSA35, which enhanced lettuce growth via VOC also inhibits the growth of pathogenic strains of *F. oxysporum* (Minerdi et al. 2011).

11.8 Conclusion

Volatiles are only a minor proportion of the entire number of metabolites produced by existing organisms. Nevertheless, because of their unique attributes they are predestined to act as infochemicals in intra- and interspecies communications in the atmosphere as well as in soil. Among microbes, endophytic filamentous fungi are an excellent platform for exploiting biosynthetic routes to hydrocarbon biofuels or biofuel precursors. In recent years, bacterial and fungal production of volatiles has emerged as a novel process by which these endophytes modulate plant growth and induce resistance against abiotic and biotic stresses. Exposure to the volatiles produced by microbes has been shown to lead to up to fivefold increased plant biomass or to plant death.

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Chapter 12
Potential of Lignin-Degrading Endophytic Fungi on Lignocellulosic Biorefineries

Úrsula Fillat, Raquel Martín-Sampedro, David Macaya-Sanz, Juan A. Martín, David Ibarra and María E. Eugenio

Abstract Renewable lignocellulosic biomass is considered as feedstocks to play a significant role in the future of biorefineries for the sustainable production of food, chemicals, materials, and biofuels. Lignin, the natural barrier that protects cellulose and hemicelluloses from microbial attack, represents an important limiting factor in these processes. Removal of lignin has a vast scope with central importance to be utilized as a source of carbohydrates in the manufacturing of cellulose-based chemicals including paper pulp and ethanol production. Enzymes produced by ligninolytic fungi acted as an alternative to develop competent and eco-friendly technologies to use biomass of lignin and cellulose. Among these microorganisms, the "white-rot" causing fungi that belong to basidiomycetes are potential contenders of efficient depolymerization and mineralization of lignin via secretion of low molecular mass oxidative enzymes. Recently, some endophytic fungi have been tested for ligninolytic enzymes and their possible biotechnological applications. This chapter highlights the recent progress that has been made in screening endophytic fungi for ligninolytic activities and their capacities for transforming lignocellulosic biomass into fermentable sugars and paper pulps in a biorefinery framework.

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12.1 Introduction

Wood is the main renewable material on Earth, and primarily composed of lignin, cellulose, and hemicellulose (Higuchi 1997). Lignin provides mechanical resistance to the plant. Lignin complex polymer with a three-dimensional structure consisting guaiacyl (G), p-hydroxyphenyl (H), syringyl (S), and phenylpropanoid as components. The unit of lignin is derived from the hydroxycinnamyl alcohols (p-coumaryl, coniferyl, and sinapyl alcohols), which gives rise to variants of C–C and ether bonds subunits (Martínez et al. 2009). Very few organisms are able to degrade lignin, due to its chemical complexity and recalcitrance (Martínez et al. 2005). Degradation of lignin becomes a central issue in biorefinery processes, such as in the production of ethanol and cellulose-based papers (Cañas and Camarero 2010). In the plant cell wall, lignin is intimately associated to carbohydrates (hemicellulose and celluloses) to prevent easy hydrolysis for bioethanol production. Thus, a step as pre-treatment (mainly physical or physico-chemical methods) is necessary for increasing fermentable sugars for hydrolysis step (Salvachúa et al. 2011; Kataaria et al. 2013; Ofori-Boateng and Lee 2013). Regarding paper pulp manufacture, the process basically consists in the separation (chemically or mechanically) of lignin from fibers. After that, residual lignin in pulps are removed by oxidative bleaching reactions, including totally chlorine-free (TCF) bleaching sequences (Fillat and Roncero 2010; Sixta 2006; Fillat and Roncero 2009b).

Biotechnological processes could provide efficient and eco-friendly biocatalysts for lignin modification or removal (Cañas and Camarero 2010). Bacteria and fungi are capable of competently depolymerizing and mineralizing lignin (Martínez et al. 2005; Kunamneni et al. 2007). Wood-decaying fungi can secrete extracellular enzymes related to lignin degradation, developing a complex system involving reductases, oxidases, peroxidases, mediators, low molecular weight compounds, etc. (Martínez et al. 2005). Laccases are the oxidoreductases most studied to be applied in biotechnological processes; these enzymes oxidize phenol and other compounds of aromatic nature (Cañas and Camarero 2010). Basidiomycota is recognized as the most relevant phylum secreting laccases. However, there are species within ascomycota, such as *Myceliophthora thermophila*, which also produce ligninolytic enzymes with high industrial interest due to thermal stabilities and activities at higher pH (Ibarra et al. 2006). In the industrial applications, the ability of ligninolytic fungi and their oxidoreductases enzymes (laccases and peroxidases) to alter or remove lignin are eco-friendly schemes for utilization of renewable lignocellulosic feedstocks. Various workers have observed that these fungi and their enzymes act for pre-treatment steps to enhance enzymatic hydrolysis of

lignocellulosic biomass (Moreno et al. 2015a; Castoldi et al. 2014; Kudanga and Le Roes-Hill 2014; Singh et al. 2014; Ghorbani et al. 2015; Moreno et al. 2013). In the same way, these biotechnological tools have been used as biological pre-treatment before pulping process improving fiber individualization or lignin removal, respectively (Bajpai et al. 2001). Moreover, laccases, mainly being used as a laccase-mediator system (LMS) to catalyze the development of efficient TCF biobleaching (Singh et al. 2015; Fillat and Roncero 2009a, 2010). Lignin degradation by fungi has been previously evaluated significant and necessary for long-term storage for the growth of desired saprophytic fungi which complicates industrial applications. Therefore, novel candidates of potential fungi become essential to find out to solve or ameliorate the hurdle of industrial implementation.

Various studies on wood-attacking fungi were based on advanced degradation stages. However, few fungal endophytes also served as potential wood-decomposers (Fukusawa et al. 2009). In nature, fungal endophytes are found living in symbiosis in asymptomatic photosynthetic tissues of all major lineages of land plants. These fungi represent an enormous fungal diversity and its geographical distribution, host range and ecological roles are yet unknown (Arnold et al. 2002; Martín et al. 2013). Some endophytic fungi remain in a dormant stage until the plant or its organs become dead and then trigger to grow to become primary colonizers (Promputtha et al. 2010a). Thus, these fungi advantageously compete on other saprophytes in early stages of decomposition (Fukusawa et al. 2009). A number of studies on fungal succession have been carried out to suggest that some of the early colonizers are fungal endophytes (Promputtha et al. 2010b), those bears complex enzymatic systems (Wang and Dai 2011; Sunitha et al. 2013), become able to degrade tissues of their host plant. On the other hand, other fungi such as vesicular and arbuscular mycorrhizal (VAM) interact with living plant roots modifying lignin biosynthesis in the plant and then altering their resistance to pest and pathogens (Bennett et al. 2014). Other studies focused on the environmental effect on the nonstatic interaction amid plant and endophyte (Faeth and Fagan 2002; Lehtonen et al. 2005). However, temporal and spatial distribution of endophytes in the bulky and ancient forest is still poorly understood.

