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Chetan Keswani · M. S. Reddy
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# Secondary Metabolites of Plant Growth Promoting Rhizomicroorganisms

## Discovery and Applications

Springer

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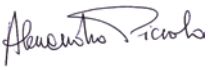
# Foreword

Rhizosphere biology is approaching a century of investigations wherein plant growth promoting rhizomicroorganisms (PGPRs) have attracted special attention for their beneficial traits. Considering the priorities of food security and enhancing the productivity, profitability, and sustainable rural livelihoods at farm level, developing new order of farm inputs has become imperative. Strategies for the management of phytopathogens in the modern systems cannot be a single approach but a multiple of promising disease management strategies. The prospective use of PGPR especially those that producing antimicrobial metabolites against phytopathogens could be a wise choice for the management of soil-borne diseases of crop plants. Currently, we are far away of being able to understand and exploit the full potential of PGPR as an effective disease management strategy at field scale. PGPR produces a wide array of secondary metabolites such as siderophores, antibiotics, volatile metabolites, and other allelochemicals. In this perspective, bioinputs either directly in the form of microbes or their by-products are gaining tremendous momentum. The global market for biopesticides was valued at $1,796.56 million in 2013 and is expected to reach $4,369.88 million by 2019, growing at a CAGR of 16.0% from 2013 to 2019. The PGPR industry is just coming out of its infancy. Its potential is being tested, realized, and used. The antimicrobial metabolites of the PGPRs have received much attention in the past few decades. While it is always a challenge to maintain a desirable population of PGPRs in the bioformulations, it is envisaged that the secondary metabolite-based formulations could be the potential alternative for the management of plant diseases. Harnessing the potential of agriculturally important microorganisms could help in providing low-cost and environmentally safe technologies to the farmers especially those who cannot afford expensive technologies. In this context, the volume entitled *Secondary Metabolites of Plant Growth Promoting Rhizomicroorganisms: Discovery and Applications* includes contributions from

vastly experienced, global experts in PGPR research. I congratulate the editors for synchronizing with global authorities on the subject to underline the upcoming challenges and present most viable options for translating commercially viable ideas into easily affordable products and technologies.



June 01, 2018

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# Preface

Recent changes in the pattern of agricultural practices from the use of hazardous pesticides to natural (organic) cultivation have brought into focus the use of agriculturally important microorganisms for carrying out analogous functions. The reputation of plant growth promoting rhizomicroorganisms (PGPRs) is due to their antagonistic mechanisms against most of the fungal and bacterial phytopathogens. The biocontrol potential of agriculturally important microorganisms is mostly attributed to their bioactive secondary metabolites. However, low shelf life of many potential agriculturally important microorganisms impairs their use in agriculture and adoption by farmers. The focal theme of this book is to highlight the potential of employing biosynthesized secondary metabolites (SMs) from agriculturally important microorganisms for the management of notorious phytopathogens, as a substitute of the currently available whole organism formulations and also as alternatives to hazardous synthetic pesticides. Accordingly, we have incorporated a comprehensive rundown of sections which particularly examine the SMs synthesized, secreted, and induced by various agriculturally important microorganisms and their applications in agriculture.

Part I includes discussion on biosynthesized antimicrobial secondary metabolites from fungal biocontrol agents. This part will cover the various issues such as development of formulation of secondary metabolites, genomic basis of metabolic diversity, metabolomic profiling of fungal biocontrol agents, and novel classes of antimicrobial peptides. This part also covers the role of these secondary metabolites in antagonist-host interaction and application of biosynthesized antimicrobial secondary metabolites for the management of plant diseases.

Part II discusses the biosynthesized secondary metabolites from bacterial PGPRs, strain-dependent effects on plant metabolome profile, bioprospecting various isolates of bacterial PGPRs for potential secondary metabolites, and nontarget effects of PGPR on microbial community structure and functions.

Part III encompasses the synthesis of antimicrobial secondary metabolites from beneficial endophytes, bioprospecting medicinal and aromatic hosts, and effect of endophytic SMs on plants under biotic and abiotic stress conditions.

The most distinguishing feature of this book is that it discusses in detail the most recently conceived idea of employing biosynthesized SMs from agriculturally important microorganisms in crop protection as a potential alternative to hazardous pesticides and relatively slow-performing biocontrol agents.

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Auburn, AL, USA — M. S. Reddy
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# Contents

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**Harikesh Bahadur Singh** is a Professor in the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, India. Professor Singh has been decorated with several national and international awards for his key role in popularizing organic farming and translating agriculturally important microorganisms from lab to land. To his credit, he has 20 US patents which have been successfully transferred for commercial production of biopesticides to several industrial houses in India.

**Chetan Keswani** is a Postdoctoral Fellow in the Department of Biochemistry, Institute of Science, Banaras Hindu University, India. He has keen interest in regulatory and commercialization issues of agriculturally important microorganisms. He is an Elected Fellow of the Linnean Society of London, UK. He received the Best Ph.D. Thesis Award from the Uttar Pradesh Academy of Agricultural Sciences, India, in 2015. He is an Editorial Board Member of several reputed agricultural microbiology journals.

**M. S. Reddy** is a Professor in the Department of Entomology & Plant Pathology at Auburn University, USA. He is the Founder Chairman of the Asian PGPR Society for Sustainable Agriculture. He is a recipient of many prestigious awards from the USA, Canada, Saudi Arabia, Indonesia, the Philippines, China, India, etc. He has been successful in generating several millions of dollars funding from federal, state, private, and international agencies for his research to commercialize biofertilizers and biofungicides. Currently, he is an Entrepreneur and Consultant for several national and international agencies. He has authored over 300 publications.

**Estibaliz Sansinenea** joined the Chemistry faculty in the Benemérita Universidad Autónoma de Puebla, Facultad de Ciencias Químicas, Puebla, Pue. México, in 2012. Her current research interest is "secondary metabolites from microorganisms" with special emphasis on *Bacillus* spp. She has published 31 research articles and 4 book chapters and edited 1 book.

**Carlos García-Estrada** received his D. Phil. from the University of León (Spain) in 2003 after completing his training at the University of Mississippi, MS (USA). In 2004, he received the Extraordinary Award in a Doctorate and started his postdoctoral studies at Instituto de Biotecnología de León (INBIOTEC). Since 2011, he is the Head of the Biopharma and Biomedicine Area of INBIOTEC and Adjunct Professor at the University of León. He has published more than 50 scientific articles and 13 book chapters and has edited 2 books.

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# Part I

# Fungal PGPRs

# 1 Bioactive Secondary Metabolites of Basidiomycetes and Its Potential for Agricultural Plant Growth Promotion

Irina Sidorova and Elena Voronina

## 1.1 Introduction

Fungi are a major source of bioactive natural compounds with high chemical structure diversity. Tens of thousands of natural products are derived from fungi for medicinal, nutritional, agricultural, and industrial application (Bérdy 2012; Keswani et al. 2014). The ability to produce secondary metabolites (SM) is essential for the most of fungi, but a comparative small number of commonly applied in biotechnology producers may reflect not the highest activity, but rather the large-scale culture simplicity. Further progress in screening for novel compounds and novel producers is necessary in the light of both target organisms acquired resistance and the perspective of the more effective and lesser expensive treatment recognizing (Keswani 2015; Singh et al. 2016).

The sustainable agriculture is a hot spot of modern biology for environmental hazard created by agrochemicals is well-known. The role of fungi in plant growth promotion encompasses nutrient facilitation, plant pathogen and pest biocontrol, and many other effects discussed in a number of current reviews (Mishra et al. 2015; Singh et al. 2017; Varma et al. 2017). Fungal bioactive SMs contribute to plant fitness prominently, thus having a strong agricultural potential (Loiseleur 2017), but different fungal groups are studied in this field rather irregularly.

Fungal class *Agaricomycetes* of *Basidiomycota* phylum (Hibbett et al. 2014), further addressed as basidiomycetes, or basidial fungi, represents a source for perspective novel producers and novel compounds evidently underestimated in agriculture. Wide array of bioactive SM derived from basidiomycetes will be discussed below with focus on its agricultural applications. Some metabolites considered as primary (e.g., fatty acids) with particular SM properties will be mentioned too.

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## 1.2 Bioactive Secondary Metabolites of Basidiomycetes: An Overview

In the environment basidial fungi meet a number of foes and competitors. Basidiomycete mycelia inhabit the soil, litter, and wood and are able to occupy a range of different substrates existing in multicomponent communities, often under the press of nutrient limitation. Multiple groups of living organisms, such as fungicolous fungi and bacteria, fungivorous insects, mites, nematodes, and others, feed upon basidiomycetes. Over the many million year-long history, basidial fungi have evolved protective mechanisms including chemical defense. Bioactive SMs act as a weapon against competing organisms, occupying and consuming the same substrate, and as signal molecules for inter- and intraspecies communication (Spiteller 2008). These crucial functions facilitated SM production by both basidiomycete fruit bodies and mycelia. A number of SM possess a strong potential for medicine and sustainable agriculture, and some compounds are already exploited at global scale.

### 1.2.1 The Brief Historical Background

Basidiomycete bioactive SMs were regularly studied since the 1940s. The research was initiated by the mycology laboratories in New York and Oxford. By the early 1950s, about 2000 basidiomycete species were explored, and many of them proved to be active against bacteria and/or fungi; the results are summarized in Florey et al. (1949). Two perspective compounds were detected: pleuromutilin from *Clitopilus scyphoides* and *C. passeckerianus* active against Gram-positive bacteria, further incorporated in veterinary and, later, human therapy (Kavanagh et al. 1951; Novak and Shlaes 2010), and antifungal biformin from *Trichaptum biforme*, the first of basidiomycete polyacetylenes discovered (Zjawiony 2004).

In the 1950s the bioactive compound screening switched to actinomycetes as producers of novel promising antibiotics (streptomycin, chloramphenicol, tetracyclines, polyenes), thus retarding studies in the field of fungi (Bérdy 2005). However there was a revival of scientific interest toward basidiomycete active compounds, and the numbers given below can rather expressively indicate that. Only 300 basidiomycete antibiotics (23% of all fungi-derived) were revealed in 1940–1974, but in 1975–2000 its number grew up to 1500 (46%) and in 2001–2010 – to 1800 (61%) (Bérdy 2012). Undoubtedly, this "basidiomycete boom" was induced by many advances in medicinal fungi, the group embracing mostly basidial ones (De Silva et al. 2013; Chen and Liu 2017; Gargano et al. 2017).

Antifungal natural $\beta$-methoxyacrylic acid derivatives, strobilurins and oudemansins, produced by different basidiomycetes were originally isolated from *Strobilurus tenacellus* (Anke et al. 1977). These SMs became lead compounds for chemical synthesis of widely applied agricultural and industrial fungicides (Clough 2000). The achievements of basidiomycete bioactive SM research are reviewed in Anke

(1989), Lorenzen and Anke (1998), Schüffler and Anke (2009), De Silva et al. (2013), and Chen and Liu (2017).

### 1.2.2 Chemical Structure of Basidiomycete Bioactive Compounds and Producing Species

There are about 90,000 natural bioactive metabolites known by present; 15,600 (nearly 17%) are fungi-derived. Fungi are the champions among all microorganisms producing 45.8% of all microbial-derived SM. Basidiomycetes' contribution is notable, namely, 3600 (23%) high diverse bioactive compounds (Bérdy 2012). The most comprehensive recent review on the topic is authored by Chen and Liu (2017).

Contrary to primary metabolites, SMs are individually produced compounds, often specific for a single species or a limited species group (Turner and Aldridge 1983). By the way, polyacetylenes in fungi are detected only in some basidiomycetes (Hanson 2008). At the same time, some SMs are produced by members of different families or even orders (Table 1.1). Besides, SM diversity is enlarged by multiple chemical derivatives produced by the same species or a number of related ones. The modifications differ in some functional groups, activity, and other traits. Some examples are $\beta$-methoxyacrylate derivatives such as strobilurins A–F; oudemansins A, B, and X; 9-methoxystrobilurin K and L; etc. (Zakharychev and Kovalenko 1998) and numerous sesquiterpenoids of *Granulobasidium vellereum*, viz., illudane, illudalane, and protoilludane (Kokubun et al. 2016).

Carbon backbone in SM consists from glucose-derived C entering the biosynthesis via several routes. Despite enormous diversity, SMs are created through a rather few biogenetic pathways (Turner and Aldridge 1983). The similar in its early steps polyketide synthesis and fatty acids and terpene synthesis involves acetyl-CoA. Other biogenetic pathways, not based on acetate, include nonribosomal peptide synthesis and shikimate pathway. Contrary to plant and actinomycete compounds, the incorporation of intact glucose C backbone is very rare in fungal SM. Some compounds are derived from amino acids, through trioses and pyruvate, or through shikimic acid.

Terpene biosynthesis is the most important SM pathway in fungi and plants (Turner and Aldridge 1983). Terpene chemical structure is derived from isoprene C5 units linked together "head to tail." Isopentenyl pyrophosphate, the original chemical unit, derives from acetate through mevalonate. Isopentenyl pyrophosphate and its derivative dimethylallyl pyrophosphate condensation results in creation of different terpenes. According to C numbers per molecule, basidiomycete terpenes are classified into monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes, and steroids (C30). Basidial fungi produce a range of different terpenoids – terpenes containing additional functional groups – and the sesquiterpenoids are the most abundant. These SMs are distributed in nearly all basidiomycete orders examined (Hanson 2008; Schmidt-Dannert 2014). One of the most promising diterpenoids is antibacterial pleuromutilin (Kavanagh et al. 1951; Schüffler and Anke 2014). Triterpenoids are scarce in basidiomycetes, but

**Table 1.1** Secondary metabolites with antifungal activities detected in basidiomycetes

| Groups of chemical compounds | Bioactive secondary metabolites | Producer fungal species[a] | Producer species position in taxonomy (family, order)[a] | References |
|---|---|---|---|---|
| Terpenoids | Enokipodins A–D | *Flammulina velutipes* (Curtis) Singer | *Physalacriaceae*, *Agaricales* | Ishikawa et al. (2001) |
| Sesquiterpenoids | 1(10),4-Germacradiene-2,6,12-triol | *Hohenbuehelia leightonii* (Berk.) Watling ex Courtec. et Roux | *Pleurotaceae*, *Agaricales* | Eilbert et al. (2000) |
| | Hyphodontal | *Hyphodontia* sp. | *Schizoporaceae*, *Hymenochaetales* | Erkel et al. (1994) |
| | Hyphodontal | *Mycoacia uda* (Fr.) Donk | *Meruliaceae*, *Polyporales* | Schüffler et al. (2012) |
| | Marasmene B, marasmals B, C | *Marasmius* sp. | *Marasmiaceae*, *Agaricales* | Liermann et al. (2012) |
| | Melleolides | *Armillaria mellea* (Vahl.) P.Kumm. | *Physalacriaceae*, *Agaricales* | Bohnert et al. (2014) |
| | Nebularic acids A, B, nebularilactones A, D | *Clitocybe nebularis* (Batsch) P.Kumm. | *Tricholomataceae*, *Agaricales* | Wangun et al. (2006) |
| | Penarines A–F | *Hygrophorus penarius* Fr. | *Hygrophoraceae*, *Agaricales* | Otto et al. (2014) |
| | Rufuslactone | *Lactarius rufus* (Scop.) Fr. | *Russulaceae*, *Russulales* | Luo et al. (2005b) |
| | Sterelactones | *Stereum* sp. | *Stereaceae*, *Russulales* | Opatz et al. (2008) |
| | Udalactaranes A, B | *Mycoacia uda* (Fr.) Donk | *Meruliaceae*, *Polyporales* | Schüffler et al. (2012) |
| Diterpenoids | Heptemerones A–G | *Coprinellus heptemerus* (M. Lange et A.H. Sm.) Vilgalys, Hopple et Jacq. Johnson | *Psathyrellaceae*, *Agaricales* | Kettering et al. (2005) |
| | Herical | *Hericium coralloides* (Scop.)Pers., *H. abietis* (Weir ex Hubert) K.A. Harrison | *Hericiaceae*, *Russulales* | Anke et al. (2002) |
| | Scabronines G, H | *Sarcodon scabrosus* (Fr.) P. Karst. | *Bankeraceae*, *Thelephorales* | Ma et al. (2010) |
| | Tintinnadiol | *Mycena tintinnabulum* (Paulet) Quél. | *Mycenaceae*, *Agaricales* | Engler et al. (1998b) |
| Sesterterpenoids | Aleurodiscal | *Aleurodiscus mirabilis* (Berk. et M.A.Curtis) Parmasto | *Stereaceae*, *Russulales* | Lauer et al. (1989) |

| Groups of chemical compounds | Bioactive secondary metabolites | Producer fungal species[a] | Producer species position in taxonomy (family, order)[a] | References |
|---|---|---|---|---|
| Triterpenoids | Favolon | *Favolaschia calocera* R. Heim, *Favolaschia* sp. | *Mycenaceae*, *Agaricales* | Anke et al. (1995), Chepkirui et al. (2016) |
| | Favolon B | *Mycena* sp. | *Mycenaceae*, *Agaricales* | Aqueveque et al. (2005) |
| Steroids | Laschiatrion | *Favolaschia* sp. | *Mycenaceae*, *Agaricales* | Anke et al. (2004) |
| Alkenes | Scorodonin | *Mycetinis scorodonius* (Fr.) A.W.Wilson et Desjardin | *Omphalotaceae*, *Agaricales* | Anke et al. (1980) |
| Polyacetylenes | Biformin | *Trichaptum biforme* (Fr.) Ryvarden | *Incertae sedis*, *Hymenochaetales* | Zjawiony (2004) |
| | 1-Hydroxy-2-nonin-4-on | *Ischnoderma benzoinum* (Wahlenb.) P. Karst. | *Fomitopsidaceae*, *Polyporales* | Anke et al. (1982) |
| Prenylphenols | Grifolin | *Polypus dispansus* (Lloyd) Audet | *Incertae sedis*, *Russulales* | Luo et al. (2005a) |
| Benzoquinone derivatives | Mycenon | *Mycena* sp. | *Mycenaceae*, *Agaricales* | Hautzel et al. (1990) |
| Isocoumarin derivatives | Gymnopalynes A, B | *Gymnopus* sp. | *Omphalotaceae*, *Agaricales* | Thongbai et al. (2013) |
| Cyclopentenone derivatives | Hygrophorones A–G | *Hygrophorus* spp. | *Hygrophoraceae*, *Agaricales* | Lũbken et al. (2004) |

(continued)

**Table 1.1** (continued)

| Groups of chemical compounds | Bioactive secondary metabolites | Producer fungal species[a] | Producer species position in taxonomy (family, order)[a] | References |
|---|---|---|---|---|
| Methoxyacrylate derivatives | Oudemansin | *Mucidula mucida* (Schrad.) Pat. | *Physalacriaceae*, *Agaricales* | Anke et al. (1979) |
| | Oudemansin A | *Gymnopus vernus* (Ryman) Antonín et Noordel. | *Omphalotaceae*, *Agaricales* | Engler et al. (1998a) |
| | Oudemansin X | *Hymenopellis radicata* (Relhan) R.H. Petersen | *Physalacriaceae*, *Agaricales* | Anke et al. (1990) |
| | 9-Oxostrobilurins A, G, K, and I | *Favolaschia calocera* R. Heim | *Mycenaceae*, *Agaricales* | Chepkirui et al. (2016) |
| | Strobilurins | *Gymnopus vernus* (Ryman) Antonín et Noordel. | *Omphalotaceae*, *Agaricales* | Engler et al. (1998a) |
| | Strobilurins | *Strobilurus tenacellus* (Pers.) Singer | *Physalacriaceae*, *Agaricales* | Anke et al. (1977) |
| Peptides | Alveolarin | *Neofavolus alveolaris* (DC.) Sotome et T. Hatt. | *Polyporaceae*, *Polyporales* | Wang and Ng (2004b) |
| | Eryngin | *Pleurotus eryngii* (DC.) Quél. | *Pleurotaceae*, *Agaricales* | Wang and Ng (2004a) |
| | Pleurostrin | *Pleurotus ostreatus* (Jacq.) P. Kumm. | *Pleurotaceae*, *Agaricales* | Chu et al. (2005) |

[a]The fungal species names and their position in taxonomy are provided according to the Index Fungorum database (http://www.indexfungorum.org/, accessed on 2018/01/28)

antifungals favolons were detected in *Favolaschia* and *Mycena* species (Anke et al. 1995; Aqueveque et al. 2005) (Table 1.1).

Polyketide and fatty acid metabolic routes are homologous both in the process of chain elongation via the common pool of simple precursors and in the synthases' types (Turner and Aldridge 1983). Polyketides derive from repetitive decarboxylative condensation of the primer (acetyl-CoA) with several units of malonyl-CoA. The products created are instable and stabilize by aromatization with one or several ring buildings. Polyketide formation is arranged by polyfunctional enzymes, polyketide synthases (PKS). Basidiomycete polyketides include antifungals isolated from *Hygrophorus* species – hygrophorones (Lũbken et al. 2004) and chrysotriones (Gilardoni et al. 2007) and numerous methoxyacrylate derivatives (Clough 1993). The fatty acid biosynthesis differs in reduction accompanying repetitive condensation of acetyl-CoA with malonyl-CoA through the action of acyl carrier protein. Basidiomycetes are rich in fatty acids and their derivatives, in particular, bioactive ones (Stadler et al. 1994b). Polyacetylenes – linear compounds with double or triple conjugated bonds – are fatty acid derivatives known only from higher plants and basidial fungi (Hanson 2008). Thorough examination of 300 basidiomycete species revealed that 10% of them are able to produce significant amounts of polyacetylenes under culture conditions. Many compounds demonstrated biological activity, such as the first discovered biformin (Zjawiony 2004).

The lesser significant for fungal secondary metabolism shikimate pathway starts with the condensation of phosphoenolpyruvate and erythrose-4-phosphate. These precursors are derived from glucose conversion via glycolysis and pentose phosphate pathway. Further metabolic route involves shikimate and chorismate. This pathway is typical for plants and fungi, but is absent in animals (Hanson 2008). Basidiomycete SMs derived from it include terphenyls of *Thelephora* spp. and *Sarcodon* spp. (*Thelephoraceae*) (Schüffler and Anke 2009) and pulvinic acids of *Boletales* (Turner and Aldridge 1983).

The last but not least SM pathway is nonribosomal peptide synthesis (Finking and Marachiel 2004). Nonribosomal peptides are originated through the action of specialized nonribosomal peptide synthetases (NPRS) consisting of a series of functional units. They are able to bind amino acids, to activate them in the form of thioesters, and to join them to elongated peptide chain. This way results in a colossal diversity of peptides derived. Ribosomal synthesis allows operation with standard array of *L*-amino acids only, but nonribosomal peptides can contain unusual structural units, such as non-proteinogenic amino acids (*D*-isomers) and standard amino acids modified by methylation, hydroxylation, and glycosylation. The research of basidiomycete bioactive peptides was initiated later than of other SM groups. Nematicidal cyclic dodecapeptide omphalotin was the first revealed (Mayer et al. 1997). By present several effective antifungal peptides have been discovered, including eryngin (Wang and Ng 2004a), alveolarin (Wang and Ng 2004b), and pleurostrin (Chu et al. 2005).

Nearly all basidiomycete taxa produce bioactive SM, but producing species numbers are distributed unevenly for several reasons. A range of species, especially, symbiotrophs, are recalcitrant to isolation and management in axenic culture, thus

impeding their involvement into biotechnology processes. Many fungal groups were examined for SM only at the fruitbody stages with mycelial phase remained totally unexplored. Basidiomycetes are an extremely large group, and biological activity was examined in relatively small proportion of species. Regular fungal surveys are only starting in many regions, and one can expect new undescribed species detection. Moreover, the system of *Basidiomycota* recently underwent drastic changes (Hibbett et al. 2014), thus giving rise to misinterpretation of identity and taxonomy position of SM-producing species studied in the previous years.

However, the analysis of bioactive SM producer distribution within taxa is a promising challenge both for novel compound screening and basidiomycete chemotaxonomy investigation. Ranadive et al. (2013) analyzed data on antibacterial and antifungal activity of 281 basidiomycete species of 122 genera and 45 families and tried to rank these taxa according to detected producers' numbers. Family *Polyporaceae* (64 species active) was in lead; a "bad second" and so on were *Agaricaceae*, *Hymenochaetaceae*, and *Tricholomataceae* (22, 21, and 16 species, respectively). Unfortunately, the sample analyzed was quite small compared to known number of bioactive SMs – 3600 (Bérdy 2012). In addition, families compared are sharply unequal in their volume, and for future screening, plotting this ranking is disadvantageous for perspective species from small families. A similar research concerned analysis of bioactive compounds' producers from all groups of biota assembled as a tree of life (Zhu et al. 2011). Here basidiomycetes obtained rather lowly position, but orders *Agaricales* and *Polyporales* were pointed out as promising groups. So, "the size matters" again: the larger taxa got the more privileges without any attention to taxonomic divisions (here, the families). The examples given demonstrate the perspectives of bioactive SM producers' taxonomic analysis for novel compound screening, but this approach requires activity target detailing, representative samples of species arranging, and standardization of taxonomic structure within the sample.

### 1.2.3 Biological Activity of Basidiomycete Secondary Metabolites

Reviewing the complete array of basidiomycete chemical potential is beyond the scope of this chapter. For it is aimed to discuss basidial fungi perspectives in agriculture, the SMs outlined below are either antifungals (in particular, inhibiting plant pathogenic fungi) or nematicidal, insecticidal, and acaricidal compounds affecting plant pests.

However, discussing the bioactivity, it is necessary to mention the antibacterial pleuromutilin illustrating biotechnological and bioengineering potential of basidial SMs. The tricyclic diterpene was originally isolated from the cultures of *Clitopilus scyphoides* and *C. passeckerianus*, and its natural derivatives were detected too (Kavanagh et al. 1951; Knauseder and Brandl 1976; Hartley et al. 2009). The antibiotic is a protein synthesis inhibitor active against Gram-positive bacteria, including methicillin-resistant staphylococci and mycoplasmas (Poulsen et al.

2001). Semisynthetic analogues were implicated in veterinary since 1979 (tiamulin) and entered human medicine in 2007 (retapamulin) as a treatment for superficial skin infections caused by *Staphylococcus aureus* and *Streptococcus pyogenes* (Daum et al. 2007). Novel pleuromutilin analogues were synthesized for wider medicinal application (Yang and Kean 2008; Tang et al. 2012). All the enzymes contributing to pleuromutilin biosynthesis were characterized, the metabolic pathway was proposed, and the cluster of seven genes operating the process was identified. The biotransformation was carried out in the heterologous host (*Aspergillus oryzae*), thus allowing 2106% increase in the antibiotic production (Bailey et al. 2016; Yamane et al. 2017). These achievements can surely encourage the researchers in the laborious work with basidiomycete SM-producing species.

Various antifungal SMs derived from basidiomycetes are summarized in Table 1.1. It should be noted that activity was often revealed in preliminary experiments, while the main goal was the chemical structure recognition. Terpenoids, mainly sesquiterpene derivatives, are produced by diverse fungal taxa with a slight predominance of some *Agaricales* families. Melleolides, protoilludene alcohols esterified with orsellinic acid, proved to be both active against micromycetes (activity is based on the double bond in the protoilludene moiety) and cytotoxic compounds. It is noteworthy that antifungal melleolides interfere with metabolite-related gene transcription in their targets (Bohnert et al. 2014). Sesquiterpenoids recognized by present are not antifungals only, but can affect nematodes, mites, and multiple insects, in the latter case exhibiting both insecticidal and deterrent activities. Cheimonophyllons A–E and cheimonophyllal, bisabolane-type sesquiterpenoids, isolated from *Cheimonophyllum candidissimum*, showed weak antifungal and antibacterial activities, but were toxic for nematodes (Stadler et al. 1994a) (Table 1.2). Granulolactone and granulodione (derivatives from illudalane and 15-norilludane, respectively) isolated from *Granulobasidium vellereum* exhibit acaricidal and insecticidal activities (Kokubun et al. 2016). Some basidiomycete sesquiterpenoids are known as direct plant growth promoters. Protoilludene sesquiterpenes from *Lactarius repraesentaneus*, repraesentins A–C, stimulated radicle elongation in the lettuce seedlings (Hirota et al. 2003).

Diterpenoides scabronines G and H have an ability to affect in low concentrations plant pathogenic fungi and, to a lesser extent, bacteria (Ma et al. 2010). *Mycena tintinnabulum* growing on the nutritional medium and on wood possesses a complex of antifungals, comprising strobilurins and a new diterpenoid tintinnadiol. The latter was detected only in fruit bodies and exhibited cytotoxicity (Engler et al. 1998b). Diterpenoides heptemerones A–G were derived from *Coprinellus heptemerus* culture while screening for antagonists to deleterious rice pathogen *Magnaporthe grisea*. These compounds inhibited pathogen spore germination, but not affected the mycelial growth. Four heptemerones showed plant protective activity against pyriculariosis in the experiment with leaf segments. SM had a wide range of action, inhibiting yeasts and bacterial growth and demonstrating a strong cytotoxical effect. Phytotoxicity, however, was detected for heptemerone D only (Kettering et al. 2005).

Antifungal sesterterpenoids are rare within basidiomycetes. Aleurodiscal derived from *Aleurodiscus mirabilis* causes abnormal apical branching in *Mucor miehei*

hyphae in very low concentrations (Lauer et al. 1989) (Table 1.1). Chrysotriones A and B, 2-acylcyclopentene-1,3-dione derivatives, were detected in *Hygrophorus chrysodon* fruit bodies (Gilardoni et al. 2007). Preliminary data pointed at activity against widespread plant pathogen *Fusarium verticillioides*. Chrysotriones were suggested to protect their producer's fruit bodies against fungicolous fungi. Antifungal *β*-methoxyacrylic acid derivatives, strobilurins and oudemansins, are basidiomycete-derived active compounds most widely applied in agriculture by present and will be discussed in the second subchapter.

Screening for the novel natural nematicidal SM active against *Meloidogyne incognita* revealed the cyclic peptide omphalotin in the mycelium of *Omphalotus olearius*, known as producer of sesquiterpenoids illuidines M and S with high antimicrobial activity and cytotoxicity (Table 1.2). Omphalotin exhibits remarkable, outmatching the commercial ivermectin, activity against the pathogenic nematode *M. incognita*, but affects the saprobic species *Caenorhabditis elegans* far lesser and has no antimicrobial and phytotoxic properties (Mayer et al. 1997). Subsequently four new omphalotin variations (E–I) were revealed and recognized as cyclic dodecapeptides (Liermann et al. 2009). Omphalotins are promising bioactive SMs with highly selective action against nematodes.

Peptides and proteins with molecular weight 7–28 kDa were isolated from fruit bodies of some basidiomycete species (*Pleurotus eryngii*, *P. ostreatus*, *Ganoderma lucidum*, *Neofavolus alveolaris*, etc.). These compounds inhibit plant pathogenic fungi *Botrytis cinerea*, *Fusarium oxysporum*, *Mycosphaerella arachidicola*, and *Physalospora pyricola* growth by mechanism not elucidated yet (Wang and Ng 2004a, b, 2006; Chu et al. 2005). Protease-inhibiting proteins were detected in *Clitocybe nebularis* (Avanzo et al. 2009), *Macrolepiota procera* (Sabotič et al. 2009), and some other species. These SMs act both as regulators and protectors; insecticidal activity of cnispin against the model dipteran *Drosophila melanogaster* was demonstrated (Avanzo et al. 2009). The current opinion on fungal toxic proteins, e.g., mycospin and mycocypin families, and perspectives of their research and agricultural application are outlined in Sabotič et al. (2016).

## 1.3 Potential and Application of Basidiomycete Bioactive Secondary Metabolites for Agricultural Plant Growth Promotion

Different aspects of sustainable agriculture are now in the focus of research because of a global urgent need to create an alternative for toxic, expensive, and ecologically non-friendly agrochemicals. It is widely acknowledged that even registered commercial "-cides" have multiple side effects and can be harmful for nontarget beneficial living organisms. Thus biocontrol method implying natural or closely related synthetic bioactive compounds for plant growth promotion could be considered far more preferable. Various organisms can contribute in many ways to plant growth promotion, and the most popular and well recognized are bacteria, particularly rhizobial and soil-borne microfungi, predominantly of ascomycete

**Table 1.2** Secondary metabolites with nematicidal activities detected in basidiomycetes

| Groups of chemical compounds | Bioactive compounds | Producer fungal species[a] | Producer species position in taxonomy (family, order)[a] |
|---|---|---|---|
| Terpenoids | Illinitone A | *Limacella illinita* (Fr.) Maire | *Amanitaceae*, *Agaricales* |
| Monoterpenes | 1,2-Dihydroxymintlactone | *Cheimonophyllum candidissimum* (Sacc.) Singer | *Cyphellaceae*, *Agaricales* |
| Sesquiterpenes and derivatives | Cheimonophyllal cheimonophyllons A, B, C, D, and E | *Cheimonophyllum candidissimum* (Sacc.) Singer | *Cyphellaceae*, *Agaricales* |
| | Cheimonophyllon E | *Pleurotus eryngii* (DC.) Quél. | *Pleurotaceae*, *Agaricales* |
| | 2*β*,13-Dihydroxyledol | *Dichomitus squalens* (P. Karst.) D.A. Reid | *Polyporaceae*, *Polyporales* |
| | Isovelleral | *Lactarius vellereus* (Fr.) Fr. | *Russulaceae*, *Russulales* |
| | Isovelleral | *Russula cuprea* J.E. Lange | *Russulaceae*, *Russulales* |
| | Lactarorufins A and B, furantriol | *Lactarius aurantiacus* (Pers.) Gray | *Russulaceae*, *Russulales* |
| | Marasmic acid | *Lachnella villosa* (Pers.) Donk, *Lachnella* sp. 541 | *Niaceae*, *Agaricales* |
| | Marasmic acid | *Strobilurus conigenus* (Pers.) Gulden | *Physalacriaceae*, *Agaricales* |
| | Marasmic acid | *Peniophora laeta* (Fr.) Donk | *Peniophoraceae*, *Russulales* |
| | Stereumins A, B, C, D, and E | *Stereum* sp. CCTCC AF 207024 | *Stereaceae*, *Russulales* |
| Simple aromatic compounds | *p*-Anisaldehyde, *p*-anisyl alcohol, 1-(4-methoxyphenyl)-1,2-propanediol, 2-hydroxy-(4′-methoxy)-propiophenone | *Pleurotus pulmonarius* (Fr.) Quél. | *Pleurotaceae*, *Agaricales* |
| | 3,5-Dihydroxy-4-(3-methyl-but-2-enyl)-benzene-1,2-dicarbaldehyde, butyl 2,4-dihydroxy-6-methylbenzoate | *Stereum* sp. 8954 | *Stereaceae*, *Russulales* |
| | Methyl 3-*p*-anisoloxypropionate | *Irpex lacteus* (Fr.) Fr. | *Meruliaceae*, *Polyporales* |

(continued)