The study of the wood-associated fungi in lignin degradation, as well as their oxidative enzymes, is necessary, as they could advance current bioconversion processes.

12.2 Lignin-Degrading Enzymes

In nature, basidiomycetes cause white-rot with a wide array of enzymes that are effective for lignin degradation (Wong 2009). Peroxidases and laccases are the major groups of ligninolytic enzymes (Fig. 12.1) (Alcalde 2015). Ligninolytic peroxidases are hemeperoxidases with high redox potential that can oxidize phenolic and nonphenolic units of lignin using hydrogen peroxide mediated hydrolysis as a co-substrate. Lignin peroxidases can directly oxidize the substrates, whereas

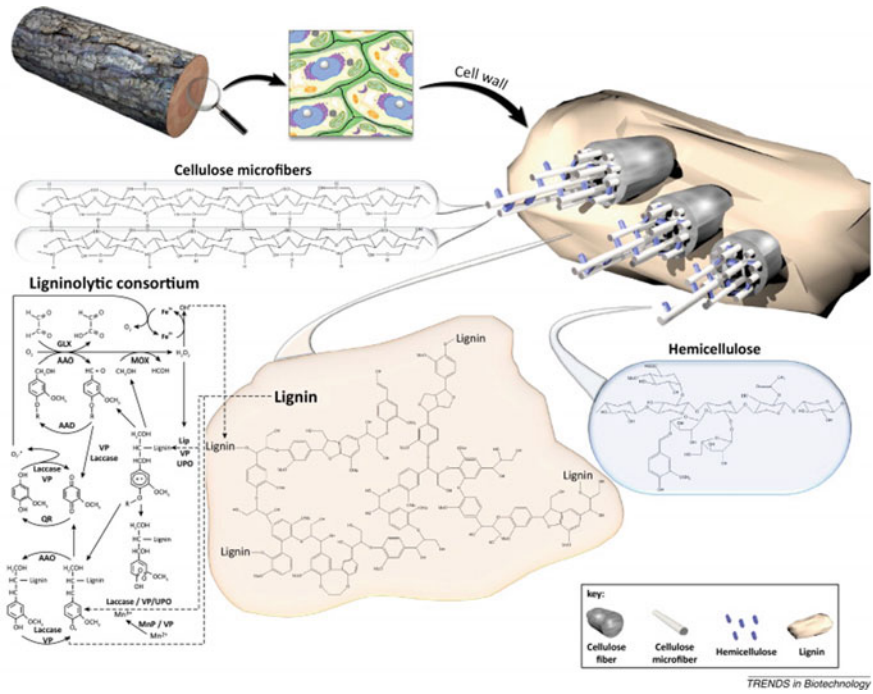

Fig. 12.1 Overview of the place of lignin in cellulosic biomass and the reactions catalyzed by lignoenzymes. Figure extracted from Alcalde (2015)

manganese peroxidases produce Mn^{3+} and act mainly on phenolic units; both were discovered in *Phanerochaete chrysosporium* (Martínez et al. 2005). *Pleurotus eryngii* produces versatile peroxidase that shows catalytic properties similar to lignin and manganese peroxidases (Ruiz Dueñas et al. 1999). On the other hand, oxidades, such as glyoxal and aryl-alcohol oxidases described in *Phanerochaete chrysosporium* and *P. eryngii*, respectively (Guillén et al. 1992), produce the hydrogen peroxide necessary for the catalytic action of the peroxidases (Kersten 1990), Finally, to avoid the repolymerization of the derived phenols produced during lignin oxidation, aryl-alcohol dehydrogenases and quinone reductases catalyze their reduction (Guillén et al. 1997).

Laccases oxidize several phenolic and nonphenolic substrates using four Cu^{+} ions on the active site. Type 1 copper acts as an electron acceptor from substituted phenols or amines and is liable for the development of blue color. While type 2 copper transfer electrons to the molecular oxygen which further reduces water molecule (Martínez et al. 2005). Laccases also generate radicals, and then nonenzymatic reactions were also produced, such as polymerization and hydrogen abstraction. Although they have been isolated from ascomycetes and deuteromycetes, lignin degradation studies have not been a focus as much on the basidiomycetes (Madhavi and Lele 2009). Most common laccase producers are

wood-rotting fungi from the genus *Trametes*, *Pleurotus, Pycnoporus, Coriolopsis,* and *Cerrena* (Morozova et al. 2007; Kunamneni et al. 2007; Madhavi and Lele 2009). Laccase activity has also been reported in bacteria including *Azospirillum lipoferum, Bacillus subtilis, Bordetella campestris, Caulobactercrescentus, Escherichia coli, Mycobacterium tuberculosum, Pseudomonas syringae, Pseudomonas aeruginosa, Yersinia pestis*, *Stenotrophomonas maltophilia, Streptomyces cyaneus,* and *Streptomyces ipomoea* (Diamantidisa et al. 2000; Chandra and Chowdharya 2015; Arias et al. 2003; Eugenio et al. 2011).

12.3 Ligninolytic Enzymes in Endophytic Fungi

Most endophytic fungi are ascomycetes, though other phyla are also represented. Literature about ligninolytic enzymes production by endophytes is limited and, in general, although few ascomycetes have shown the ability for lignin degradation largely being ignored as liable for the degradation of wood-biomass (Pointing 1999; Liers et al. 2006; Shary et al. 2007).

Most studies carried out to screen ligninolytic enzymes from fungi were culture-procurement based beside, culture-dependent (Levin et al. 2004; Järvinen et al. 2012). However, recently some microorganisms have been isolated from the decayed wood of forests in Zimbabwe (Tekere et al. 2001), Tunisia (Dhouib et al. 2005) and Spain (Barrasa et al. 2009; Fillat et al. 2016) have been reported. Solid media facilitate a fast selection of diverse fungi for enzymatic activity (Sunitha et al. 2013; Niku-Paavola et al. 1988). Different substrates as ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)), naphthol and Poly R-478 have been assayed for the search of lignin-degrading enzymes in endophytes isolated from living plants (Table 12.1). A solid screening suggests that fungal endophytes possess enzymatic machinery, which could produce decay of wood under certain conditions (Oses et al. 2006). Ligninolytic activities in basidiomycetous fungi associated with woody trees, isolated from the Chilean tree species *Drymiswinteri* and *Prumnopitysandina,* were found using Poly R-478 (Oses et al. 2006). Only two endophytes, *Xylaria* sp. from Xylariaceae and *Curvularia brachyspora* from Pleosporaceae, were positive in naphthol from twelve different species isolated from four medicinal plants (*Adhatodav asica* Nees, *Costus igneus* N.E.Br. *Coleus aromaticus* Benth and *Lawsonia inermis* Linn) (Amirita et al. 2012b). Fifty fungal strains, isolated from medicinal plants (*Alpiniacalcarata, Bixaorellana, Calophyllum inophyllum,* and *Catharanthusroseus*) were selected for extracellular enzymes and thirty percent of the fungi screened showed naphthol oxidation from different families (Table 12.1) (Sunitha et al. 2013). Endophytic fungi community of *Acer truncatum* trees was investigated and seventeen from twenty isolates oxidized the substrate naphthol, as indicated in Table 12.1 (Sun et al. 2011).

A new screening study with an enormous amount of strains isolated from eucalyptus trees in Spain has recently been published (Fillat et al. 2016) (Fig. 12.2). In this study, strains of endophytic fungi isolated from *E. globulus* trees in different

Table 12.1 Endophytic fungi with potential for producing ligninolytic enzymes assayed in solid plates

Substrate	Accession family	Accession species	References
ABTS	Lophiostomataceae	*Lophiostoma corticola*	Fillat et al. (2016)
ABTS	Dothioraceae	*Hormonema* sp.	Fillat et al. (2016)
ABTS	Dothioraceae	*Pringsheimia smilacis*	Fillat et al. (2016)
ABTS	Montagnulaceae	*Paraconiothyrium variabile*	Fillat et al. (2016)
Naphtol	Montagnulaceae	*Paraconiothyrium brasiliense*	Sun et al. (2011)
ABTS	Botryosphaeriaceae	*Neofusicoccum luteum*	Fillat et al. (2016)
ABTS	Botryosphaeriaceae	*Neofusicoccum australe*	Fillat et al. (2016)
ABTS	Botryosphaeriaceae	*Dothiorella sarmentorum*	Fillat et al. (2016)
ABTS	Botryosphaeriaceae	Botryosphaeria sp.	Cruz et al. (2012)
Naphtol	Botryosphaeriaceae	*Fusicoccum* sp.	Sunitha et al. (2013)
ABTS	Pleosporaceae	*Ulocladium* sp.	Fillat et al. (2016)
Naphtol	Pleosporaceae	*Curvalaria brachyspora*	Amirita et al. (2012a)
Naphtol	Pleosporaceae	*Curvularia* sp.	Patel et al. (2013)
Naphtol	Pleosporaceae	*Drechslera biseptata*	Sun et al. (2011)
Naphtol	Pleosporaceae	*Alternaria alternata*	Sun et al. (2011)
Naphtol	Pleosporaceae	*Alternaria arborescens*	Sun et al. (2011)
ABTS	Amphisphaeriaceae	*Leiosphaerella praeclara*	Fillat et al. (2016)
Poly R-478	Xylariaceae	*Xylaria* sp.	Urairuj et al. (2003)
Naphtol	Xylariaceae	*Xylaria* sp.	Amirita et al. (2012a)
Naphtol	Diaporthaceae	*Phomopsis longicolla*	Sunitha et al. (2013)
Naphtol	Trichosphaeriaceae	*Nigrospora* sp.	Patel et al. (2013)
Naphtol	Nectriaceae	*Fusarium* sp.	Patel et al. (2013)
Naphtol	Chaetomiaceae	*Chaetomium* sp.	Sunitha et al. (2013)
Naphtol	Trichocomaceae	*Aspergillus niger*	Sunitha et al. (2013)
Naphtol	Trichocomaceae	*Penicillium* sp.	Sunitha et al. (2013)
Naphtol	Pestalotiopsidaceae	*Pestalotiopsis* sp.	Sunitha et al. (2013)
Naphtol	Cordycipitaceae	*Isaria* sp.	Sunitha et al. (2013)
Naphtol	Amphisphaeriaceae	*Pestalotiopsis disseminata*	Sunitha et al. (2013)
Naphtol	Leptosphaeriaceae	*Leptosphaeria*sp.	Sun et al. (2011)
Naphtol	Leptosphaeriaceae	*Coniothyrium olivaceum*	Sun et al. (2011)
Naphtol	Diaporthaceae	*Diaporthe* sp.	Sun et al. (2011)
Naphtol	Glomerellaceae	*Glomerella miyabeana*	Sun et al. (2011)
Naphtol	Gnomoniaceae	*Gnomoniella* sp.	Sun et al. (2011)
Naphtol	Melanconidaceae	*Melanconis* sp.	Sun et al. (2011)
Naphtol	Montagnulaceae	*Microsphaeropsis arundinis*	Sun et al. (2011)
Naphtol	Incertae sedis	*Ascochytopsis vignae*	Sun et al. (2011)
Naphtol	Incertae sedis	*Coelomycetes* sp.	Sun et al. (2011)

(continued)

Table 12.1 (continued)

Substrate	Accession family	Accession species	References
ABTS	Incertae sedis	*Phaeomoniella effusa*	Fillat et al. (2016)
ABTS	Incertae sedis	*Phaeomoniella niveniae*	Fillat et al. (2016)
Naphtol	Incertae sedis	*Discosia* sp.	Sunitha et al. (2013)
Naphtol	Incertae sedis	*Phoma* sp.	Sunitha et al. (2013), Sun et al. (2011)
Naphtol	Incertae sedis	*Phoma glomerata*	Sun et al. (2011)
Naphtol	Incertae sedis	*Sirococcus clavigignenti juglandacearum*	Sun et al. (2011)
Poly R-478	Meruliaceae[a]	*Bjerkandera* sp.[a]	Oses et al. (2006)

[a]All are Ascomycete fungi except the Basidiomycete *Bjerkandera* sp.