**Table 1.2** (continued)

| Groups of chemical compounds | Bioactive compounds | Producer fungal species[a] | Producer species position in taxonomy (family, order)[a] |
|---|---|---|---|
| *O*-containing heterocyclic compounds | 4,6-Fimethoxyisobenzofuran-1(3*H*)-one, 5-methylfuran-3-carboxylic acid, 5-hydroxy-3,5-dimethylfuran-2(5*H*)-one, 4,6-dihydroxybenzofuran-3(2*H*)-one, 5-hydroxy-3-(hydroxymethyl)-5-methylfuran-2(5H)-one, 4,6-dihydroxyisobenzofuran-1,3-dione, 3-formyl-2,5-dihydroxybenzylacetate | *Coprinus comatus* (O.F. Müll.) Pers | *Agaricaceae*, *Agaricales* |
| | 7,8,11-Drimanetriol | *Coprinellus xanthothrix* (Romagn.) Vilgalys, Hopple et Jacq. Johnson | *Psathyrellaceae*, *Agaricales* |
| | 5-Hydroxymethyl-furancarbaldehyde | *Pleurotus eryngii* (DC.) Quél. | *Pleurotaceae*, *Agaricales* |
| | 5-Pentyl-2-furaldehyde, 5-(4-pentenyl)-2-furaldehyde | *Irpex lacteus* (Fr.) Fr. | *Meruliaceae*, *Polyporales* |
| Benzoquinone derivatives | Mycenon | *Mycena* sp. | *Mycenaceae*, *Agaricales* |
| *N*-containing heterocyclic compounds | Xanthothone, 2-(1H-pyrrol-1-yl)-ethanol | *Coprinellus xanthothrix* (Romagn.) Vilgalys, Hopple et Jacq. Johnson | *Psathyrellaceae*, *Agaricales* |
| Alkaloids | 2-Aminoquinoline | *Leucopaxillus albissimus* (Peck) Singer | *Tricholomataceae*, *Agaricales* |
| | Phenoxazone | *Calocybe gambosa* (Fr.) Donk | *Lyophyllaceae*, *Agaricales* |
| | Phenoxazone | *Pycnoporus sanguineus* (L.) Murrill | *Polyporaceae*, *Polyporales* |
| Alkynes | 2,4,6-Triacetylenic octane diacid | *Wolfiporia cocos* (F.A. Wolf) Ryvarden and Gilb. | *Polyporaceae*, *Polyporales* |

(continued)

**Table 1.2** (continued)

| Groups of chemical compounds | Bioactive compounds | Producer fungal species[a] | Producer species position in taxonomy (family, order)[a] |
|---|---|---|---|
| Fatty acids[b] | *Trans*-2-decenedioic acid | *Pleurotus ostreatus* (Jacq.) P. Kumm. | *Pleurotaceae*, *Agaricales* |
| | S-coriolic acid, linoleic acid | *Pleurotus pulmonarius* (Fr.) Quél. | *Pleurotaceae*, *Agaricales* |
| | Linoleic acid, oleic acid, palmitic acid | *Hericium coralloides* (Scop.) Pers. | *Hericiaceae*, *Russulales* |
| Peptides | Beauvericin | *Laetiporus sulphureus* (Bull.) Murrill | *Fomitopsidaceae*, *Polyporales* |
| | Omphalotins A, B, C, and D | *Omphalotus olearius* (DC.) Singer | *Omphalotaceae*, *Agaricales* |
| | Phalloidin | *Conocybe apala* (Fr.) Arnolds | *Bolbitiaceae*, *Agaricales* |

Modified from Li and Zhang (2014), references to the original research see in Li and Zhang (2014), Askary and Martinelli (2015)

[a]The fungal species names and their position in taxonomy are provided according to the Index Fungorum database (http://www.indexfungorum.org/, accessed on 2018/01/28)

[b]Fatty acids are traditionally ascribed to primary metabolites, but the compounds outlined here exhibit traits essential for secondary metabolism

affinity (Singh et al. 2017). Basidiomycetes didn't get much attention from the researchers because of the range of obstacles interfere their exploration and application, but there are evidences for the encouraging progress in their SM research both with examples of successful implication in agriculture and industry.

### 1.3.1 Strobilurins, Oudemansins, and Their Derivatives as Biopesticides Protective Against Plant Pathogens

Fungi play an important role in the agriculture as a rich source of plant defensive bioactive compounds. They can be applied as a base for commercial preparations, but more often they act as leads for structural modifications aimed at increasing or changing their activities and target group resistance reduction. Such products share advantages both from biotechnology and chemistry approaches (Loiseleur 2017).

The route "from mushroom to molecule to market" was successfully marched by strobilurin fungicides derived from basidiomycete bioactive SMs (Clough 2000; Balba 2007). The first active compound from this group, strobilurin A, was isolated in 1977 in Germany from *Strobilurus tenacellus* (Anke et al. 1977). Lately oudemansin was generated in the same laboratory (Anke et al. 1979). The compounds possessed high and selective antifungal activity along with low toxicity and no

antibacterial effects. Their chemical structure was rather uncommon for basidiomycete SM; the compounds contain methoxyacrylate moiety in the form of methyl ether or amide linked through carbon atom to the rest of the molecule (Clough 1993).

Now plenty of natural strobilurins' variations are recognized, differing in the structure of aliphatic chain and in the presence/position of functional groups. The strobilurins' mode of action was uncommon as well; they inhibit cell respiration, thus disrupting electron transport at complex III in the mitochondrial membrane (Von Jagow et al. 1986).

Strobilurins, oudemansins, and their numerous modifications are produced by several families of basidial fungi. The producers are *Strobilurus*, *Oudemansiella*, *Hydropus*, *Mucidula*, *Merismodes*, *Favolaschia*, *Mycena* spp., and others (Anke 1995). They are common litter or wood dwellers with wide distribution, reported from all continents. Strobilurin production was observed not only at laboratory media but in the natural environment too (Engler et al. 1998a). The compounds considered to provide effective protection for their producers.

Comparatively simple chemical structure, stable high activity despite significant structural variations, principally new mechanism of action implying the absence of cross resistance in pathogens resistant to registered fungicides, and low toxicity of some strobilurin modifications facilitate chemical analogue synthesis. There was another challenge: to obtain photostable compounds without loss of fungicidal activities, for the natural SMs were subject to rapid light degradation. A bulk body of research articles and several reviews describe strobilurin derivative synthesis (Zakharychev and Kovalenko 1998; Clough 2000). The first synthetic strobilurins were introduced to the market in 1996. Soon they ranked with the most asked-for commercial fungicides at global scale (Balba 2007). Azoxystrobin (Syngenta) is one of the most popular. Nearly all the largest world pesticide-producing companies accomplish fundamental research of strobilurins, and over 70,000 compounds of this group are recognized by now. Synthetic analogues of natural compounds are patented as agricultural and industrial fungicides with wide-range activities, as nematicides, insecticides, and acaricides. Redox reactions in cytochrome system are common for many groups of living organisms, so respiration inhibitors are effective against various pests and pathogens. Many synthetic strobilurin analogues are active not only against the fungi but against insects, mites, and nematodes too (Balba 2007).

One of the key points of azoxystrobin's outstanding commercial success is its ability to destroy both ascomycete and basidiomycete fungi along with oomycete pseudofungi. Nearly all strobilurins are highly effective against downy mildews, rust fungi, powdery mildews, and various blights (alternariosis, cercosporosis, etc.). Azoxystrobin is able to inhibit such co-occurring plant pathogen groups (e.g., downy and powdery mildews of grapevine), which previously required a complex treatment including two or more fungicides. Another significant advantage is strobilurins' high activity against a complex of plant pathogens specialized for a range of crops. By the way, there are compounds with narrow-ranged activity, e.g., for the rice treatment only.

### 1.3.2 Bioactive Metabolites of Ectomycorrhizal Fungi and Its Potential in Sustainable Agriculture

Mycorrhiza is a widely acknowledged beneficial plant-fungus symbiosis, so mycorrhizal fungi represent in many ways promising guild for sustainable agriculture and forestry. There is a large body of literature concerning the multifaceted role of mycorrhiza in plant growth promotion. Most of these functions go beyond the scope of this chapter, and information on them can be found in a range of comprehensive up-to-date reviews (Smith and Read 2008; Varma et al. 2017). The plant protection by mycorrhizas can be based upon several mechanisms (Whipps 2004), and the direct plant pathogens inhibition by fungal-derived bioactive SM is the objective of this subchapter.

Basidiomycetes form several mycorrhizal types with the most important ectomycorrhiza, typical for the majority of tree and shrub species playing key roles in the boreal and temperate biomes (Smith and Read 2008). In vitro studies of ectomycorrhizal (EM) basidiomycete bioactivity and biocontrol potential were popular in 1970–1990s, when the most data on the topic were obtained (Whipps 2004). Unfortunately, recently such type of research became somewhat neglected. A range of EM basidiomycetes, such as species of *Suillus*, *Laccaria*, *Lactarius*, *Pisolithus*, *Rhizopogon*, *Scleroderma*, and *Thelephora*, in vitro exhibited production of active soluble SMs against plant deleterious fungi and pseudofungi. Polyacetylene diatretyne nitrile was the main active compound against *Phytophthora* and *Pythium*, and it was shown that pine roots colonized with its producers turned out to be less vulnerable for pathogen zoospore infection compared to EM with other mycobionts or non-mycorrhizal ones. The axenic culture of *Suillus variegatus* was shown to produce other antifungal SM, volatile isobutanol and isobutyric acid (Curl and Truelove 1986).

*Pisolithus arhizus* (formerly known as *P. tinctorius*), the most widely applied commercial EM agricultural inoculum, is remarkable for producing antibiotics pisolithins A (*p*-hydroxybenzoylformic acid) and B ((R)-(−)-*p*-hydroxymandelic acid). Along with a few related compounds, these SMs were active against the significant number of phytopathogenic fungi both at spore and mycelial phases (Tsantrizos et al. 1991). The fungicidal mechanism suggested is cell turgor disruption. Two synthetic *S* enantiomers of mandelic acid obtained were the most effective in the pathogenic fungal growth arrest (Kope et al. 1991).

The most well-studied EM species with antifungal potential so far are *Laccaria* species and *Paxillus involutus* due to their comparatively easy maintenance under laboratory conditions and common occurrence (Whipps 2004). However, the research of their protective potential and its mechanisms are far from complete. *Paxillus involutus* was shown to induce 47% increase in colonized *Pinus resinosa* seedling resistance to the pine damping-off causative agent *Fusarium oxysporum* via some antifungal compound releasing (Duchesne et al. 1988). Oxalic acid turned out to be one of the compounds contributing to the antifungal effect (Duchesne et al. 1989), but other potential antifungals of *P. involutus* are still obscure. *Laccaria laccata* culture filtrate strongly inhibited spore germination in *Fusarium oxysporum* (Chakravarty and Hwang 1991), but the SM involved was not elucidated yet too.

However, the most possible hypotheses suggested in later studies, the plant production of antifungals induced by mycorrhization (Machón et al. 2009) is not suitable for the case of in vitro fungal activity detected.

A promising bioactive lactarane sesquiterpene rufuslactone was derived from *Lactarius rufus* fruit bodies (Luo et al. 2005b). As antifungal it outmatched the commercial fungicide carbendazim against plant pathogenic *Alternaria* strains, thus suggesting a prospect for analogue synthesis and future application.

It is obvious that EM fungi-derived bioactive compounds should not be disregarded in sustainable agriculture. To facilitate its proper usage in biocontrol ad hoc and as lead compounds, the greatest challenges to be addressed are recognizing the corresponding SM identity and its focused screening and not so easy delimitation of fungal chemical direct antagonistic effect against pathogens from EM-induced plant intrinsic resistance and the general plant performance improvement under natural conditions.

### 1.3.3 Nematicidal Metabolites of Basidiomycetes and Its Potential in Sustainable Agriculture

More than 4000 nematode species are plant pathogenic (predominantly soil-borne root pathogenic). These pests are of extreme economic importance being a cause of at least 12% worldwide food production annual losses (Askary and Martinelli 2015). Provided that a number of registered chemical nematicides affect a range of nontarget organisms and jeopardize soil ecosystems' normal functioning, the environment-friendly biocontrol method should be a promising alternative for the toxic chemical's usage. Fungal-nematode natural antagonism is based on the fungal ability to attack nematodes to prevent mycelium grazing or either to consume nematodes compensating nitrogen limitation. Nematode-preying (and consuming their prey with extracellular enzymes) and nematode-parasitic fungi are known as nematophagous, while nematode toxic (nematicidal) ones exhibit toxicity against nematodes without obligate further utilization of their victims. It is naturally enough to expect nematode toxicity in nematophagous fungi, but the recent studies have shown the presence of nematicidal SM in far wider spectrum of fungi, thus considering the activity against nematodes as a fungal defense strategy (Li and Zhang 2014; Askary and Martinelli 2015; Degenkolb and Vilcinskas 2016a).

Nematode-toxic fungi are numerous, comprising about 280 species of *Ascomycota* and *Basidiomycota* (Li and Zhang 2014), but some nematicidal ascomycetes are phytopathogenic or phytotoxic themselves (Degenkolb and Vilcinskas 2016a). Nematicidal SM are represented in 77 basidiomycete genera with about 160 species lacking plant deleterious effects in reasonable concentrations (Li and Zhang 2014). The most well-known and promising for biocontrol nematicidal basidial fungi SMs are summarized in Table 1.2.

White-rot-causing genus *Pleurotus* is the most well-studied nematophagous basidiomycete group by now, comprising 23 species with nematicidal activity (Li and Zhang 2014). It includes common edible cultivated species such as *P. ostreatus*

and, along with this, is notable for excreting toxins to prey nematodes, such as the first detected SM (E)-2-decenedioic acid. Further compounds followed, and *S*-coriolic and linoleic acids derived from *P. pulmonarius* are considered to be the most potent and promising for application against phytopathogenic nematodes (Degenkolb and Vilcinskas 2016b). Herb-associated *P. eryngii* both with wood-inhabiting non-nematophagous *Cheimonophyllum candidissimum* are producers of nematicidal sesquiterpenoids cheimonophyllons (Table 1.2). It is notable that this *Pleurotus* species possesses an effective antifungal peptide eryngin too, thus representing a promising biocontrol agent for integrated management.

Some terrestrial basidiomycetes are known to be nematophagous too. Besides an ability to damage nematodes mechanically, *Coprinus comatus* was shown to produce seven nematicidal compounds under culture conditions. Two of them, 5-methylfuran-3-carboxylic acid and 5-hydroxy-3,5-dimethylfuran-2 (5H)-one, are highly effective against *Meloidogyne incognita*, the root-knot nematode pathogenic for a range of crops worldwide (Degenkolb and Vilcinskas 2016b).

Non-nematophagous, predominantly wood-inhabiting, basidiomycetes can exhibit notable nematicidal activity too. *Cheimonophyllum candidissimum* produces nontoxic cheimonophyllons for plants which became lead compounds for synthesis. Sesquiterpene dichomitin B from polyporoid *Dichomitus squalens* can be considered as an excellent lead SM with pronounced activity against plant pathogenic nematodes (Degenkolb and Vilcinskas 2016b). Effective and stable nematicidal cyclic dodecapeptides omphalotins from *Omphalotus olearius* mycelium and overmatching the commercial actinomycete-derived preparation ivermectin are discussed earlier. Terpenoid illinitone A derived from terrestrial *Limacella illinita* is considered as a promising agricultural nematicide too, but its activity was shown against the model free-living nematode species *Caenorhabditis elegans*, known for its sensitivity toward diverse SM (Degenkolb and Vilcinskas 2016b).

At present there are no widely applied commercial fungi-derived nematicides comparable to actinomycete-derived ivermectin (Li and Zhang 2014), but a number of basidiomycetes, listed above, have a strong potential for novel nematicide development. The nematode toxicity may be more widespread among basidial fungi than it was previously thought; and the culture collection screening with focus on the species proved bioactive in different ways, such as a popular medicine fungus *Wolfiporia cocos*, is a future challenge for urgent discovery of new active species and probably novel undescribed compounds too.

## 1.4 Screening for Novel Plant-Protective Basidiomycete Bioactive Metabolites

The strobilurins' triumph encourages the screening for novel promising compounds for plant protection. The enormous diversity of basidial fungi themselves and their SM as well provide an inexhaustible "Klondike" for researchers. The regions with mycobiota totally unexplored or surveyed "with half an eye" can be the most perspective in this field. There the biodiversity inventory could be coupled with

assessment of fungal biochemistry potential. The situation has some in common with that at the dawn of novel actinomycete antibiotic search. This bacterial group was nearly unknown in 1950–1960s, and the disclosure of a novel compound suggested a description of a new species as well. Thereby the screening facilitated actinomycete taxonomy studies and biodiversity assessment.

Basidiomycete screening for bioactive SM became regular many years ago. Often the investigations of fruit bodies' chemical composition and products of basidial fungal cultures displaced the bioactivity research. Probably this led to the loss of many interesting and economically valuable natural compounds. SM screening can be accomplished by two different approaches. The first one implies the detection of the bioactive substance with standard test-organism set: bacteria, yeasts, and filamentous fungi. The second is focused upon SM targeting certain groups: plant pathogenic fungi, parasitic nematodes, insect pests, etc. The fungal material for screening procedure can vary too from field samples to axenic cultures, the latter causing a little disadvantage for symbiotrophs and some other fungal groups. Screening protocols usually consider the ecology of potential producers or concentrate at groups with poorly known biosynthetic potential. The latter supports the urge for marine, coprophilous, and stress-tolerant fungi. The publications dealing with the screening for basidiomycetes SM producers are rather numerous, so we can apply only to some typical ones.

Antifungal basidiomycete species active against plant pathogens were revealed in the Yunnan province, China, within the frame of bioactive SM research. The compounds diverse in their chemical structure were detected. One species has produced the bioactive SM grifolin, which was examined in details for its effects on pathogens' spores germination and mycelial growth. The experiments on plant defense were conducted (Liu 2002; Luo et al. 2005a).

Chilean basidiomycete culture collection (148 strains) was screened for antibacterial and antifungal SM producers. Activity was detected in 60% of species studied, and *Agaricales* and *Polyporales* orders proved to be the most promising groups (Aqueveque et al. 2010).

Wood-inhabiting fungi (51 cultures) from eucalypt plantations in Uruguay were examined for antifungal and antibacterial activity against plant pathogens. As a result eight cultures proved to be active (Barneche et al. 2016).

The interesting approach to plant pathogen inhibitor exploration was suggested by Thines et al. (2004). Many plant pathogenic fungi pass several stages during their life cycle, and if these differ significantly from vegetative mycelium, then they are potentially subject to fungitoxic plant protection treatment. In the case of *Magnaporthe grisea*, the stages preliminary to plant infection were examined, and the selective bioactive SMs inhibiting the signal transduction associated with appressoria formation were listed, while SMs inhibiting the pathogen growth within plant tissues or its sporulation were not recognized. The authors consider this approach as a perspective future trend.