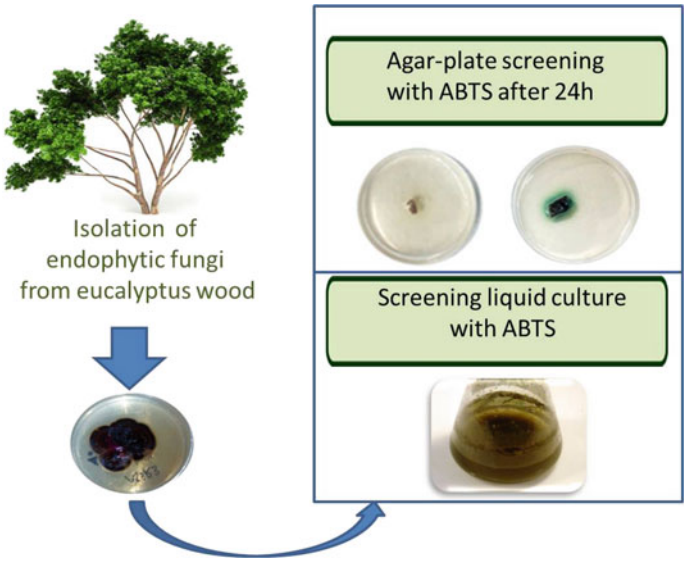

Fig. 12.2 Scheme of the experimental procedure for screening of ligninolytic enzymes from eucalyptus wood endophytes. Figure extracted from Fillat et al. (2016)

regions of Spain were screened on agar medium containing ABTS. Among more than one hundred strains of endophytic fungi tested, twenty-one fungal strains oxidized ABTS at some extent. High ABTS oxidation was observed in eight strains after 48 h with a higher ratio of green halo and diameter of the colony (DH/DC) and other five strains were observed to produced medium oxidation (DH/DC ratio 1.5–1.2). However, *Pycnoporus sanguineus* and *Trametes* sp. I-62 strains (DH/DC

ratio) used as a model of white-rot fungi known to be a good producer of laccases (Martín-Sampedro et al. 2013; Martín-Sampedro et al. 2013; Eugenio et al. 2013). Endophytic strains showing positive oxidation were identified a member of ascomycetes such as *Neofusicoccum, Ulocladium, Lophiostoma, Pringsheimia, Hormonema, Dothiorella, Pyrenochaeta,* and *Coniothyrium*) and Sordariomycetes (genus *Leiosphaerella*). Four strains classified as genera *Phaeomoniella* and *Tumularia* and were of *Incertae sedis* classes. It should be noted that the majority of endophytic strains found a member of Dothideomycetes and to the orders of Pleosporales, Dothideales, and Eurotiomycetes. This predominance of Dothideomycetes in this screening can be due to different facts (i) more common in the endophytic community; (ii) their isolation is easier than that of others endophytes or (iii) produce more oxidoreductases.

Endophytic strains of Xylariaceae shown ligninolytic activity on agar medium using Poly R-478 as indicator (Urairuj et al. 2003; Cruz et al. 2012); and a positive result has been found with naphthol in *Curvularia* sp., *Nigrospora* sp. and *Fusarium* sp. from Pleosporaceae and Nectriaceae families, respectively (Patel et al. 2013).

Various workers have observed laccase activity in liquid medium for some endophytic fungi (Anderson et al. 2005; Fillat et al. 2016; Shary et al. 2007) (Table 12.2). These include members of the class Sordariomycetes, and family Xylariaceae (Urairuj et al. 2003), *Fusarium proliferatum* of the family Nectriaceae (Anderson et al. 2005; Muthezhilan et al. 2014), *Podospora anserina* of the family Lasiosphaeriaceae (Durand et al. 2013), *Chaetomium globosum* of the family Chaetomiaceae (Benhassine et al. 2016; El-Zayat 2008), *Colletotrichum gloesporioides* of Glomerellaceae (Xie and Dai 2015; Zhou et al. 2014) and *Phomosis liquidambari* of Diaporthaceae (Xie and Dai 2015; Zhou et al. 2014). Similarly, laccase activity has been demonstrated for members of class Dothideomycetes, *Monotospora* sp. of the family Hysteriaceae (Wang et al. 2006), few genera such as *Neofusicoccum australe, N. luteum* and *Botryosphaeria* sp. of Botryosphaeriaceae (Barbosa et al. 1996; Srivastava et al. 2013; Cruz et al. 2012; Sunitha et al. 2013; Fillat et al. 2016), *Ulocladium* sp. of Pleosporaceae (Atalla et al. 2010) and *Hormonema* sp. and *Pringsheimia smilacis* of Dothioraceae (Fillat et al. 2016; Zifcáková et al. 2011). Laccase activity has been measured in endophytic basidiomycetes *Peniophora* sp. of Peniophoraceae family (Zifcáková et al. 2011). To the best of our knowledge, four laccases from endophytes have been purified and characterized from *Trichoderma harzianum* (Sadhasivama et al. 2008), *Podospora anserine* (Durand et al. 2013), *Cladosporium cladosporioides* (Halaburgi et al. 2011), and *Paraconiothyrium variabile* (Forootanfar et al. 2011).

On the other hand, ligninolytic activities have been detected in endophytes cultures: manganese peroxidase and independent manganese peroxidase in several species of the family *Xylariaceae* (Urairuj et al. 2003) and aryl-alcohol oxidadase in *F. proliferatum* (Anderson et al. 2005). The peroxidase activity has been measured in the endophytic basidiomycete strain *Bjerkandera* sp. (Oses et al. 2006).

Table 12.2 Endophytic fungi with potential for producing ligninolytic enzymes in a liquid medium. Substrates used in measurements and enzymatic activity detected

Class	Accession family	Accession species	Substrate (ligninolytic activity)	References
Sordariomycetes	Diaporthaceae	*Phomopsis liquidambari*	ABTS (Laccase)	Xie and Dai (2015), Zhou et al. (2014)
Sordariomycetes	Xylariaceae	*Xylaria* sp.	DMP (Laccase, MnP, MIP (independent manganese))	Urairuj et al. (2003)
Sordariomycetes	Nectriaceae	*Fusarium* sp.	Guaiacol (Laccase)	Muthezhilan et al. (2014)
Sordariomycetes	Nectriaceae	*Fusarium proliferatum*	ABTS, DMP, VA, azure B (Laccase, AAO)	Anderson et al. (2005)
Sordariomycetes	Chaetomiaceae	*Chaetomium* sp.	ABTS (Laccase)	Benhassine et al. 2016)
Sordariomycetes	Chaetomiaceae	*Chaetomium globosum*	ABTS (Laccase)	El-Zayat (2008)
Sordariomycetes	Lasiosphaeriaceae	*Podospora anserine*	ABTS (Laccase)	Durand et al. (2013)
Sordariomycetes	Glomerellaceae	*Colletotrichum gloeosporioides*	Guaiacol (Laccase)	Sidhu et al. (2016)
Sordariomycetes	Hypocreaceae	*Trichoderma harzianum*	ABTS, guaicaol (Laccase)	Sadhasivama et al. (2008), Gao et al. (2013)
Dothideomycetes	Botryosphaeriaceae	*Botryosphaeria* sp.	ABTS (Laccase)	Barbosa et al. (1996)
Dothideomycetes	Botryosphaeriaceae	*Neofusicoccum australe*	ABTS (Laccase)	Fillat et al. (2016)
Dothideomycetes	Botryosphaeriaceae	*Neofusicoccum luteum*	ABTS (Laccase)	Fillat et al. (2016)
Dothideomycetes	Botryosphaeriaceae	*Botryosphaeria rhodina (Lasiodiplodiatheobromae)*	ABTS (Laccase)	Srivastava et al. (2013)
Dothideomycetes	Botryosphaeriaceae	*Botryosphaeria obtusa*	ABTS (Laccase)	Srivastava et al. (2013)
Dothideomycetes	Botryosphaeriaceae	*Botryosphaeria dothidea*	ABTS (Laccase)	Srivastava et al. (2013)
Dothideomycetes	Botryosphaeriaceae	*Botryosphaeriaribis (Neofusicoccumribis)*	ABTS (Laccase)	Srivastava et al. (2013)
Dothideomycetes	Hysteriaceae	*Monotospora* sp.	ABTS (Laccase)	Wang et al. (2006)
Dothideomycetes	Dothioraceae	*Hormonema* sp.	ABTS (Laccase)	Fillat et al. (2016)

(continued)

Table 12.2 (continued)

Class	Accession family	Accession species	Substrate (ligninolytic activity)	References
Dothideomycetes	Dothioraceae	*Hormonema dematioides*	ABTS (Laccase)	Zifcáková et al. (2011)
Dothideomycetes	Dothioraceae	*Pringsheimia smilacis*	ABTS (Laccase)	Fillat et al. (2016)
Dothideomycetes	Pleosporaceae	*Ulocladium chartarium*	Guaiacol, veratrylaldehide, ABTS (Laccase)	Atalla et al. (2010)
	Davidiellaceae	*Cladosporium cladosporioides*	ABTS (Laccase)	Halaburgi et al. (2011)
Dothideomycetes	Montagnulaceae	*Paraconiothyrium variabile*	ABTS, guaiacol, DMP (Laccase)	Forootanfar et al. (2011)
Agaricomycetes[a]	Peniophoraceae[a]	*Peniophora* sp.[a]	ABTS (Laccase)	Zifcáková et al. (2011)
Agaricomycetes[a]	Meruliaceae[a]	*Bjerkandera* sp.[a]	ABTS (Peroxidase)	Oses et al. (2006)

[a]All strains are Ascomycete fungi except Basidiomycetous *Peniophora* sp. and *Bjerkandera* sp.

12.4 Role of Biotechnology in Lignocellulosic Biorefineries

Biorefineries are facilities that provide fiber products, biofuels, and other chemical materials including plastics, sugar polymers, oils, and biomass derived proteins (Cañas and Camarero 2010). Biorefineries combine and integrate various technologies, among which major are biotechnical methodologies which have the potential to reduce carbon emissions through different ways; substituting petroleum as a fuel and as a starting material, increasing process efficiency, closing loops, and diminishing wastes (Erickson et al. 2012). In nature, lignin oxidation performed by wood-rotting fungi is an important issue for carbon cycling, and its implementation in the industry can improve the accessibility of chemicals or enzymes to cellulose. Application of ligninolytic fungi and its enzymes has been extensively studied in the pulp and paper industry and the production of biofuels (Camarero et al. 2014).

Cellulose and hemicelluloses can be hydrolyzed by acid treatments or enzymatic hydrolysis after pre-treatment, conversion into bioethanol by a microbial fermentation. In contrast, lignin is not constituted of fermentable sugars and due to their structural properties has an unruly structure challenging to discompose. The introduction of a pre-treatment step is indispensable to improve digestion ability of lignocellulose and sugars production (Parawira and Tekere 2011; Salvachúa et al. 2011; Kataaria et al. 2013; Ofori-Boateng and Lee 2013). Several physico-chemical and chemical pre-treatment processes exist for ammonia fiber explosion to improve lignocellulose saccharification thereby (Kumar et al. 2009). However, these technologies include high high-capital investment with energy demand and also produce certain sugars that influence the subsequent fermentation (Moreno et al. 2015b). In order to overcome this disadvantages produced by the physico-chemical pre-treatment, different eco-friendly approaches using biotechnology have been studied to degrade lignocellulose (biodelignification) and to decrease the quantity of inhibitors (biodetoxification) (Parawira and Tekere 2011; Moreno et al. 2015b). These biotreatments exhibit higher product yield and few side reactions (Moreno et al. 2015b). Moreover, biotechnology processes use mild reaction conditions that reduced reactor requirements to resist pressure and corrosion and also energy costs (Moreno et al. 2015b). White-rot fungi and their oxidative enzymes avoid the formation of inhibitors and are an alternative or an additional pre-treatment step to physico-chemical methods for bioethanol production (Moreno et al. 2015a; Castoldi et al. 2014; Castoldi et al. 2014; Kudanga and Le Roes-Hill 2014; Singh et al. 2014; Ghorbani et al. 2015; Moreno et al. 2013; Ruíz-Dueñas and Martínez 2009).

On the other hand, the pulping process consists of the separation of pulp fibers from wood for papermaking. One of the less harmful and more promising alternatives to improve conventional pulping processes is the use of microorganisms (such as white-rot fungi) and their enzymes for biotreatment wood chips to reduce lignin content (Fonseca et al. 2014). This process was industrialized to reduce the electrical energy required for pulping wood chips and to economize active alkali charge or cooking time for chemical pulping (Mendonça et al. 2002; Villalba et al. 2006). As mentioned above endophytic fungi have been widely studied as

biotechnological pre-treatments. Recently, the role of five ascomycete endophytic strains *Neofusicoccum luteum, Ulocladium* sp., *Pringsheimia smilacis, Hormonema* sp. and *Neofusicoccum australe* in lignin degradation has been studied extensively. Application of the endophytic fungal strain *Hormonema* sp. CECT 13092 for the mentioned applications has been patented (Martín et al. 2014).