## 1.5 Conclusion and Future Research Prospects

Basidiomycete bioactive SMs are undoubted headliners of modern medicine exhibiting enormous numbers of antiviral, antibacterial, antifungal, antitumor, and immunomodulating effects (Gargano et al. 2017). But in the field of plant growth promotion, they undeservedly obtained rather little attention from researchers and are generally underestimated. Basidiomycete-derived antifungals have a great potential for agriculture, and strobilurins have already proved their advantages at global scale. The nematicidal SM should be considered too, not rare being derived from the species considered active against fungi. One of the limitations for basidial fungi exploration and application, their recalcitrance to culture methods, now could be evaded via analogue synthesis lead by natural SM. In the field of plant protection, ectomycorrhizal guild SMs deserve more attention, for they are presumably required for competing with other fungi for root tips. Besides, regretfully artificial experimental conditions can mask perspective results and lead to misinterpretation of data obtained. So, the future research prospects and challenges can be outlined as:

- Regular biodiversity surveys, especially for regions previously ignored, for new fungal species
- Extensive screening for novel bioactive SM using the culture collections available with attention to small previously neglected taxa and, contrary, to species already exhibiting any activity
- Detailed analysis of all kinds of activities, for basidiomycetes tend to possess multiple effects (e.g., the route from medicine toward the agriculture or from fungicides to nematicides), and broadening the range of test objects
- Recognizing the SM-based bioactive effects per se and delimitation those from other forms of antagonism, especially in EM research
- Considering the species- and even strain-level variability of the bioactive effects and advances in the taxonomy to interpret phylogenetic relationship correctly
- Considering the ecology specificity and plasticity of potential producers and seek for close to natural experimental conditions

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# 2 Secondary Metabolites of *Metarhizium* spp. and *Verticillium* spp. and Their Agricultural Applications

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## 2.1 Introduction

The practice of cultivation soil and growing of the crops has been one of the major reasons for the adoption of the civilized lifestyle of humans. The practice of agriculture has always been a source of food production as well as a livelihood means. It is estimated that almost one-third of the total crop yield is lost due to the infestation of crop pests, the infection of the pathogens and the competition from the weeds. With the advent of the synthetic pesticides, the loss of the total crop yield was reduced, and the agricultural productivity was increased as it provided protection to the crops against the pests and the diseases. Although the haphazardous use of these synthetic pesticides has led to serious problems such their persistance in environment, residual effects in the food products and development of resistance in pests (Shelton et al. 2002). Over the past few years, there has been an increased concern in the people about the potential adverse effects which are associated with the imperceptive use of the synthetic pesticides, which has, in turn, led to the urge for development of an alternative method for the control of the crop pests (Keswani et al. 2016; Mishra et al. 2015). In this context, microbial secondary metabolites of the reported entomopathogenic fungi are deemed to be employed as one of the finest alternatives.

In general, all microorganisms produce a variety of compound which are structurally related but are found in the different magnitude relatively and are classified as the primary or the secondary metabolites (Singh et al. 2016, 2017). The primary

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metabolites are the microbial products which are made during the log or exponential phase of the growth and whose synthesis is an integral part of the normal growth process on a microbe. These include the end products and the intermediaries of the anabolic metabolism, which are used by the cell as the building blocks for essential macromolecules (such as amino acids, nucleotides) or are converted to coenzymes (such as vitamins). They also include the resultant products from the catabolic metabolism, which leads to the production of energy and utilization of the substrate and thus ultimately to the growth (Sanchez and Demain 2008). The secondary metabolites, on the other hand, are products of the secondary metabolism which are diverse in nature and don't have a role in the basic life processes. They are not involved in either cell metabolism or in the growth of the microorganism. They are produced at the stationary phase of the microbial growth stage and are the source of the therapeutics, insecticides, drugs, flavours and fragrances (Donadio et al. 2002). The concept of the secondary metabolism was first introduced by Kossel in 1891 (Hartmann 1985; Haslam 1986; Seigler 1998; Turner 1971). The application of the secondary metabolites as the insecticide against the crop pests has emerged to be advantageous to other alternatives as they are biodegradable, non-toxic to nontarget organisms and highly selective and have low resistance development in the target pest population (Deepa et al. 2014; Keswani 2015a, b).

A key pest can be described on the basis of economic injury level (EIL), general equilibrium population (GEP) and damage boundary (DB). Pests whose GEP always lies above EIL are persistent, severly damaging and the spray of the insecticides brings their population below EIL. The estimated annual crop loss in India by insect pests is Rs. 29,240 crores (Dhaliwal and Arora 1996).

Most of the studies on the entomophagous fungi are on *Metarhizium anisopliae* and *Beauveria bassiana* and less on equally important species of commercial importance such as *Verticillium lecanii*, *Paecilomyces fumosoroseus*, *Tolypocladium* spp. and *Hirsutella* spp. These fungi produce an array of secondary metabolites, of which some are restricted to specific genera, while others are more ubiquitous (Keswani et al. 2013). These secondary metabolites originate as a derivative from various intermediaries in primary metabolism. In general, most of the secondary metabolites emerge from the five metabolic sources, viz.:

(i) Amino acids
(ii) The shikimic acid pathway for the biosynthesis of aromatic amino acids
(iii) The polyketide biosynthesis pathway from acetyl coenzyme A (CoA)
(iv) The mevalonic acid pathway from acetyl coenzyme A
(v) Polysaccharides and peptido-polysaccharides (Griffin 1994)

This chapter reviews about the different secondary metabolites secreted by the fungi *Metarhizium* spp. and *Verticillium* spp. that act against the crop's insect pests.

## 2.2 The Hypothesis Suggesting the Role of Secondary Metabolites

There are mainly three hypotheses which suggest the role of the secondary metabolites in organisms which are as follows:

### 2.2.1 The "Waste Product" Hypothesis

The role of secondary metabolites has been rather uncertain and was initially thought to be just the waste materials. The relatively large number and amount of secondary metabolites which are observed in nature and the concept that these compounds have arisen from the "errors" in the primary metabolisms in plants led to the idea that the secondary metabolic compounds originate and accumulate as "waste products". Although taking into consideration their nonmotility and lack of sophisticated immune system, plants have to develop their own defence system against the pathogens and predators along with the systems to lure the motile organisms, for fertilization and dissemination (Luckner 1972, 1990; Mothes 1976; Seigler 1998).

### 2.2.2 The Shunt or Overflow Hypothesis

For some, secondary metabolites are envisaged as the shunt metabolic compounds which are produced in a state of unbalanced growth for reducing the abnormal concentration of the normal cellular constituents. The synthesis of enzymes designed to carry out the secondary metabolism allows the primary metabolic enzymes to continue to function until such time as circumstances are propitious for renewed metabolic activity and growth. However, this could be linked to the depletion of nutrients such as phosphorous or nitrogen (Bu'Lock 1980; Haslam 1986).

### 2.2.3 The Increased Fit Hypothesis

The hypothesis takes into account that many natural products trigger very specific physiological responses in other organisms and in many cases bind to the receptors which have a remarkable complementarity, which means that the natural products may aid in an organism's survival in the absence of an immune system. This fact, in turn, supports the hypothesis that the secondary metabolites increase the fitness of those individuals which possess them and they are favoured in the process of natural selection. The secondary metabolites thus have an important ecological role in the interaction with the environment and act like the communication interface between the plants and its friends and foes in the environment (Harborne 1986; Rosenthal and Janzen, 1979; Swain 1977; Torssell 1997).

Many of those secondary metabolites are fungicidal, bactericidal, repellent or poisonous to insect pests and the herbivores. The flower pigments give attracting colours for insects that help in fertilization or warning colours against the predators. Some of the secondary compounds also perform in signalling pathway as plant hormones (Haslam 1985). In addition to these, many of them are initially meant for defence against herbivores such as insect pests which would soon come up with the metabolic pathways to detoxify and even use these defence compounds.

## 2.3 The Secondary Metabolites of *Metarhizium* Species

### 2.3.1 Destruxins

They are a class of cyclic hexapeptides that were originally isolated from the entomophagous fungus, *Metarhizium anisoplae* (Kodaira 1961a, b, 1962; Roberts 1966, 1969). The discussions of the secondary metabolites of *Metarhizium* start and stop with the destruxins. After their first report as insect toxins (Kodaira 1961a, b), several papers and reviews describing their chemistry and biological activities have been published (Hu and Dong 2015; Liu and Tzeng 2012; Pedras et al. 2002).

They are composed of five amino acid residues and a single α-hydroxycarboxylic acid moiety (Suzuki et al. 1970; Suzuki and Tamura 1972; Pais et al. 1981), whose exact nature differentiates the major destruxins into subclasses A to F. The two of the five amino acids are N-methylated amino acids: N-methyl-L-alanine (replaced by L-alanine in protodestruxin) and N-methyl-L-valine (replaced by L-valine in desmethyldestruxin B and protodestruxin). The remaining three amino acids are β-alanine, L-leucine (e.g. in destruxin A and A1 but is replaced by L-valine in destruxin A2) and L-proline (e.g. in destruxin A and A2 but is replaced by L-pipecolic acid in destruxin A1).

The variable structural residue of destruxins is the α-hydroxycarboxylic acid unit. For example, in destruxin A it is 2-hydroxy-4-pentanoic acid; in destruxin B it is 2-hydroxy-4-methylpentanoic acid; in destruxin E it is 2-hydroxy-4,5-methylpentanoic acid; and in destruxin F it is 2,4-dihydroxy pentanoic acid (Wahlman and Davidson 1993). The destruxin analogues obtained from other fungi include destruxins A4 and A5 and homodestruxin B from an entomophagous fungus *Aschersonia* sp. (Krasnoff et al. 1996); roseocardin and roseotoxin B from a plant pathogenic fungus *Trichothecium roseum* (Springer et al. 1984; Tsunoo et al. 1997); bursaphelocides from a *Mycelia sterilia* (Kawazu et al. 1993); pseudodestruxins A and B from a coprophilous fungus *Nigrosalbulum globosum* (Che et al. 2001) and *Beauveria felina* (Lira et al. 2006); and isaridins A and B from an undescribed *Isaria* strain isolated from rat dung (Ravindra et al. 2004; Sabareesh et al. 2007).

Destruxin exhibits an array of amazing biological properties which include insecticidal activity, cytotoxic activity and moderate antibiotic (antituberculotic) activity (Pedras et al. 2002). Apart from these, destruxins have also been shown to possess immunodepressant activity in insect model systems (Vey et al. 1985; Huxham et al. 1989; Cerenius et al. 1990). They cause membrane depolarization by

opening calcium channel leading to the tetanic paralysis in the insects (Samuels et al. 1988). Destruxin E seemed to be the most potent destruxin with the insecticidal activity having repellent and aphicidal properties (Robert and Riba 1989), contact insecticidal activity (Poprawski et al. 1994) and antifeedant properties (Amiri et al. 1999).

### 2.3.2 Serinocyclins

The serinocyclins were first identified from the conidia of fungus *Metarhizium robertsii* ARSEF 2575, and its structure was elucidated from the isolates obtained from *Metarhizium acridum* (Krasnoff et al. 2007). They are the cyclic heptapeptides which feature many non-proteinogenic amino acids and composed of 1-aminocyclopropane-1-carboxylic acid (ACC) which acylates 4-hydroxyproline followed by the amidation of 1-aminocyclopropane-1-carboxylic acid with L-serine, D-4-hydroxylysine, β-alanine, D-serine and L-serine to form a 22-membered macrocycle. Serinocyclin B has D-lysine in place of D-4-hydroxylysine.

Serinocyclin A showed entomophagous activity as the exposed mosquito larvae to this compound exhibited abnormal swimming as they were unable to control the position of their heads (Krasnoff et al. 2007). The compound is believed to have a neurophysiological effect on the hair tufts which are used as the rudders to adjust the head position while swimming (Brackenbury 1999, 2001). A virtual docking study in 2014 has suggested that the serinocyclin binds to glutathione S-transferase (Sanivada and Challa 2014).

### 2.3.3 Metachelins

It is a group of coprogen-type hydroxamate siderophores that were isolated first from *Metarhizium robertsii* ARSEF 2575 when it was grown in iron-exploited medium (Krasnoff et al. 2014). The isolated medium included $N^{\alpha}$-dimethyl coprogen and dimerumic acid which were known earlier to be obtained from *Alternaria longipes*, *Fusarium dimerum* (Jalal et al. 1988), *Alternaria brassicicola* (Oide et al. 2006), *Verticillium dahliae* (Harrington and Neilands, 1982) and *Gliocladium virens* (Jalal et al. 1986), respectively. Apart from these known compounds, four novel siderophores were also reported from *M. robertsii*.

Dimerumic acid is synthesized by the condensation of two molecules of 5-anhydromevalonyl-*N*-5-hydroxyornithine to form a diketopiperazine ring, and further $N^{\alpha}$-dimethyl coprogen is synthesized by the head-to-tail esterification of the third molecule of 5-anhydromevalonyl-*N*-5-hydroxyornithine to one of the terminal hydroxyl group. One of the four novel siderophores which is also the major component of the mixture, metachelin A, is derived from *N*-dimethyl coprogen molecule after the glycosylation of both terminal hydroxyl groups by D-mannose and N-oxidation of the dimethyl nitrogen.

Metachelin forms the hexadentate chelating complexes with $Fe^{+3}$ and other trivalent metal cations like $Al^{+3}$ and $Ga^{+3}$. Metachelins and related compounds from *Me. robertsii* showed approximately equal activity to that of the bacterial siderophore, ferrioxamine, in a CAS plate assay (Krasnof et al. 2014).

### 2.3.4 Ferricrocin

It is an intracellular hexapeptide of the ferrichrome-type siderophore that was produced in its ferrated form by *M. robertsii* 2575 (Jalal et al. 1988). It was first reported in *Aspergillus* spp. (Zähner et al. 1963). It is presumed to receive environmental iron scavenged by extracellular siderophores and to transport it to its target sites in the cell (Wallner et al. 2009). Ferricrocin has the sequence Ser-Ser-Gly-Orn1-Orn2-Orn3-Orn4 where the Orn units are all $N^{\delta}$-acetyl-$N^{\delta}$-hydroxyornithines.

### 2.3.5 Tyrosine Betaine

It was isolated and characterized from *Metarhizium anisopliae* var. *anisopliae* strain ESALQ 1037 (Carollo et al. 2010). It is a dipeptide molecule having a molecule of betaine that is conjugated with tyrosine whose structure is identified as 2-{[1-carboxy-2-(4-hydroxyphenyl)ethyl] amino}-*N*,*N*,*N*-trimethyl-2-oxoethanammonium (Carollo et al. 2010). It was then also identified in an HPLC screening of the conidial extracts of *Metarhizium acridum* (ARSEF 324, ARSEF 3391 and ARSEF 7486) and *Metarhizium brunneum* (ARSEF 1095, ARSEF 5626 and ARSEF 5749) (Carollo et al. 2010). It is also observed to co-occur with serinocyclins and ferricrocin in extracts of conidia of *Metarhizium guizhouense* (ARSEF 683), *Metarhizium pingshaense* (ARSEF 2106 and ARSEF 5197) and *M. robertsii* (ARSEF 2575 and ARSEF 4123) during mass spectrometric analysis (Donzelli and Krasnoff 2016). The biological activity of this compound has not been reported yet.

### 2.3.6 Metacytofilin

It is an immunosuppressive compound that was obtained from *Metarhizium* spp. TA2759 which is a two-residue depsipeptide having the structure 3α-hydroxy-6β-methylamino-6α-(−methyl propyl)-3β-phenylmethyl-4H-2,3,5,6-tetrahydro-1,4-oxazine-2,5-dione (Iijima et al. 1992).

### 2.3.7 Fungerins

Isolated from *Metarhizium* spp. FKI-1079, fungerin, which was initially identified from a *Fusarium* spp. (Singh et al. 2001), along with two novel analogues, namely, hydroxyfungerin A and its regioisomer hydroxyfungerin B, has an imidazole core

(Uchida et al. 2005). The potentiality of the new compound which is unique to the *Metarhizium* strain was 1/12 in acute toxicity assay against brine shrimp (*Artemia*) and was inactive at 10 μg/disk against *Caenorhabditis elegans* or against a panel of microbe which included nine bacteria and five fungi as compared to fungerin (Donzelli and Krasnoff 2016).

### 2.3.8 Aurovertins

In 2008, three new analogues of aurovertins (F–H) were isolated along with previously described aurovertin D from *M. anisopliae* HF260 (Azumi et al. 2008). Aurovertins were isolated first from *Calcarisporium arbuscula* with the structural elucidation first for aurovertin B (Mulheirn et al. 1974). They are known to inhibit the mitochondrial, bacterial and chloroplast ATPases (F1) and so are used for probing these critical enzymes (Donzelli and Krasnoff 2016).

### 2.3.9 Metacridamides

The two compounds, metacridamides A and B, were isolated from spores of *Metarhizium acridum* ARSEF 3341 composed of 19-membered macrocyclic lactones (Krasnoff et al. 2012). They neither showed the insecticidal activity nor the antimicrobial activity (Donzelli and Krasnoff 2016).

### 2.3.10 JBIRs

The compounds JBIR-19 and JBIR-20 were isolated from *Metarhizium* spp. fE61 having two 24-membered macrolides differing from each other by one hydroxyl substitution (Kozone et al. 2009). JBIR-19 showed weak antimicrobial activity against *Saccharomyces cerevisiae* at MICs of 200 μM, but JBIR-20 did not show any antimicrobial activity at this concentration, although both of them induced cell elongation of the same at the concentrations of 3.1 μM and 13 μM, respectively (Kozone et al. 2009).