12.4.1 *Effect of Fungal Pre-treatments on Enzymatic Hydrolysis*

Wood chips were inoculated with individual preinoculum of each fungal strain. After the biotreatments and before enzymatic saccharification, a mild alkali treatment was performed on solid samples, in order to improve the hydrolysis yields (Salvachúa et al. 2011). An autohydrolysis (AH) pre-treatment was carried out prior or after the fungal (and alkali) pre-treatment (Fig. 12.3) (Martín-Sampedro et al. 2015b). The AH pre-treatment augmented glucose yield from 4 to 11% more than xylose in comparison to that of untreated control (Fig. 12.4). Pre-treated material presents a major accessibility due to the decrease of hemicelluloses and the increased porosity achieved in the biotreatment (Alvira et al. 2010). Earlier enhanced sugar yields using more severe pre-treatment conditions were observed and Martin-Sampedro et al. (2014) and Romaní et al. (2010) have been reported glucose yields of 23–51% after AH pre-treatment with 3.1–3.8 severity factor. When fungal pre-treatment was carried out before or after AH pre-treatment saccharification improved in all samples. It should be pointed out that a synergistic effect of both pre-treatments could be observed and it was more noticeable when *Ulocladium* sp. or *Hormonema* sp. were used: 8.5 and 8.0 times increases in total sugar yields for both fungi (31–34% glucose and 24–29% xylose yields) regarding no pre-treated control sample (4% glucose and 3% xylose yields) (Fig. 12.4). When the white-rot fungus *Trametes* sp. I-62 used as a reference was inoculated after AH pre-treatment, total sugar yields were 2–3 times higher than that of the yields increased by autohydrolyzed *E. globulus* wood without fungal treatment.

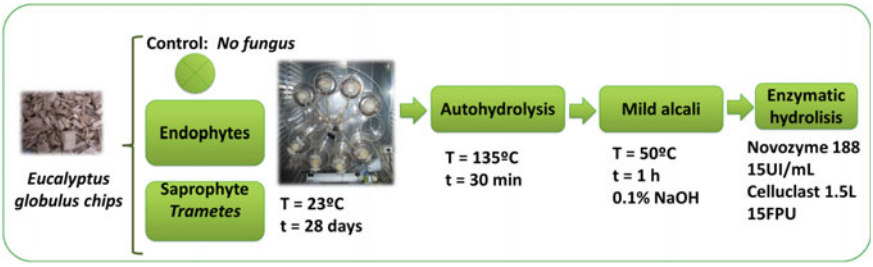

Fig. 12.3 Scheme of the experimental procedure of biological pre-treatments to enhance enzymatic saccharification. Figure extracted from Martín-Sampedro et al. (2015b)

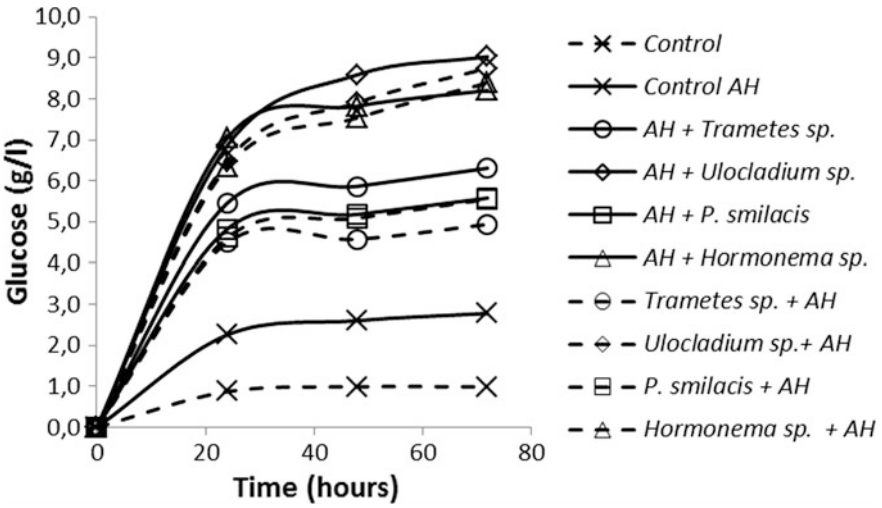

Fig. 12.4 Glucose concentrations during the enzymatic hydrolysis of the samples after fungal treatments and those subjected to autohydrolysis (AH) before or after fungal pre-treatments. Figure extracted from Martín-Sampedro et al. (2015b)

Interestingly, when AH pre-treatment was performed after fungal treatment, the increase in saccharification produced by this reference fungus was lower than that observed for the control sample. The endophytic fungi caused a higher boost of hydrolysis than the white-rot fungi, probing their high potential for enhancing saccharification of *E. globulus* wood. Other studies combined fungal pre-treatments with white-rot or brown-rot fungi with chemical and physical methods so as to improve saccharification yields (López-Abelairas et al. 2013; Wang et al. 2013; Gui et al. 2013) and/or to decrease biotreatment times (Fu et al. 2013; Yu et al. 2009). However, increases in saccharification were lower than that of observed with endophytic strains. López-Abelairas et al. (2013) observed a glucose yield 3.9 and 4.6 times higher in wheat straw pre-treated with a thermal treatment and a fungal treatment with *Pleorotus eryngii* or *Irpex lacteus*. Gui et al. (2013) obtained glucose yields 1.7 times higher using *Pycnoporus chrysosporium* combined with 2.5% sulphuric acid treatment than in acid-treated *Glycyrrhiza uralensis* under the similar conditions. Wang et al. (2013) reported that fungal pre-treatment of poplar wood with *Trametes orientalis* or *Fomitopsis palustris* before a $FeCl_3$ treatment increased sugar yields 1.4 and 1.6 times more than $FeCl_3$ treatment without fungi. Yang et al. (2013) reported a similar value to that found in the endophytic fungi mentioned before when poplar was treated with *Trametes velutina* D10149 and alkaline fractionation.

12.4.2 *Effect of Fungal Pre-treatments on Kraft Pulping*

Fungal pre-treated chips with endophytes and saprophytic fungi were subjected to kraft pulping (Fig. 12.5). Lower Kappa number and higher brightness values were obtained for all the samples treated with fungi compared to control pulp (without fungal treatment). However, no significant differences in polymerization degree were found. Moreover, higher delignification was observed with endophytic strains during kraft pulping compared to *Trametes* sp. I-62, except *P. smilacis*. The highest increment in delignification (27% compared to control) was found when *Hormonema* sp. was applied compared with 9% obtained by *Trametes* sp. I-62. Moreover, *Hormonema* sp. and *N. australe* provided higher brightness (46.0 and 44.3% ISO, respectively) than *Trametes* sp. I-62 (41.6% ISO). Other white-rot fungi have improved delignification during kraft pulping in previous works, but the results were worse than that observed for this endophytic fungus in most of the cases: when chips from hardwood (eucalyptus and poplar) were pre-treated with *Ceriporiopsis subvermispora* an increase of 14% was obtained (Yadav et al. 2010); increases around 18% were produced from pine pre-treated with *Pycnoporus sanguineus*, *Pycnoporus* sp. and *Stereumhirsutum* (Wolfaardt et al. 2004). Higher increases were measured when soda pulping was performed in rice straw pre-treated with *P. chrysosporium* and *Pleorotus ostreatus*: 26 and 35%, respectively (El-Din et al. 2013).