### 2.3.11 Helvolic Acid

Helvolic acid was isolated from *M. anisopliae*, and its 6-deacetyl analogue, helvolinic acid, was isolated from *M. anisopliae*, *Metarhizium brunneum* and eight other fungi (Turner and Aldridge 1983). It was originally isolated as an antibacterial "fumigacin", from *Aspergillus fumigatus* and *Aspergillus clavatus*, but was not structurally elucidated (Waksman et al. 1943). The full structure was finally solved in 1970 as a fusidane similar to fusidic acid which is built on the skeleton of cyclopentanoperhydrophenanthrene (Iwasaki et al. 1970; Okuda et al. 1964, 1967).

Helvolic acid along with its 1,2-hydro analogue isolated from *M. anisopliae* strain HF293 was shown to have antibacterial activity against *Staphylococcus aureus* (Lee et al. 2008).

### 2.3.12 Metarhizins

Metarhizins A and B are the two functionalized diterpenes which are produced by *Metarhizium flavoviride* and are similar to viridoxins (Donzelli and Krasnoff 2016). Metarhizin A has (2R, 3S)-2-hydroxy-3-methylpentanoate at C3 as in viridoxin A, but metarhizin B has (R)-2-hydroxy-3-methylbutanoic acid (deaminated Val) (Kikuchi et al. 2009).

### 2.3.13 Ovalicins

The type of compound of this group, ovalicin, was isolated from *Pseudeurotium ovalis* (Sigg and Weber 1968). Its hydroxylated analogue, Mer-f3 or 12-hydroxy-ovalicin, was obtained from *Metarhizium* spp. f3 (Kuboki et al. 1999). The ovalicins are monocyclic sesquiterpenoids having highly oxygenated cyclohexane ring and two epoxide groups (Donzelli and Krasnoff 2016). 12-Hydroxy-ovalicin showed immunosuppressive activity in a mixed lymphocyte culture reaction assay and leukaemia cells of L-1210 mouse (Kuboki et al. 1999). It has also shown potent cytotoxicity against four human cancer cell lines and human umbilical vein endothelial cells (Donzelli and Krasnoff 2016).

### 2.3.14 Taxanes

The overwhelmingly effective chemotherapeutic to cancer, placitaxel, and the other related taxanes were isolated originally from various species of yew trees' bark. Subsequently, placitaxel was reported from an endophyte, *Taxomyces andreanae*, living on Pacific yew (*Taxus brevifolia*) (Stierle et al. 1993). Among the more than 200 reported placitaxel-producing endophytic fungi, the highest yield is obtained from *M. anisopliae* (H-27 Accession FJ375161) (Donzelli and Krasnoff 2016). A controversy attached to the compound is that whether it is indeed a product of fungi at all (Heinig et al. 2013) and, if so, whether it is the result of a fungal version of the accepted plant pathway (Croteau et al. 2006).

### 2.3.15 Cytochalasins

These molecules are the subset of the "cytochalasans" which were thoroughly reviewed by Scherlach et al. (2010). The first cytochalasans which were described in 1966 are cytochalasins A and B that were obtained from *Phoma* strains S 298

(Rothweiler and Tamm 1966) and *Helminthosporium dematioideum* (Aldridge et al. 1967), respectively, and later cytochalasins C and D were isolated from the cultures of *M. anisopliae* (Roberts 1981). Cytochalasin D is also known to additionally occur in the fungi *Zygosporangium mansonii* and *Helminthosporium* species (Zimmermann 2007). In subsequent years many subclasses of compounds have been put together under the cytochalasins which include scoparisins, chaetoglobosins, penochalasins, aspochalsins, phomacins and alachalasins (Scherlach et al. 2010). In 2000, two new cytochalasin analogues were isolated from *M. anisopliae* in a screen for plant growth retardants, viz. diacetyl-cytochalasin C and an unnamed isomer (Fujii et al. 2000).

Cytochalasans constitute a perhydro-isoindolone molecule which is fused typically with a macrocyclic ring which may be a carbocycle, a lactone or a cyclic carbonate. The cytochalasins bear a benzyl group to the hydrogenated isoindolone moiety. The cytochalasins act as the inhibitors of the actin-cofilin interaction (Roberts, 1981; Strasser et al. 2000). When the plasmatocytes of greater wax moth (*Galleria melanoleuca*) were treated with the cytochalasins obtained from *M. anisopliae*, it was found that it caused the inhibition of attachment and also showed morphological alterations to the untreated ones (Vilcinskas et al. 1997a, b). This inhibition indicates the impairment in the plasmatocytes of the greater wax moth to perform the cell movements required for proper functioning of the cytoskeleton. Despite the basic biological activities of the cytochalasins, they are overshadowed by the destruxins in the collective effort of the secondary metabolites against insects.

### 2.3.16 Swainsonine

The compound was discovered after the observations of neurological symptoms and weight loss in livestock feeding on *Swainsona* spp. (Family Fabaceae), in *Swainsona canescens*, which inhibited lysosomal α-mannosidase (Dorling et al. 1978). The compound was named swainsonine, and its structure was elucidated as indolizidine-1,2,8-triol (Colegate et al. 1979). It was revealed to be isolated first from the fungus *Rhizoctonia leguminicola* and not from the plant after the complete structural elucidation of a compound that was previously obtained from the aforementioned fungus identical to swainsonine (Guengerich et al. 1973). Swainsonine was then subsequently isolated from *M. anisopliae* F-3622 (Hino et al. 1985). It is an indolizidine alkaloid moiety containing a fused piperidine and pyrrolidine ring system. They act as an aphid-feeding deterrent (Dreyer et al. 1985).

### 2.3.17 Viridoxins

Isolated from *M. flavoviride* (ARSEF 2133), viridoxins A and B are composed of a diterpenoid core with a 6-methoxy-2,3-dimethyl-γ-pyrone moiety that is attached to the 19th carbon and with (2R,3S)-2-hydroxy-3-methyl pentanoate and

(R)-2-hydroxy-4-methyl pentanoate, respectively, at the third carbon (Gupta et al. 1993). They have shown insecticidal activity against the Colorado potato beetle (*Leptinotarsa decemlineata*) as leaf contamination (Gupta et al. 1993).

#### 2.3.18 *N*-(Methyl-3-Oxodec-6-Enoyl)-2-Pyrroline and *N*-(Methyl-3-Oxodecanoyl)-2-Pyrroline

These are the two substituted pyrrolines that were reported from *Metarhizium flavoviride* HF698 as a weak plant pathogenic oomycete inhibitor (Putri et al. 2014). They were previously reported from *Penicillium brevicompactum* as the juvenile hormone inhibitors and also showed insecticidal activity against *Oncopeltus fasciatus* (Cantin Sanz et al. 1999; Moya et al. 1998) (Table 2.1).

### 2.4 The Secondary Metabolites of *Verticillium* Species

#### 2.4.1 Bassianolide

It is a toxic metabolite which is obtained from *Beauveria bassiana* and *Verticillium lecanii* (Suzuki et al. 1977), and it was originally isolated from both fungi which were entomophagous on the cadavers of *Bombyx mori* pupae (Murakoshi et al. 1978). The bassianolide is an octadepsipeptide with a 24-membered macrolactone ring which is formed as the cyclic tetrameric ester of the dipeptidol monomer D-hydroxyisovaleric acid-N-methylleucine (Xu et al. 2008). The insecticidal activity of bassianolide was shown by Suzuki et al. in 1977, and it also inhibits acetylcholine-induced smooth muscle contraction (Nakajyo et al. 1983). They are proven to induce atony to the *Helicoverpa* (*Heliothis*) *zea* larvae (Champlin and Grula 1979).

#### 2.4.2 Cyclosporines

They are also called as cyclosporines and were discovered in the 1970s obtained from *Trichoderma polysporum* and *Cylindrocarpon lucidum* (Borel et al. 1977; Dreyfuss et al. 1976). They are a series of cyclo-undecapeptide that were also reported to be produced by the *Verticillium* species by Jegorov and Weiser in 1990. They have insecticidal activities and were reported effective against larvae of mosquito (Matha et al. 1988; Podsiadlowski et al. 1998). Apart from that, cyclosporin A has the immunosuppressive effect on insect humoral immune response (Fiolka 2008) and cellular immune response (Vilcinskas et al. 1999).

**Table 2.1** Secondary metabolites from *Metarhizium* spp.

| Secondary metabolites class | Metabolite name | Occurrence | References |
|---|---|---|---|
| Peptides | Destruxins | *Metarhizium* | Kodaira (1961a, b) |
| | Serinocyclins | *Metarhizium robertsii* ARSEF 2575 | Krasnoff et al. (2007) |
| | Metahelins | *M. robertsii* ARSEF 2575 | Krasnoff et al. (2014) |
| | Ferricrocin | *M. robertsii* | Jalal et al. (1988) |
| Dipeptides and didepsipeptides | Tyrosine betaine | *Metarhizium brunneum* ARSEF 1095 | Carollo et al. (2010) |
| | Metacytofilin | *Metarhizium* sp. TA2759 | Iijima et al. (1992) |
| Amino acid derivatives | Swainsonine | *Swainsona canescens* | Dorling et al. (1978) |
| | Fungerins | *Metarhizium* sp. | Uchida et al. (2005) |
| Polyketides | Aurovertins | *M. ansiopliae* | Azumi et al. (2008) |
| Polyketide/peptide hybrids | Cytochalasins | *M. anisopliae* | Scherlach et al. (2010) |
| | NG-391 and NG-393 | *M. robertsii* ARSEF 2575 | Krasnoff et al. (2006) |
| | Metacridamides | *M. acridum* ARSEF 3341 | Krasnoff et al. (2012) |
| Other polyketide hybrids | JBIR-19 and JBIR-20 | *M. anisopliae* var. anisopliae | (Kozone et al. (2009) |
| Terpenoids | Helvolic acid and related compounds | *M. anisopliae* | Espada and Dreyfuss (1997) |
| | Viridoxins | *Metarhizium flavoviride* (ARSEF 2133) | Gupta et al. (1993) |
| | Metarhizins | *M. flavoviride* | Kikuchi et al. (2009) |
| | Ovalicins | *Metarhizium* sp. | Kuboki et al. (1999) |
| | Taxol | *M. anisopliae* (H-27 accession FJ375161) | Gu et al. (2015) |

### 2.4.3 Enniatins

They were first discovered in the 1940s (Gäumann et al. 1947). The analogues of enniatin are produced by various species of fungi including *Verticillium* (Herrmann et al. 1996; Supothina et al. 2004). Enniatin molecule is an N-methylated cyclohexadepsipeptides which comprise of three units each of N-methylated branched-chain L-amino acid and D-2-hydroxy acid that are arranged in an alternate fashion (Firakova et al. 2007). They are reported to inhibit ABC transporters (Hiraga et al.

2005), act as ionophores (Levy et al. 1995; Doebler 2000) and suppress acyl-CoA: cholesterol acyltransferase (Tomoda et al. 1992). They have the insecticidal properties (Monma et al. 2006) and are shown to act against the larvae of spruce budworm (*Choristoneura fumiferana*) (Strongman et al. 1988), *Galleria mellonella* (Mule et al. 1992) and adult of the blowfly (*Calliphora erythrocephala*) (Grove and Pople 1980).

### 2.4.4 Dipicolinic Acid

It is chemically known as pyridine-2,6-dicarboxylic acid. It is the metabolic product of several entomophagous fungi including *Verticillium* spp. (Shima, 1955). Dipicolinic acid was shown to have the insecticidal properties against blowfly (*Calliphora erythrocephala*) (Claydon and Grove 1982).

### 2.4.5 Verticilides

It was first isolated from the fungus *Verticillium* spp. FK-1033 (Omura et al. 2004). The compound is composed of a 24-membered ring cyclic depsipeptide containing a sequence of cyclo-[(2R)-2-hydroxyheptanonyl-*N*-methyl-L-alanyl] (Omura et al. 2004; Monma et al. 2006). The verticilides are shown to inhibit the ryanodine binding to the ryanodine receptors in cockroach and mouse (Monma et al. 2006; Shiomi et al. 2010).

### 2.4.6 Enalin

An analogue of enalin A, a coumaranone from the mangrove fungus *Verrucukina enalia* (Lin et al. 2002); 2,6-dihydroxy-2-methyl-7-(prop-1*E*-enyl)-1-benzofuran-3(2*H*)-one was obtained as one of the three compounds from *Verticillium* spp. isolated from the roots of wild *Rehmannia glutinosa* (You et al. 2009). Enalin A is widely distributed from microorganisms to higher plants and is known to have antimicrobial, antifungal, phytotoxic (Furumoto et al. 1997) and antidiabetic (Manickam et al. 1997) activities. The analogue of enalin A obtained from *Verticillium* spp. exhibited antibiotic activity against *Septoria* spp. and *Fusarium* spp. and also inhibited the growth of itself to some extent (You et al. 2009).

### 2.4.7 Massariphenone

It was originally reported from the marine-derived fungus *Massarina* spp. (Abdel-Wahab et al. 2007). Massariphenone was obtained as one of the three compounds from *Verticillium* spp. isolated from the roots of wild *Rehmannia glutinosa* (You et al. 2009). The chemical formula of the compound was as $C_{10}H_{12}O_3$ by a high-resolution mass spectrometric data, and NMR spectrum of the compound

showed signals of a 1,2,4-tri-substituted benzene ring, an aryl methyl group and an OCHCH3 unit (Abdel-Wahab et al. 2007). It has slight antibiotic activity as it inhibited the growth of *Septoria* spp. and *Fusarium* spp. only slightly (You et al. 2009).

### 2.4.8 Ergosterol Peroxide

It is reported from a wide range of fungal species and was first obtained from *Cordyceps sinensis* as an antitumor sterol (Bok et al. 1999). It is chemically 5α,8α-epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-ol and was also obtained as one of the three compounds from *Verticillium* spp. isolated from the roots of wild *Rehmannia glutinosa* along with massariphenone and analogue of enalin A. It significantly inhibited biomass accumulation of *Septoria* spp., *Fusarium* spp. and *Rhizoctonia* spp. at a low concentration of 0.97 μg/ml in liquid culture (You et al. 2009).

### 2.4.9 Radicicol (Monorden)

Radicicol was isolated from *Verticillium chlamydosporium* (= *Pochonia chlamydosporia*) in search for the nematicidal mechanisms from nematophagous fungi (Khambay et al. 2000). It was originally found as an antifungal compound by Delmotte and Delmotte-Plaquee in 1953. Monorden E and an analogue of radicicol, monorden analogue-1, were purified from the fungus *Pochonia chlamydosporia* var. *chlamydosporia* strain TF-0480 (Shinonaga et al. 2009a, b). Radicicol and monorden E were originally obtained from a mycoparasite *Humicola* spp. FO-2942 that produced amidepsines, diacylglycerol acyltransferase inhibitors (Niu 2017). They have antifungal activity only against *Aspergillus niger* (Arai et al. 2003; Yamamoto et al. 2003). Radicicol does not have any nematicidal activity against root-knot nematode *Meloidogyne incognita* (Niu 2017), although it possess antiviral activity against herpes simplex virus (Hellwig et al. 2003).

### 2.4.10 Pochonins

Pochonins were all isolated first from the strains of *V.chlamydosporium* (= *P. chlamydosporia*) (Hellwig et al. 2003; Shinonaga et al. 2009a, b). Pochonins A–F were isolated from *Pochonia chlamydosporia* var. *catenula* strain P 0297 (Hellwig et al. 2003), and pochonins G–P along with pochonins B, D, E and F were isolated and characterized from *P. chlamydosporia* strain TF-0480 (Shinonaga et al. 2009a, b). Except pochonins F and J, all are chlorine-containing resorcylic acid lactones. Pochonins G and H are the first two compounds in the radicicol family to possess a furan ring, and pochonins L–N are the first three analogues of radicicol with an E-configuration of a double bond at C5–C6. Pochonin K is a 14-aldofuranose radicicol derivative, and pochonin I has a single benzene moiety in the macrolide ring (Niu 2017). Pochonins A–F except for pochonin D showed inhibitory action against herpes simplex virus 1 (Hellwig et al. 2003).

### 2.4.11 Monocillins

Monocillins I–IV along with radicicol were isolated originally from the fungus *Monocillium nordinii*, a mycoparasite of pine-pine gall rust *Endocronartium harknessii* (Ayer et al. 1980; Ayer and Peña-Rodriguez, 1987). Monocillins are the non-chlorine-containing resorcylic acid lactones. Monocillins II–III along with radicicol, pochonin F and a novel monocillin II glycoside were isolated from *P. chlamydosporia* var. *catenulata* strain P 0297 (Hellwig et al. 2003). All the four monocillins were later isolated from *P. chlamydosporia* strain TF-0480 (Shinonaga et al. 2009a, b). Monocillin III is a dechloro analogue of pochonin A showing potent inhibitory activity against herpes simplex virus 1, and monocillin II is same of pochonin D but with no inhibitory activity to the same virus (Hellwig et al. 2003). Monocillin I has antifungal activities against a wide variety of fungi including *Phycomyces blaksleeanus*, *Pythium debaryanum*, *Ceratocystis ulmi* as the cause of Dutch elm disease and *Phellinus pini* pointing towards the nonspecific nature. However, monocillins II–IV don't show the same antifungal activities (Ayer et al. 1980; Aver and Peña-Rodriguez 1987).

### 2.4.12 Phomalactones

In 2000, the first study on isolation of phomalactone was reported from the fungus *V. chlamydosporium* (= *P. chlamydosporia*) in a bioassay against the root-knot nematode *Meloidogyne incognita* as a nematicidal metabolite (Niu 2017). It was first isolated from the phytopathogenic fungus, *Nigrospora* spp. (Evans et al. 1969), and was later purified from *Phoma minispora* (Yamamoto et al. 1970; Yamano et al. 1971), *Hirsutella thompsonii* var. *synnematosa* (Krasnoff and Gupta 1994), *Paecilomyces cateniobliquus* (= *Isaria cateniobliqua*) YMF1.01799 (Wu et al. 2012) and *Nigrospora sphaerica* (= *Khuskia oryzae*) (Kim et al. 2001). It has shown nematicidal action against *M. incognita*; dose-dependent insecticidal activity against apple maggot flies, *Rhagoletis pomonella*; and mild toxicity to tephritid fruit flies. Apart from that, it has also shown inhibitory actions to spores of *Beauveria bassiana* and *M. anisopliae* (Krasnoff and Gupta 1994). The growth inhibition of a wide range of microorganisms including fungi, bacteria and a protozoan is shown by phomalactone (Niu 2017).