Among all the strains studied, *Hormonema* sp. showed the minor NaOH consumption during pulping, 12% less than control and similar Na_2S consumption. *N. australe* pre-treated samples consumed also around 8% less NaOH than control and *Trametes* sp. I-62 samples. However, Na_2S consumption increased from 54 to 82%, and kappa number and brightness were worse than those found for pulps pre-treated with *Hormonema* sp. These reductions in the alkali dose necessary to produce a target kappa number in chemical pulps have been associated with the modifications in wood chips caused by fungi (Mardones et al. 2006).

Mechanical properties of handsheets of the obtained pulps were improved when a fungal pre-treatment was performed before pulping. Endophytic fungi, except

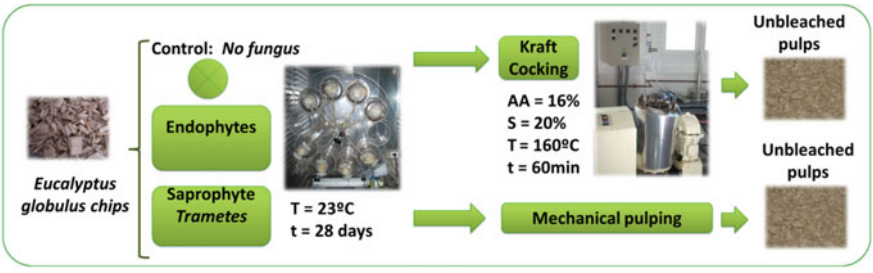

Fig. 12.5 Scheme of the experimental procedure of biological pre-treatments to enhance Kraft and mechanical pulping

P. smilacis, produced increases in tensile, tear and/or burst indices. *Hormonema* sp. again showed the best results and was the only fungus which enhances the three properties compared to control:indices. *N. australe* presented good outcomes in relation to delignification yield, also increased tensile and burst indices. Other authors have found similar results: 8% increase in tensile and 12% in burst indices and an 8% decrease in tear index after pre-treated black pine with *C. subvermispora*, (Gulsoy and Eroglu 2011). Mechanical properties as tensile and burst indices are associated with bond ability amid fibers, whereas tear index is correlated with the degradation of fibers. Those pre-treated pulps that present less lignin content can present a greater bonding amid cellulose-to-cellulose that could improve the strength in the handsheets (Ismail et al. 2005).

12.4.3 *Effect of Fungal Pre-treatments on Mechanical Pulping*

Fungal pre-treated chips with the endophytes and the saprophytic fungi were subjected to mechanical pulping (Fig. 12.5) (Martín-Sampedro et al. 2015a). After mechanical pulping of pre-treated chips, the pulps presented higher refining degree compare to an untreated pulp (48° SR), especially the one pre-treated with *Hormonema* sp. (69° SR). Gulsoy and Eroglu (2011) reported easier fibrillation in pine pulp pre-treated with *C. subvermispora* than control pulp; this enhancement was related to a higher production by fungus. As well, time reductions in refining to obtain a given fibrillation degree was observed in *Eucalyptus tereticornis* pulp pre-treated with *C. subvermispora* (Bajpai et al. 2001) and hornbeam with *P. chrysosporium* (Kasmani et al. 2012). The easier beating of fungal pre-treated pulps to obtain target wetness imply less energy consumption (Gulsoy and Eroglu 2011).

A reduction in kappa number of 8 points and 1.4% in Klason lignin was found when chips were pre-treated with *Hormonema* sp. This endophyte can modify lignin and also, remove it from the wood. Other authors have also observed kappa number reductions in pulps pre-treated with fungi and subjected to chemo-mechanical pulping (Singh et al. 2013).

Enhanced mechanical properties were found in all fungal pre-treated pulps, except for *Ulocladium* sp. According to Ferraz et al. (2008), fungal pre-treatment produces a double effect, a deep short period depolymerization of lignin and also an increase of the water saturation point due to the oxalate esterification on the polysaccharides chains mediated by the fungus. Both changes could influence the fiber bonding, and then, the physical properties of the wood and the pulp obtained after mechanical pulping. Handsheets obtained from pulp pre-treated with *Hormonema* sp. provided the highest tensile and burst indices increases of 28% in tensile, 16% in tear, and 44% in burst indices, compared with an untreated sample. whereas when using the reference fungus *Trametes* sp. I-62 increments of 23, 17, and 12% were obtained, for the same properties. Ramos et al. (2001) and Singh

et al. (2013) found less increases in tensile, tear, and brust indices during pre-treatment of oil palm trunk with *T. versicolor* and *C. subvermispora*. On the other hand, Kasmani et al. (2012) reported significant reductions in tensile and burst indices when hornbeam was pre-treated with *P. chrysosporium*. Therefore, endophytic fungi, mainly *Hormonema* sp., show a high potential in biomechanical pulping when these results were compared to other white-rot fungi. Thus, it is expected that endophytes pre-treated chips would need less energy for fibrillation target and/or similar mechanical properties than untreated pulp.

12.5 Conclusions

Endophytic fungi have been screened for the search of oxidative enzymes that could be used as an alternative to existing industrial processes of transformation of lignocellulosic biomass. Ligninolytic activity has been reported for several endophytic strains in the solid and liquid medium. Among several, endophytes isolated from eucalyptus trees have been recently applied for biotechnological applications in biorefineries. Fungal treatments with endophytes are able to enhance saccharification of *E. globulus*, as well as kraft and mechanical pulping. Moreover, some of them provided greater enhancement than various white-rot fungi. Therefore, endophytic fungi show a high potential to be applied in the lignocellulosic industry.

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Chapter 13
Conclusion

K.G. Ramawat

Abstract Endophytes form a very complex physical and physiological association with the host plant influencing many biological activities. This complex association results in several novel and beneficial activities for these hidden organisms as evident by chapters presented in the two-volume set on endophytes. All the work on endophytes can be categorized as i) isolation and identification, ii) role in agriculture, iii) production of industrially important products, and iv) production of useful secondary metabolites. The concluding remark envisioned the future beneficial role of endophytes with the use of new technology in industry and agriculture.