### 2.4.13 Aurovertins

Aurovertins D, E, F and I were first isolated from the parasitic fungus of root-knot nematode, *P. chlamydosporia* strain YMF 1.00613 (Niu et al. 2009). Aurovertin D is toxic to the free-living nematode, *Panagrellus redivivus* (Niu 2017).

### 2.4.14 Pseurotin A

It was originally isolated from *Pseudeurotium ovalis* (Bloch et al. 1976) and is a spirocyclic alkaloid containing oxygen and nitrogen atoms. It was reported as the main metabolite from most of the isolates of *P. chlamydosporia* propagated in Q6 medium (Hellwig et al. 2003). It acts as a chitin synthase inhibitor (Wenke et al. 1993) and has a moderate effect on phytopathogenic bacteria *Erwinia carotovora* and *Pseudomonas syringae* (Niu 2017).

### 2.4.15 Oosporein

It was originally obtained from a fungus *Oospora colorans* as a red-coloured pigment in 1944 (Niu 2017). It was later obtained from *Verticillium psalliotae* (= *Lecanicillium psalliotae*) that was selected as an antagonist against fungus causing late blight of tomato, *P. infestans* (Wainwright et al. 1986). It has a strong inhibitory action especially against *Phytophthora infestans* (Niu 2017).

### 2.4.16 Pyrenocines

The first pyrenocines to be described are pyrenocines A and B which were isolated as the phytotoxic metabolites of *Pyrenochaeta terrestris* (= *Setophoma terrestris*) causing pink root disease of onions (Sato et al. 1981). They both were isolated from entomophagous pathogen *Verticillium hemipterigenum* BCC 1449 (Nilanonta et al. 2003). They have several biological activities, reportedly showing phytotoxicity, cytotoxicity and antifungal, antibacterial, antimalarial and antitrypanosomal activity (Sparace et al. 1987; Krohn et al. 2008).

### 2.4.17 Vertinoids

It is a group of compounds that are obtained from *Verticillium intertextum* ATCC 46284 (Trifonov et al. 1983, 1986). The three secondary metabolites, viz. the hexaketide yellow sorbillin, its derivative 2′,3′-dihydrosorbicillin and the dimeric hexaketide yellow bisvertinoquinol, were reported in 1983, and four new dimeric hexaketides were reported in 1986, viz. bisvertinol, dihydrobisvertinol, isodihydrobisvertinol and bisvertinolone. All the compounds are hexaketide-derived secondary metabolites having two additional methyl groups, one at C2 and the other at C4 of the C12 chain (Niu 2017).

### 2.4.18 Vertinolide

It was obtained as a new tetronic acid derivative from the fungus *Verticillium intertextum* ATCC 46284 (Trifonov et al. 1982). It contains a 4-hydroxy-3,5-dimethyl-2(5H)-furanone-5-yl and an (E,E)-2,4-hexadienon-1-yl substructures with a dimethylene bridge in between.

### 2.4.19 Lowdenic Acid

It was isolated from non-sporulating cultures of an undescribed fungus *Verticillium* spp. (MYC-406 = NRRL 29280 = CBS 102427) (Angawi et al. 2003). It has shown antifungal activity against *A. flavus* (NRRL 6541), *Candida albicans* ATCC 90029, *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* ATCC 6051 (Niu 2017). Lowdenic acid possesses an unusual bicyclic structure containing a furylidene ring which is linked via a C=C double bond to a tetrahydrofurandione ring (Angawi et al. 2003).

### 2.4.20 Asteltoxins

They have a trienic α-pyrone structure and are related to citreoviridin and aurovertins. Asteltoxin was the first identified compound as a mycotoxin metabolite of the fungus *Aspergillus stellatus* (Kruger et al. 1979). Later on, four asteltoxin-type metabolites along with two new asteltoxins were isolated from the fungus *Pochonia bulbillosa* (=*Metapochonia bulbilosa*) 8-H-28, which was obtained from the fruiting body of *Elaphocordyceps capitata* (= *Tolypocladium capitatum*) (Adachi et al. 2015). Asteltoxin has shown inhibitory action against *Escherichia coli* BF1-ATPase (Satre 1981).

### 2.4.21 Bigutol

Bigutol along with its derivative methylbigutol was isolated from the mycoparasitic fungus *Verticillium biguttutum* (Morris et al. 1995). They both have prenylated 4-(hydroxymethyl)benzene-1,2-diol moiety in their structure. Bigutol and methylbigutol both inhibit the growth of *Rhizoctonia solani* and other plant pathogenic fungi (Morris et al. 1995).

### 2.4.22 Ascochlorin

Ascochlorin-type compounds were first isolated from the fungus *Ascochyta viciae* (= *Septoria viciae*) (Tamura et al. 1968), and later on it has been purified from an array of fungus which includes *Fusarium* sp. LL-Z1272 (Ellestad et al.

1969), *Cylindrocladium ilicicola* (= *Calonectria pyrochroa*) MFC-870 (Hayakawa et al. 1971; Minato et al. 1972), *Nectria coccinea* (= *Neonectria coccinea*) (Aldridge et al. 1972), *Colletotrichum nicotianae* (= *Colletotrichum tabacum*) (Kosuge et al. 1973), *Ascochyta viciae* (Sasaki et al. 1974), *Acremonium luzulae* (= *Gliomastix luzulae*) (Cagnoli-Bellavita et al. 1975), *Cephalosporium diospyri* (= *Nalanthamala diospyri*) IFO 6118 (Kawagishi et al. 1984), *Cylindrocarpon lucidum* (= *Thelonectria lucida*) (Singh et al. 1996), a sponge-derived fungus *Acremonium* sp. (Zhang et al. 2009) and a leafhopper pathogenic fungus, *Microcera* sp. BCC 17074 (Isaka et al. 2015). The metabolites are a class of a 2,4-dihydroxy-5-chloro-6-methylbenzaldehyde (or 5-chloroorclaldehyde) having a sesquiterpene side chain at C5. In 1994, a series of ascochlorin-type compounds which included a new ascochlorin, 8′,9′-dehydroascochiorin, and five known ascochlorins were identified from *Verticillium* spp. FO-2787 (Takamatsu et al. 1994). Again in 2004, a new ascochlorin, 8′-hydroxyascochlorin, and a novel ascochlorin glycoside, vertihemipterin A, together with six known ascochlorins, were isolated from the entomophagous fungus *Verticillium hemipterigenum* (= *Torrubiella hemipterigena*) BCC 2370 (Seephonkai et al. 2004). The members of ascochlorin-type compounds are known to exhibit antifungal activity (Bal Tembe et al. 1999), antiviral activity and antitumour activity (Takatsuki et al. 1969) (Table 2.2).

## 2.5 The Fate of Secondary Metabolites of *Metarhizium* spp. and *Verticillium* spp.

The two fungi are accessible in the market in both solid and liquid formulations containing the spore and the mycelium of the fungus. When these two entomophagous fungi are applied to a crop ecosystem, it comes in direct contact with humans and target insects and to the crop on which it is applied. The indirect interaction of these fungi happens to occur by drifting to soil, water and atmosphere (Hu et al. 2016). Humans are the first ones to come into contact with the cultures of these fungal entomopathogens whether be it the people who are producing the formulation or the people who are applying it on to their field. There are reports of fungal spore allergy caused by the entomopathogenic fungi including *M. anisopliae* to the workers producing it (Zimmermann 2007), although there are no reports of any sort of things because of the secondary metabolites.

When applied to the crop, the two fungi reach to the proximity of the target insect pests. The spores and mycelium of the fungi adhere to the external surface of insect and then start its infection process. The fungi penetrate through the cuticle of the insect, and various metabolites including various enzymes such as cutinases produced by the fungal mycelia or spores are known to aid in this step of the process. After the penetration, the fungi proliferate itself inside the target insect pest body and then carry on with its life cycle and in the process produce various primary and secondary metabolites. After a successful establishment, the fungi are known to produce the secondary metabolites of which many have the insecticidal effect on the

**Table 2.2** Secondary metabolites from *Verticillium* spp.

| Secondary metabolites class | Metabolites name | Occurrence | References |
|---|---|---|---|
| Aromatic compounds | ES-242-1 | *Verticillium* sp. SPC-15898 | Toki et al. (1992a, b) |
| | ES-242-2 | *Verticillium* sp. SPC-15898 | Toki et al. (1992b) |
| | ES-242-3 | *Verticillium* sp. SPC-15898 | Toki et al. (1992b) |
| | ES-242-4 | *Verticillium* sp. SPC-15898 | Toki et al. (1992b) |
| | ES-242-5 | *Verticillium* sp. SPC-15898 | Toki et al. (1992b) |
| | ES-242-6 | *Verticillium* sp. SPC-15898 | Toki et al. (1992b) |
| | ES-242-7 | *Verticillium* sp. SPC-15898 | Toki et al. (1992b) |
| | ES-242-8 | *Verticillium* sp. SPC-15898 | Toki et al. (1992b) |
| | Oosporein | *Verticillium psalliotae* | Wainwright et al. (1986) |
| Vertinoids | Sorbicillin | *V. intertextum* ATCC 46284 | Trifonov et al. (1983) |
| | 2,3-Dihydrosorbicillin | *V. intertextum* ATCC 46284 | Trifonov et al. (1983) |
| | Bisvertinoquinol | *V. intertextum* ATCC 46284 | Trifonov et al. (1983) |
| | Bisvertinol | *V. intertextum* ATCC 46284 | Trifonov et al. (1986) |
| | Dihydrobisvertinol | *V. intertextum* ATCC 46284 | Trifonov et al. (1986) |
| | Isodihydrobisvertinol | *V. intertextum* ATCC 46284 | Trifonov et al. (1986) |
| | Bisvertinolone | *V. intertextum* ATCC 46284 | Trifonov et al. (1986) |
| Furanone and pyranone | Vertinolide | *V. intertextum* ATCC 46284 | Trifonov et al. (1986) |
| | Lowdenic acid | *Verticillium* sp. (MYC-406 = NRRL 29280 = CBS 102427) | Angawi et al. (2003) |
| | Canescin | *Verticillium* sp. (MYC-406 = NRRL 29280 = CBS 102427) | Angawi et al. (2003) |
| | Pyrenocin A | *V. hemipterigenum* (teleomorph: *T. hemipterigena*) BCC 1449 | Nilanonta et al. (2003) |

(continued)

**Table 2.2** (continued)

| Secondary metabolites class | Metabolites name | Occurrence | References |
|---|---|---|---|
| | Pyrenocin B | *V. hemipterigenum* (teleomorph: *T. hemipterigena*) BCC 1449 | Nilanonta et al. (2003) |
| *Phenol-terpenoid hybrids* | *Bigutol* | *V. biguttatum* | Morris et al. (1995) |
| | *Methylbigutol* | *V. biguttatum* | Morris et al. (1995) |
| | *LL-Z1272*β | *Verticillium* sp. FO-2787 | Takamatsu et al. (1994) |
| | *8′,9′-Dehydroascochlorin* | *Verticillium* sp. FO-2787 | Takamatsu et al. (1994) |
| | *Ascochlorin/LL-Z1272*γ | *Verticillium* sp. FO-2787 | Takamatsu et al. (1994) |
| | *8′-Acetoxyascochlorin/ LL-Z1272* | *Verticillium* sp. FO-2787 | Takamatsu et al. (1994) |
| | *8′-Hydroxyascochlorin* | *V. hemipterigenum* BCC 2370 | Seephonkai et al. (2004) |
| | *Vertihemipterin A* | *V. hemipterigenum* BCC 2370 | Seephonkai et al. (2004) |
| | *Ascofuranone* | *V. hemipterigenum* BCC 2370 | Seephonkai et al. (2004) |
| | *Ascofuranol* | *V. hemipterigenum* BCC 2370 | Seephonkai et al. (2004) |
| Terpenoids | *β*-Apo-4′-carotenoic acid | *V. agaricinum* | Valadon and Mummery (1977) |
| | *β*-Apo-4′-carotenoic acid methyl este | *V. agaricinum* | Valadon and Mummery (1977) |
| | *Dahliane A* | *V. dahlia* | Wu et al. (2016) |
| | *Dahliane B* | *V. dahliae* | Wu et al. (2016) |
| | *Dahliane C* | *V. dahliae* | Wu et al. (2016) |
| | *Dahliane D* | *V. dahliae* | Wu et al. (2016) |
| *Nitrogen-containing phenolic compound* | *Balanol* | *V. balanoides* | Kulanthaivel et al. (1993) |
| *Cyclodepsipeptides* | *Bassianolide* | *V. lecanii* (*Lecanicillium* sp.) | Suzuki et al. (1977) |
| | *Enniatin B* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |

(continued)

**Table 2.2** (continued)

| Secondary metabolites class | Metabolites name | Occurrence | References |
|---|---|---|---|
| | *Enniatin B4* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Enniatin C* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Enniatin G* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Enniatin MK1688* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Enniatin H* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Enniatin I* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Enniatin O1* | *V. hemipterigenum* BCC 1449 | Supothina et al. (2004) |
| | *Enniatin O* | *V. hemipterigenum* BCC 1449 | Supothina et al. (2004) |
| | *Enniatin O3* | *V. hemipterigenum* BCC 1449 | Supothina et al. (2004) |
| *Diketopiperazines* | *1-Demethylhyalodendrin tetrasulfde* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Vertihemiptellide A* | *V. hemipterigenum* BCC 1449 | Minato et al. (1973) |
| | *Vertihemiptellide B* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Demethylhyalodendrin* | *V. hemipterigenum* BCC 1449 | Nilanonta et al. (2003) |
| | *Verticillin A* | *Verticillium* sp. TM-759 | Minato et al. (1973) |
| | *Verticillin B* | *Verticillium* sp. TM-759 | Minato et al. (1973) |
| | *Verticillin C* | *Verticillium* sp. TM-759 | Minato et al. (1973) |
| *Polyhydroxylated pyrrolizidin* | *Pochonicine* | *P. suchlasporia* var. *suchlasporia* TAMA 87 | Usuki et al. (2009) |

insect pests that are still living up to this stage of fungal infection cycle. So at last, the fungal establishment and its secondary metabolites along with the cadavers of the target insect pests enter the environment. Till now, estimating the number of the fungal metabolites getting released in the environment is very difficult (Hu et al. 2016), although a few of the research point out that the number of metabolites of the entomopathogenic fungi reaching the environment is scarce. As, for example, destruxins, a secondary metabolite of *Metarhizium* spp., targets insect pests, as the compound decomposes shortly after the death of the host insect pest. The decomposition of destruxin is presumed to be due to the activity of the hydrolytic enzymes in

the cadaver, being independent of host or soil and biota, apparently. Thus, destruxins are restricted essentially to the pathogen and the target host and are unlikely to contaminate the environment or enter the food chain (Skrobek et al. 2008).

The target crop along with the weeds also comes in direct contact with the applied entomopathogenic fungus. As the entomopathogenic species of these two fungi are not phytopathogenic, the fungal mycelial and spores in the suspension just only get deposited on the applied plant's surface but not in those cases where the species of the *Metarhizium* have been shown to possess endophytic characteristics (Mantzoukas et al. 2015).

Next in the line is the indirect interaction that is caused by the drifting of entomopathogenic fungal formulation to the soil, water and atmosphere while applying. The soil is believed to be the reservoir of the microorganisms including the entomopathogenic fungi. The fungus can survive in the soil as a spore or mycelium and/or in form of any dormant or active structures. Drift from the application and the dropping from the target pest cadavers, fungal spores, mycelia and the metabolites can reach the soil system, but there are no such reports of metabolites of fungal entomopathogen being detected in soil (Hu et al. 2016). Although beauvericins have been detected in drainage water after *Fusarium* spp. was inoculated on the wheat plants (Schenzel et al. 2012), there are no reports showing metabolites from these two entomopathogenic fungi reaching the water system, and neither are there reports of the same in the atmosphere (Hu et al. 2016).

## 2.6 Secondary Metabolites of *Metarhizium* spp. and *Verticillium* spp. as Potent Insecticidal Agents

As discussed earlier, the secondary metabolites are the organic compounds which do not play a direct role in organisms' growth and metabolism (Andersson 2012). Various species of the entomopathogenic *Metarhizium* and *Verticillium* fungi along with the other entomopathogenic fungi have been investigated as a source of a wide range of secondary metabolites which possess bioactivities against a broad range of the insect pests. As a result, diversified metabolites have been reported that display insecticidal properties against insect pests (Khan et al. 2012). Destruxins (A and B) (Kodaira 1961a, b), serinocyclin A (Krasnoff et al. 2007), cytochalasins (Vilcinskas et al. 1997a, b), swainsonine (Dreyer et al. 1985) and viridoxins (Gupta et al. 1993) produced from *Metarhizium* spp. and bassianolides (Champlin and Grula 1979), cyclosporines (Matha et al. 1988; Podsiadlowski et al. 1998), enniatins (Grove and Pople 1980; Strongman et al. 1988; Mule et al. 1992; Monma et al. 2006), dipicolinic acid (Claydon and Grove 1982), verticilides (Monma et al. 2006), phomalactones (Krasnoff and Gupta 1994) and oosporein (Eyal et al. 1994; Wilson 1971) produced from the *Verticillium* spp. are the secondary metabolites that have shown the insecticidal properties apart from the metabolites of other entomopathogenic fungi that have also shown the insecticidal properties. Secondly, there are also certain extracellular enzymes that are produced by *Metarhizium* spp. and *Verticillium* spp. such as chitinase, protease and lipases that also possess certain insecticidal

properties (da Silva et al. 2010). So, proper attention is given on the isolation and purification of such enzymes from their producing entomopathogenic fungal species and their utilization in formulations of biopesticides. Some of these formulations also have been patented by their inventors such as the following: an enzyme preparation composed of at least one protease derived from *Metarhizium*, *Beauveria*, *Verticillium* and *Aschersonia* was formulated and patented (US4987077) (Charnley et al. 1991) and a technology of controlling insect pest prepared with chitinolytic enzymes was patented (US6069299) (Broadway et al. 2000). Similarly, the formulations of the secondary metabolites of *Metarhizium* spp. and *Verticillium* spp. that have shown the insecticidal properties can be engineered and used as target-specific green pesticides.

## 2.7 Conclusion and Future Perspectives

In this chapter, the secondary metabolites of various *Metarhizium* spp. and *Vetricillium* spp. have been exemplified. Most of these compounds exhibited a profound range of biological activities including antifungal, antibacterial, antitumoral, insecticidal and enzyme-inhibiting abilities. In many cases of the secondary metabolites from these two entomopathogenic fungi, only a superficial research is done except for a few of the metabolites. As the new facets of the secondary metabolites are yet to be explored, there is a wide scope of discovering many new compounds as well as the biological activities of the already discovered compounds. There is also a whole new area of using the secondary metabolites of these two fungi along with the other entomopathogenic fungi as pesticide formulations as only few of them are presently available in the market. Although entomopathogenic fungal formulations are present in the market as suspension of mycelia and spore, there is no any prominent product that uses a secondary metabolite as a pest control. There is a need of a full-fledged research that is focused on finding the novel secondary metabolites, proving the different biological activities of the metabolites, standardization of the effective quantity of the metabolites in their biological activities, finding ways to use the metabolites for human welfare, demarcation of the metabolite use in diverse field of science and technology and adverse effect that may occur due to the metabolites. The major problem of the natural product research is its randomness which is obligated to be rectified. Many new technologies in this particular area are waiting to be revealed and put to use in agricultural and agri-allied sectors and also in other sectors. The specificity in the mode of action of these metabolites makes them eco-friendly and, thus, helps in the sustainable development. With the use of these products, we can maintain the balance in nature while meeting the human demands. It is time we should take keen interest in identification of the natural products of the entomopathogenic fungi as the promising new source of bioactive natural compounds because the time has never been more suitable to do so as now we have all the analytical and the molecular tools at our disposal.

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# 3 Secondary Metabolites of Non-pathogenic *Fusarium*: Scope in Agriculture

Laith Khalil Tawfeeq Al-Ani

## 3.1 Introduction

*Fusarium* is a very interesting genus compared with other genera of fungi. *Fusarium* in the pathogenic state can cause very hazardous diseases in plants and human. *Fusarium* and other fungi produced very dangerous secondary metabolites such as mycotoxins (Attitalla et al. 2010a, b; Nor Azliza et al. 2014). While the genus can be useful in avirulent state, in this state it is known as non-pathogenic *Fusarium*. Many species of non-pathogenic *Fusarium* inhabit the tissues of plants as endophytic fungus or as saprophytes in soil. Non-pathogenic *Fusarium* can live for a long time in soil, in rhizosphere and in planta (Singh et al. 2016, 2017).

Non-pathogenic *Fusarium* are endophytes in many crops in agricultural ecosystems (Burgess 1981; Leslie et al. 1990; Kuldau and Yates 2000). Non-pathogenic *Fusarium* can invade internal plant tissues without causing any symptoms (Burgess 1981). Some non-pathogenic *Fusarium* isolated from healthy rakkyo roots (*Allium chinense*) after 97 days including *F. fujikuroi*, *F. solani* and *F. oxysporum* (Honda and Kawakubo 1999). Some non-pathogenic or endophytic *Fusarium* were isolated from five species of the medicinal plants in the Western Ghats of India (Raviraja 2005). The fungal biomass of non-pathogenic *Fusarium* strains could differ from other pathogenic *F. oxysporum* strains present in the root cortex (Validov et al. 2011). Three species of non-pathogenic *Fusarium*, viz. *F. oxysporum*, *F. solani* and *F. fujikuroi*, were reported to be dormant in the rhizosphere of tomato (Imazaki and Kadota 2015). Non-pathogenic *Fusarium* are highly diverse in soil, in rhizosphere and in the roots of tomato (Demers et al. 2015). The non-pathogenic *Fusarium*, viz. *F. fujikuroi*, *F. solani*, *F. proliferatum* and *F. polyphialidicum*, were among the fungal flora isolated from the roots of banana (Cao et al. 2002; Al-Ani 2017b). Many

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strains of non-pathogenic *F. oxysporum* were also isolated from healthy banana (Nel et al. 2006b; Al-Ani 2017b).

Some antimicrobial compounds produced by plants affect the growth of pathogenic *Fusarium*, exclusively (Mishra et al. 2015). Landa et al. (2002) reported phytoanticipins including biochanin A and tomatine, which inhibit the growth of pathogenic *Fusarium*, while enhancing the growth of non-pathogenic *Fusarium.* Also, coumarin could inhibit the growth of both non-pathogenic and pathogenic *Fusarium*. Different species of non-pathogenic *Fusarium* could be efficiently used for the management of various phytopathogens by reducing infection of plant-parasitic nematodes, bacteria and fungi (Bisen et al. 2015; Keswani et al. 2016). Indeed, non-pathogenic *Fusarium* can utilize as biocontrol agent such as other biocontrol agents. Many biocontrol agents with natural products were used in controlling the plant pathogens and pests (Al-Ani 2006; Al-Ani and Salleh 2013b; Mohammed et al. 2011, 2012, 2013, 2014; Al-Ani and Al-Ani 2011; Al-Ani et al. 2012; Al-Ani et al. 2013a, b; AL-Ani 2017a, b; Al-Ani and Albaayit, 2018a, b; Al-Ani et al. 2018; AL-Ani 2018a, b; Al-Ani 2019a, b, c, d, e; Al-Ani et al. 2019). Other methods are including several methods such natural products (Mohammed et al. 2012; Al-Ani et al. 2012). Non-pathogenic *Fusarium* have been effectively employed for management of *Fusarium* wilt of many important agricultural crops, including banana (Nel et al. 2006a; Al-Ani 2010, 2017b; Al-Ani et al. 2013a), tomato (Lemanceau and Alabouvette 1991; Larkin and Fravel 1998), chickpea (Hervás et al. 1995), cucumber (Mandeel and Baker 1991; Wang et al. 2013), watermelon (Larkin et al. 1996; Freeman et al. 2002), basil (Fravel and Larkin 2002), celery (Schneider 1984), strawberry (Tezuka and Makino 1991), muskmelon (Freeman et al. 2002), cyclamen (Minuto et al. 1995) and flax (Lemanceau and Alabouvette 1991). Also, non-pathogenic *Fusarium* is compatible with other biocontrol agents and be very efficiently involved in integrated pest management. Belgrove et al. (2011) used non-pathogenic *F. oxysporum* with *Pseudomonas fluorescens* WCS 417 against pathogenic *F. oxysporum* f. sp. *cubense* race 4 demonstrating effective suppression and protection of banana cultivar from Panama disease (*Fusarium* wilt). Also, application of consortia of non-pathogenic *Fusarium* and *Trichoderma* proved to be highly effective in reducing the vanilla shoot rot disease (Taufiq et al. 2017).

The efficacy of non-pathogenic *Fusarium* in the production and secretion of diverse and bioactive secondary metabolites was contributed to the management of phytopathogens (Jayaprakashvel and Mathivanan 2011), while other mechanisms include mycoparasitism, competition and induced resistance in host (Fravel and Larkin 2002; Kaur et al. 2010; Shishido et al. 2005).

## 3.2 Secondary Metabolites

Secondary metabolites produced by biocontrol agents are highly effective in controlling phytopathogens. Non-pathogenic *Fusarium* produces an array of chemically diverse, bioactive secondary metabolites. Non-pathogenic *Fusarium* secretes

low molecular weight volatile organic compounds (VOCs) (Weikl et al. 2016). Non-pathogenic *Fusarium* produces several secondary metabolites which were absent in its pathogenic counterpart. Nawar (2016) reported that GC-MS analysis of the cultural filtrate of non-pathogenic *Fusarium* had as many as 30 secondary compounds compared with 22 for the pathogenic isolate.

Two antifungal compounds of *F. chlamydosporum* were able to inhibit the growth of uredospore of *Puccinia arachidis* (Mathivanan and Murugesan 1998). α-Pyrones, viz. fusapyrone (FP) and deoxyfusapyrone (DFP), were reported to be produced by *F. semitectum* (Evidente et al. 1999). DFP and FP inhibited the growth of many filamentous fungi such as *Alternaria alternata*, *Penicillium verrucosum*, *P. brevicompactum*, *Ascochyta rabiei*, *Aspergillus flavus*, *Cladosporium cucumerinum*, *Phoma tracheiphila*, *Botrytis cinerea*, *Candida albicans*, *C. glabrata* and *Cryptococcus neoformans* (Altomare et al. 2000; Bartelt and Wicklow 1999; Garret and Robinson 1969; Mathivanan and Murugesan 1999). Non-pathogenic *F. oxysporum* MSA35 strain produces many VOCs which are highly effective against pathogenic *F. oxysporum* f. sp. *lactucae* Fuslat10 (Minerdi et al. 2009). Volatile compounds of MSA35 strain such as α-humulene efficiently reduced the mycelial growth and inhibited the virulence gene of pathogenic Fuslat10 strain (Minerdi et al. 2009). α-Humulene extracted from non-pathogenic MSA35 was effective on pathogenic Fuslat10 strain at 25–100 mM but at 5–20 mM was completely ineffective (Minerdi et al. 2009). The strain CanR-46 of *F. oxysporum* was producing four VOCs including limonene, octanoic acid, 3,4-2H-dihydropyran and 5-hexenoic acid effective against *V. dahliae* (Zhang et al. 2015).

For control of the plant parasitic nematodes, non-pathogenic *F. solani* produced secondary metabolites affecting the juveniles of *Meloidogyne javanica* (Siddiqui and Shaukat 2003). Two species of endophytic *Fusarium* such as *F. oxysporum* and *F. solani* secrete some secondary metabolites as nematicidal agents against second-stage juveniles of *Meloidogyne javanica* (Qureshi et al. 2012). Non-pathogenic *F. oxysporum* produced VOCs against second-stage juveniles of *Meloidogyne exigua* causing high mortality and immobility after 72 h (Costa 2014). For control of bacterial plant pathogens, endophytic *F. oxysporum* NRRL26379 in *A. thaliana* (A) reduced the disease severity of *Pseudomonas syringae* (Col-0), (B) improved the plant growth and (C) increased salt tolerance by producing volatile compounds (Li and Kang 2018).

For control of the plant parasitic weeds, endophytic *Fusarium* produces some toxins that can be highly beneficial for field applications. Zonno and Vurro (2002) isolated endophytic *Fusarium* secreting several toxins such as nivalenol, T-2, neosolaniol, HT-2 and diacetoxyscirpenol that were able to inhibit 100% plant parasitic weed *Orobanche ramosa*. Two endophytic *Fusarium* could produce some secondary metabolites as mycoherbicidal agents that are highly effective for growth inhibition of *Orobanche aegyptiaca* (Egyptian broomrape) of tomato (Cohen et al. 2002a). These species including *F. oxysporum* produced fusaric acid and fumonisin-like ceramide synthase inhibitors (Cohen et al. 2002a). Beauvericin (as toxin) could significantly improve the secondary metabolite content and plant growth in plant *Dioscorea zingiberensis* (a traditional Chinese medicinal herb), which was

produced by endophytic *F. redolens* Dzf2 (Campos et al. 2011; Yin et al. 2011). *Fusarium* sp. KF611679 strain of Brazilian tree *Caesalpinia echinata* Lam. was secreting a trypanocidal metabolite as beauvericin (Campos et al. 2015). The LC-MS analysis of secondary metabolites for four endophytic *Fusarium* species such as *F. oxysporum*, *F. solani*, *F. subglutinans* and *F. verticillioides* isolated from symptomless weeds produced some main compounds comprising beauverin, cyclosporines, enniatins, equisetin, fusaric acid, integracide A and trichosetin (Ilic et al. 2017). Among these compounds equisetin, fusaric acid, beauvericin and enniatins acted as mycotoxins, while trichosetin was an efficient antibacterial compound.

Endophytic *Fusarium* isolated from the inner bark of *Taxus baccata* L. was found to be producing some antimicrobial compounds (Tayung and Jha 2010). Endophytic fungi *Fusarium* were secreting antibacterial compounds active against several pathogenic bacteria including *Staphylococcus epidermis*, *S. aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Shigella flexneri*, as well as some antifungal compounds active against pathogenic fungi *Candida tropicalis* and *C. albicans* (Tayung and Jha 2010). In addition, endophytic *F. oxysporum* of plant rhizome *Acorus calamus* was found to be producing secondary metabolites with antimicrobial activity against many pathogenic microorganisms (Barik et al. 2010). Endophytic *F. solani* showed high inhibition of six bacteria such as *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Shigella flexneri* and *E. coli* and two fungi, viz. *Candida tropicalis* and *C. albicans*, by secreting antimicrobial secondary metabolites (Tayung et al. 2011a, b). These antimicrobial compounds were analysed using GC-MS for the crud metabolites of *F. solani* including (1) dodecene, (2) hexylcyclohexane, (3) 1-tetradecene, (4) tetradecane, (5) octylcyclohexane, (6) 10-nonadecanone, (7) 8-pentadecanone and (8) 8-octadecanone (Tayung et al. 2011a). For antibacterial activity, endophytic strain BH-3 of *F. oxysporum* from the bulbs of *Lilium lancifolium* produced secondary metabolites as antibacterial against *Leuconostoc mesenteroides* (Liu et al. 2012).

Several secondary compounds such as ergosterol-5,8-peroxide, triterpene acetate and cerebroside were isolated from endophytic *Fusarium* (Effendi 2004). Strain K178 of *Fusarium maire* was able to produce the anticancer compound paclitaxel (Taxol) (Xu et al. 2006). Endophyte *F. solani* was also reported to produce paclitaxel (Chakravarthi et al. 2008; Deng et al. 2009). Endophyte *F. arthrosporioides* also secreted a Taxol compound (Li et al. 2008). Two secondary metabolites, viz. camptothecin and 10-hydroxycamptothecin, were produced by two endophytic strains MTCC 9667 and MTCC 9668 of *F. solani* isolated from plant *Apodytes dimidiata* (Icacinaceae), and these compounds are used as anticancer drugs, topotecan and irinotecan (Shweta et al. 2010). An anticancer compound rohitukine was secreted by endophytic *F. proliferatum* (MTCC9690) from plant *Dysoxylum binectariferum* (Kumara et al. 2012). A Taxol compound with anticancer activity was isolated from *F. solani* (Tayung et al. 2011a). Endophytic *F. oxysporum* from mangrove leaves *Rhizophora annamalayana* was also secreting a Taxol compound (Elavarasi et al. 2012). Endophytic *F. oxysporum* isolated from the root bark of *G. biloba* was reported to produce ginkgolide B (Cui et al. 2012). A podophyllotoxin as anticancer was produced by some endophytic *F. oxysporum* that was isolated

from medicinal plant *Juniperus recurva* (Kour et al. 2008). Endophytic *F. solani* strain P1 was producing 29.0 μg/g of podophyllotoxin, this strain isolated from roots of *Podophyllum hexandrum* in Himalayan region (Nadeem et al. 2012). A Taxol compound was secreted by endophytic *F. redolens* that was isolated from plant *Taxus baccata* L. subsp. *wallichiana* (Garyali and Reddy 2013).

In additional, *F. oxysporum* NFX06 strain from plant *Nothapodytes foetida* (Musavi et al. 2015), and endophytic *F. solani* MTCC 9668 (Venugopalan et al. 2016), could produce anticancer compound camptothecin. However, the endophytic strain ZZF60 of *Fusarium* from mangroves forest secreted several secondary compounds including (1) 5-hydroxy-7-methoxy-40-O-(3-methylbut-2-enyl) isoflavone, (2) vittarin-B, (3) 3,6,7-trihydroxy-1-methoxyxanthone, (4) eriodictyol, (5) cyclo(Phe-Tyr) and (6) 1,3,6-trihydroxy-8-methylxanthone (Huang et al. 2012). Endophytic *F. oxysporum* could produce anticancer drug vincristine by converting vinblastine to vincristine (Kumar and Ahmad 2013; Kumar et al. 2013). Endophytic *Fusarium* isolated from the fresh bulbs of *Fritillaria unibracteata* var. *wabensis* produced some medicinal compounds such as peiminine and peimisine (Pan et al. 2014). *F. redolens* 6WBY3 isolated from bulbs of *Fritillaria unibracteata* var. *wabuensis* was secreting imperialine-3β-D-glucoside and peimisine (Pan et al. 2015). Five isolates of endophytic *Fusarium* such as *F. oxysporum* (one isolate), *F. incarnatum* (two isolates) and *F. solani* (two isolates) produced cinchona alkaloids, such as quinine, quinidine, cinchonine and cinchonidine (Hidayat et al. 2016). Weikl et al. (2016) demonstrated the ability of non-pathogenic *Fusarium* to produce the complex VOCs such as sesquiterpenes.

## 3.3 Other Mechanisms

Honda and Kawakubo (1998) used simultaneously two isolates of non-pathogenic *Fusarium* from healthy root of rakkyo (*Allium chinense*), viz. *F. oxysporum* and *F. moniliforme* against *F. oxysporum* f. sp. *allii* causing basal rot of rakkyo. The mode of action for non-pathogenic *Fusarium* could include several mechanisms including competition for nutrients and infection sites, induced resistance, etc., but the efficacy of these mechanisms depends on the kind of strain and isolates (Fravel et al. 2003).

### 3.3.1 Mycoparasitism

Benhamou et al. (2002) reported the ability of non-pathogenic *F. oxysporum* Fo47 strain to attack other fungal pathogens. The Fo47 strain could inhibit the mycelial growth of *Pythium ultimum* causing damping-off of cucumber and reported the ability of Fo47 to grow inside the cells of *P. ultimum* (Benhamou et al. 2002). Non-pathogenic *F. oxysporum* S6 could attack the sclerotia of *Sclerotinia sclerotiorum*, considered as a mycoparasite (Rodrıguez et al. 2006). Also, Tsapikounis (2015) reported several isolates of *Fusarium* able to mycoparasitism on the sclerotia of *Sclerotinia sclerotiorum*.