Keywords Endophytes · Molecular tools · Future of endophytes

Endophytes, as their name suggests, are organisms living within the plants without any noticeable symptom. These form a very complex physical and physiological association influencing the host and other microorganisms present in plant on the one side and all the host's pests on the other side. This complex association results in several novel and beneficial activities for these hidden organisms as evident by data presented in these two volumes on endophytes. Information about endophytic actinobacteria for sustainable agricultural application, their role in phosphate solubilisation, beneficial effects of bacterial endophytes on forest tree species, plant growth promotion by endophytic bacteria in non-native crop hosts, harnessing endophytic microbial volatile organic compound for sustainable agroecosystem, endophytic fungi as source of biologically active secondary metabolites, potential of lignin-degrading endophytic fungi on lignocellulosic biorefineries, endophytic fungi and bioremediation, extra-cellular enzymes, and plant disease control is presented in the chapters.

All the work on endophytes can be categorized as (i) isolation and identification, (ii) role in agriculture, (iii) production of industrially important products, and (iv) production of useful secondary metabolites. Production of useful metabolites

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and applications in sustainable agriculture are important areas of research on endophytes producing a plethora of secondary metabolites and growth promoting substances of diverse chemical nature from wide variety of species of plants and endophytes. Endophytes have been reported from various crop plants, grasses, tree species, and several other plants. It is envisioned that plants of extreme habitat such as cold and hot conditions, carnivores of marshy lands, and all sorts of parasites should be explored to find out more diverse endophytes and hitherto lesser known properties and metabolites. This might increase the inventory of useful metabolites. Involvement of endophytes has also been evaluated in fields as diverse as phytoremediation and biofuels production. Very limited options are available for treating polluted water from agriculture and landfills. This area needs more attention to use endophytes for developing this technology (Redfern Lauren and Gunsch Claudia 2016).

Endophyte and host relationship is complex and not clearly understood. This creates a further complex situation when a pathogen is present in the same host plant. Cell-to-cell communication between different entities is not clearly understood and this area will provide more understanding about the complex relationship, which may provide clue for novel traits for plant breeding.

Reduction in dependence on chemical pesticides and fertilizers for sustainable agriculture is an important area to meet the food security. Endophytes are beneficial to crops directly by producing growth promoting compounds or indirectly by inhibiting the growth of pathogens (Passari et al. 2016). Therefore, it is necessary to characterize the endophytes and their products for crop improvement and sustainable environment. Their effective use will reduce the dependence on chemical fertilizers and pesticides (Le Cocq et al. 2016).

Symbiotic nitrogen fixing endophytes is important class of endophytes studied in great detail in leguminous plants and trees. Efforts to transfer *nif* gene to non-leguminous plants were not successful. In this case also, characterisation of endophytes using genomics and tools of molecular biology would result useful information. Characterization of endophytic bacterial and fungal endophytes will also help in finding non-native hosts. Internal transcribed spacer (ITS) barcoding along with classical taxonomic characterization are important areas of research on endophytes (Tanney et al. 2016). Characterization of endophytes using genomic tools can help in finding more bacterial biocontrol agents and understanding the mechanism of action (Eljounaidi et al. 2016).

All microorganisms, pathogenic, non-pathogenic and endophytes, produce various types to enzymes to dissolve host cell wall and other barriers to establish themselves. These enzymes are useful in many scientific and industrial processes and thus are explored for their optimum production system (Goyal et al. 2017). Mycorrhyzal fungi occur as ecto- or endo-mycorrhyza in plant roots and recognized as phosphate solubilising fungi. In light of recent works these fungi need to work out for many more plant beneficial traits. This will benefit plant as well as ecosystem.

Production of secondary metabolites of interest to pharmaceutical industry is very attractive field of research using biotechnological methods of plant cell

cultures grown in bioreactors. This scale up technology of growing cells at large scale up to 75,000 l has been established and methods of isolation and identification as well as downstream processing of secondary metabolites has been developed for many products. Thus technology is available for industrial-scale production of secondary metabolites of interest and finding a compound of interest from endophytes can leads to its industrial production quickly (Goyal et al. 2015, 2017). In many cases, precursors from plants are converted to final product by endophyte. The complex relationship between endophyte and its host thus is evaluated carefully to establish the biosynthetic process of secondary metabolites. The research work on endophytes applications and use of new technology along with development of industrial level production system is summarized in Fig. 13.1.

Modern tools and techniques of molecular biology including metagenomes, proteomes and transcriptomes will help in defining characteristics of endophytes and finding novel products for development of industrial products. Generally plant breeding is carried out to develop resistant plants with focus on its pathogen. Once endophytes and their relationship are established, plant breeder can take in account the endophyte of the host for breeding for pathogen resistance. This will enable better crop plants from health and productivity point of view.

It is evident from the literature surveys presented in the chapters that future of endophytes research is bright as demand for pharmaceutical products and agricultural produce is increasing day by day with ever-increasing population. Use of

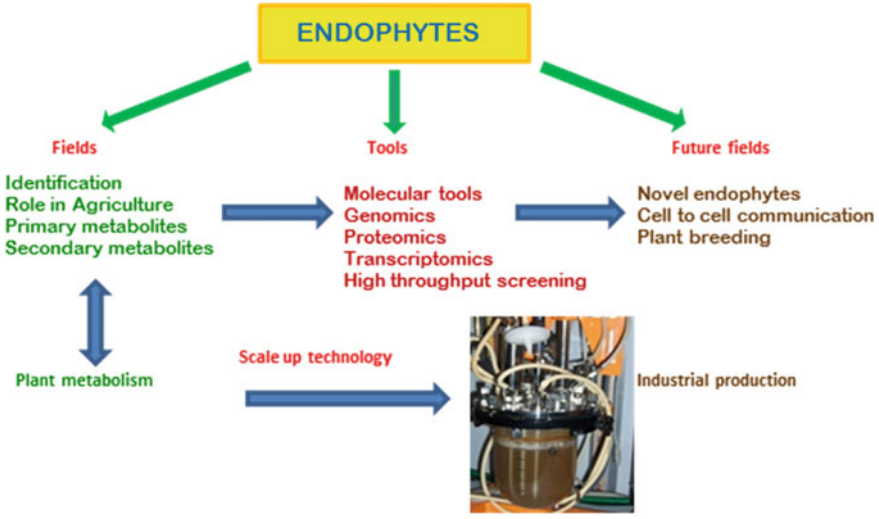

Fig. 13.1 Schematic presentation of summary of research on endophytes applications and use of new technology along for the development of industrial level production system. Scale up technology for the production of useful metabolites from plant cells is already standardized and can effectively used for the production of metabolites from endophytes

multiple technologies will produce quick results to select target compounds for human welfare. The book will be useful for agriculturists, biotechnologists and those working in the fields of pharmacy, food and feed industry, and plant breeding.

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