### 3.3.2 Antibiosis

Non-pathogenic *Fusarium* produces hydrolytic enzymes and secondary metabolites inhibiting the growth of plant pathogens without direct physical contact. Fo47 strain of non-pathogenic *Fusarium* secretes some antifungal against *P. ultimum* (Benhamou et al. 2002). Two strains of non-pathogenic *Fusarium* such as *F. solani* CS-1 and *F. oxysporum* CS-20 were inducing the systemic resistance in some vegetables such as watermelon (*Citrullus lanatus*) and tomato (*Lycopersicon esculentum*) against Fusarium wilt (Larkin and Fravel 1999). Endophytic *F. equiseti* produced two trichothecene compounds, viz. 4,15-diacetoxy-12,13-epoxy-trichothec-9-en-3-ol (diacetoxyscirpenol) and 4,15-diacetoxy-12,13-epoxy-3,7-dihydroxytrichothec-9-en-8-one (4,15-diacetylnivalenol), very effective against *Meloidogyne incognita* causing the egg-hatching inhibition and immobilization of juveniles at second stage (Nitao et al. 2001).

Additionally, cyclosporine produced by *F. oxysporum* strain S6 could inhibit the formation of sclerotia of *Sclerotinia sclerotiorum* (Rodrıguez et al. 2006). Non-pathogenic *F. oxysporum* was inducing in pepper some bioactive compounds against pathogenic *V. dahliae* including caffeic acid, ferulic acid and chlorogenic acid (Veloso et al. 2016). High antifungal activity against spore germination of some plant fungal pathogens was detected in the culture filtrate of non-pathogenic *F. oxysporum* strain F221-B (Thongkamngam and Jaenaksorn 2016). This antifungal of F221-B strain could cause damage to the spores (Thongkamngam and Jaenaksorn 2016). The cell-free culture filtrates of endophytic *F. proliferatum* I92 showed antifungal activity against fusarium crown and root rot in tomato (Nefzi et al. 2018).

### 3.3.3 Competition

Non-pathogenic *Fusarium* compete with plant pathogens for space and nutrients (Nagao et al. 1990; Couteaudier and Alabouvette 1990; Alabouvette 1990; Larkin and Fravel 1999; Benítez et al. 2004). This competition for nutrients is important for non-pathogenic *Fusarium* for growth and sporulation. Non-pathogenic *Fusarium* strain Fo47 could compete for carbon with pathogenic *F. oxysporum* (Duijff et al. 1998). Larkin and Fravel (1999) demonstrated the ability of non-pathogenic *F. oxysporum* strain Fo47 for competing for glucose with the pathogenic *F. oxysporum*. Competition was observed between pathogenic *F. oxysporum* f. sp. *lycopersici* and non-pathogenic *F. oxysporum* for root exudates on the surface of the tomato roots (Olivain and Alabouvette 1999). Non-pathogenic *F. oxysporum* Fo47 strain was competing for nutrients with pathogenic *F. oxysporum* f. sp. *lycopersici* Fo18 strain (Olivain et al. 2006). Two strains ML-5-2 and HK-5b-4-1 of non-pathogenic *F. oxysporum* were competing with *F. oxysporum* f. sp. *vanillae* for nutrients (Xia-Hong 2007).

Gizi et al. (2011) applied the F2 strain of non-pathogenic *F. oxysporum* against pathogenic *Verticillium dahlia* that F2 strain reduced 68% of *Verticillium* wilt disease incidence in eggplant by competing for nutrient and space at the surface and inside

the roots. Non-pathogenic *Fusarium* competed for nutrients with *F. oxysporum* f. sp. *niveum* that reduced *Fusarium* wilt of *V. villosa* (Himmelstein 2013). Fo47 strain was competing for nutrients with pathogenic *V. dahliae* on root surface of pepper (Veloso et al. 2016). Some isolates of non-pathogenic *Fusarium* could produce a siderophore to compete for iron with pathogenic *F. oxysporum* f. sp. *cubense* tropical race 4 (*Foc*TR4) LJ27 strain from banana of Palau Penang in Malaysia that lead to high reduction of *Fusarium* wilt disease of banana (Al-Ani 2017b).

### 3.3.4 Induced Plant Resistance

The induced resistance in plants is a mode of action that affects the pathogens indirectly (Al-Ani 2018a). Non-pathogenic *Fusarium* sp. is able to induce the plant resistance (Benhamou and Garand 2001). The plant resistance is restricted to the proliferation of non-pathogenic *Fusarium* in the inner roots (Validov et al. 2011).

The Fo47 strain of non-pathogenic *F. oxysporum* showed high efficacy against *F. oxysporum* f. sp. *lycopersici* causing *Fusarium* wilt of tomato through the split root methods (Fuchs et al. 1997). The split root system includes four methods: (A) benomyl system, (B) split root system, (C) cutting system and (D) layering system (Fuchs et al. 1997). Non-pathogenic *F. oxysporum* Fo47 strain could induce systemic resistance in tomato plant by accumulating both PR-1 proteins and chitinases (Duijff et al. 1998). The split root is a very interesting method for detecting the ability of non-pathogenic *Fusarium* isolates to induce plant resistance. Volatile compounds of non-pathogenic *Fusarium* could induce the plant resistance against *Pseudomonas syringae* in *Arabidopsis thaliana* (Bitas and Kang 2012). The process of induced resistance through non-pathogenic *F. oxysporum* Fo47 strain in cucumber against *P. ultimum* was demonstrated by Benhamou et al. (2002). The systemic resistance was induced through upregulating some defence-related gene, viz. POX, PIR7A, lectin, PR-3, PAE, PAL, catalase and PR-1, against *Radopholus similis* through treating banana (*Musa* spp.) with non-pathogenic *F. oxysporum* (Paparu et al. 2007).

### 3.3.5 Induced Plant Defences

Non-pathogenic *Fusarium* has the ability to induce plant defence (Paparu et al. 2007). The modulation of phytohormone regulators such as jasmonic acid, ethylene, abscisic acid, salicylic acid and auxin by non-pathogenic *F. oxysporum* strains leads to induction plant defence network (Di et al. 2016). Olivain et al. (2003) observed non-pathogenic *F. oxysporum* having the ability to induce the plant defence in flax plant by affecting the host physiology against pathogenic *F. oxysporum* f. sp. *lini* Foln3 strain.

Non-pathogenic *Fusarium*, viz. *F. oxysporum*, *F. moniliforme*, *F. merismoides* and *F. solani*, induced the plant defences by activating and increasing the polyphenol oxidase and peroxidase content in tomato when (A) directly treated with the

spore suspension and (B) extraction of cell wall elicitors (Patil et al. 2011). Many strains of non-pathogenic *Fusarium* could induce the plant defences enzymes such as phenylalanine ammonia lyase (PAL), β-1,3-glucanase, polyphenol oxidase (PPO), chitinase and peroxidase (POD) that inhibited the pathogenic growth in watermelon (Raghunandan 2013). Fo47 strain of non-pathogenic *F. oxysporum* could induce the defence genes such as a class II chitinase (CACHI2), basic PR-1 protein (CABPR1) and sesquiterpene cyclase (CASC1) in pepper against *Phytophthora capsici* and *Verticillium dahliae* (Veloso and Díaz 2012). Enhancement in the activities for three enzymes, viz. PPO, POD and PAL, in Chinese herbal *Dioscorea zingiberensis* was observed when treated with three oligosaccharides from endophytic *Fusarium oxysporum* Dzf17 strain (Li et al. 2012; Li et al. 2014). The gene expression of PR3, LOX1, PAL1, CsCam12, NPR1 and CsCam7 could be induced through inoculation of non-pathogenic *F. oxysporum* CS-20 in cucumber roots (Pu et al. 2014). Fo47 strain has the ability to induce plant defences through jasmonyl isoleucine and salicylic acid in pepper against the pathogen *V. dahliae* (Veloso et al. 2016). Three compounds comprising 4-hydroxybenzoic acid, gibepyrone D and indole-3-acetic acid (IAA) produced by non-pathogenic *F. oxysporum* 162 could induce plant defence against plant pathogenic nematodes (Bogner et al. 2017).

### 3.3.6 Induced Changes in Phytochemistry

Non-pathogenic *Fusarium* induce changes in phytochemistry (Huang et al. 2008). A non-pathogenic *Fusarium* strain Rs-F-in-11 was observed to be eliciting the metabolic pathway against strain Py71–1 of *Pythium ultimum* in *Lepidium sativum* (Ishimoto et al. 2004). Ishimoto et al. (2004) found that strain Rs-F-in-11 induced myrosinase enzyme in roots of *L. sativum* and this enzyme catalysed the hydrolyzation of glucosinolates to isothiocyanate, leading to the accumulation of isothiocyanates in the roots.

Non-pathogenic *Fusarium* as endophyte can alter the phenolic profile including ferulic acid, vanillic acid and caffeic acid in the leaves and roots of tomato against pathogenic *F. oxysporum* f. sp. *lycopersici* (Panina et al. 2007). The strain 162 (FO162) of non-pathogenic *Fusarium* could colonize tomato and induce the roots to produce the repellent substance against nematode *M. incognita* juveniles (Dababat and Sikora 2007). Endophytic *F. oxysporum* strain Fo162 could induce changes in the proliferation of banana root affecting the growth of nematode *Radopholus similis* (Kurtz 2010). Three polysaccharides, viz. exopolysaccharide, sodium hydroxide-extracted mycelial polysaccharide and water-extracted mycelial polysaccharide of endophytic *F. oxysporum* Dzf17, affected the biosynthesis of secondary metabolites and growth for *Dioscorea zingiberensis* (Li et al. 2011a, b). Some non-pathogenic *Fusarium* isolates were inducing the free phenol content and total protein content in tomato (Patil et al. 2011) and watermelon (Raghunandan 2013).

### 3.3.7 Non-pathogenic *Fusarium* as Biofertilizers

Non-pathogenic *Fusarium* enhance the plant growth by producing the gibberellic acid (GA) (Leslie 1996), IAA (Bogner et al. 2017) and siderophores (Al-Ani 2017b) and enhance the nutrient utilization efficiency (Zhang et al. 2012). Louter and Edgington (1990) reported the ability of non-pathogenic *Fusarium* such as *F. oxysporum* and *F. solani* in reducing the tomato root rot and increased the yield. However, endophytic *Fusarium* such as *F. arthrosporioides* and *F. oxysporum* were pathogenic for plant parasitic *Orobanche aegyptiaca* though affecting the size and number of shoots for *O. aegyptiaca* by producing IAA (Cohen et al. 2002b). The high production of IAA was for co-transforming two genes both of *iaaH* and *iaaM* in *Fusarium* that was probably increased for suppressing the appressoria formed on infected *Orobanche aegyptiaca* through attack on tomato (Cohen et al. 2002b). Some strains of non-pathogenic *Fusarium* can reduce the plant diseases and simultaneously enhance the plant growth. The KGL0401 strain of *F. proliferatum* was reported to produce several new gibberellins (GAs) (Rim et al. 2005). The conidial suspension $10^8$–$10^9$ (spores/ml) of *F. oxysporum* B6 significantly enhanced various plant growth parameters such as leaf length and leaf area, plant height and root fresh weight (Mennan et al. 2005).

In addition, endophytic *Fusarium* improved plant growth by secreting gibberellin (GA), indole acetic acid (IAA) and auxin (Dai et al. 2008). Thangavelu and Jayanthi (2009) reported a very effective strain of non-pathogenic *F. oxysporum* Ro-3 for reducing *Fusarium* wilt severity of banana. *F. oxysporum* Ro-3 also increased plant height, petiole length, leaf area, girth and the number of leaves (Thangavelu and Jayanthi 2009). Bitas and Kang (2012) reported an isolate of *F. oxysporum* producing VOCs that enhanced the plant growth by promoting root and shoot growth in *A. thaliana*. Also, non-pathogenic *Fusarium* increased the plant growth of watermelon by producing the IAA and GA with the solubilization for phosphate (Raghunandan 2013). *F. oxysporum* could enhance the plant growth of *A. thaliana* and tobacco by producing many volatile compounds (Bitas et al. 2015). Also, LeBlanc (2015) isolated many non-pathogenic *Fusarium* producing secondary metabolites such as bikaverin (BIK), IAA and GA. *F. solani* I149 isolate also improved plant growth in axenic cherry plants (Ilic et al. 2017), while endophytic *F. proliferatum* I92 enhanced plant growth in tomato (Nefzi et al. 2018).

### 3.3.8 Secreting the Enzymes

Non-pathogenic *Fusarium* produce many hydrolytic enzymes. β-D-glucuronidase (GUS) was detected by using a new method in the tomato roots that were treated with 70 T01 strain of non-pathogenic *F. oxysporum* (Bao and Lazarovits 2002). Myrosinase was produced by non-pathogenic *Fusarium* strain Ls-F-in-4-1 that inhibited the mycelial growth of *P. ultimum* strain Py71-1 (Ishimoto et al. 2004). Endophytic *F. proliferatum* I92 could produce several hydrolytic enzymes, viz.

chitinase, lipase, amylase and proteases, that may affect *Fusarium* crown and root rot in tomato (Nefzi et al. 2018).

## 3.4 Conclusion

Non-pathogenic *Fusarium* are soil-borne fungi, as well as an endophyte in the host system. This genus produces a variety of chemically diverse secondary metabolites. Some of the secondary metabolites have been identified but many are yet to be identified. Non-pathogenic *Fusarium* has other mechanisms that are very useful for agricultural production. Non-pathogenic *Fusarium* plays a huge role in agriculture through production of bioactive secondary metabolites having diverse functions. Other modes of actions include (1) induction of host defence response, induction of resistance genes and secretion of some plant activators; (2) secretion of various antibiotics against phytopathogens; (3) restricting the nutrient supply to competing microorganisms; and (4) production of hydrolytic enzymes that may be utilized against plant pathogens.

Non-pathogenic *Fusarium* can be separated from pathogenic *Fusarium* by pathogenicity/virulence testing. Also, the ability of the non-pathogenic *Fusarium* for production of mycotoxins should be tested. Finally, the importance of application of non-pathogenic *Fusarium* over chemical pesticides is far outreaching and comparatively more beneficial.

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# Non-mycorrhizal Fungal Spectrum of Root Communities

4

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## 4.1 Partners/Associated Communities of Plant Root Environment

Rhizosphere is a very interesting and complicated environment surrounding plant roots and significantly different from those of the bulk soil. This is because a continuous flux of carbon is exudated into the rhizosphere environment by plant roots, significantly affecting soil microorganisms and their competition. There are very different types of microorganisms in the soil rhizosphere interacting with the other microbes and with plant roots (Bisen et al. 2015; Mishra et al. 2015; Keswani et al. 2016). The activity and interactions of rhizotrophic microorganisms can very much influence soil conditions and hence plant growth and microorganism activities (Miransari 2011; Singh et al. 2016; Singh et al. 2017). Ma et al. (2015) reported that orchid non-mycorrhizal fungal root associates contain 110 genera of which roughly 76 belong to *Ascomycota* and 32 genera of *Agaricomycetes* in *Basidiomycota*. They also involve a few species of *Pezizomycetes*, *Eurotiomycetes*, *Chaetothyriomycetes*, *Helotiales* and *Xylariales* of *Ascomycetes*. Among all genera observed in orchid non-mycorrhizal fungi, *Colletotrichum* and *Fusarium* frequently appeared in different orchids such as *S. nepalense* and *D. nobile*. *Aspergillus*, *Trichoderma* and *Verticillium* have also been repeatedly found in orchids. They are referred to as 'core group fungi' because their frequency of occurrence is ≥10% (Sudheep and Srindar 2012).

The dominance of *Helotiales* in root-associated fungal communities has also been reported in various environments such as Arctic tundra and warm-temperate forests. Although the order *Helotiales* includes diverse fungal functional groups,

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such as ectomycorrhizal, saprotrophic and endophytic species, several clades of fungi within the order possibly benefit their plant hosts by mineralizing organic nitrogen in the rhizosphere. *Helotiales* endophytes can be major participants in belowground plant-fungal associations in various types of forests, although their ecological functions to plant hosts need to be further investigated (Toju et al. 2013).

Plant-fungal interactions are important determinants of plant community assembly and ecosystem functioning. Plant roots interact with a range of soil fungi which can influence plant growth and fitness, plant community composition as well as ecosystem functioning. Depending on the identities of the host plant and fungus, these interactions can be of a mutualistic, neutral or parasitic nature. In grasslands, arbuscular mycorrhizal fungi (AMF) are dominant symbiotic fungal partners and are known to increase nutrient status, improve water relations and protect host plants against pathogens. In addition to AMF, which extend in the rhizosphere, plant roots are often colonized by fungal endophytes that reside completely within plant tissues. However due to their microscopic nature and the difficulty of isolating many root-associated fungi, a large fraction of species remains unknown and molecular methods often essential to describe these fungal communities. Root-associated fungal community composition also seems to depend on dispersal limitation, and on host species identity, many fungal pathogens of plants are host specific. In semiarid grasslands, *Ascomycota* are commonly found to be the dominant root-colonizing fungal group. *Phialophora* species are known to form a complex group of fungi with endophytes, saprobes and plant pathogens. The second most abundant fungus was identified as related to *Paraphoma*. A relatively large number of *Basidiomycota* (18.3%) are typically more frequent in forest soils. The most abundant genus of *Basidiomycota* and the third most abundant overall was *Sebacina*. *Sebacinalean* fungi are common endophytes in many plant roots and may enhance plant growth and pathogen protection (Wehner et al. 2014).

Specifically, root endophytic fungi (REF) in the *Ascomycota* are a group of symbiotic partners that may be important players driving plant community structure in grassland ecosystems (Aguilar-Trigueros et al. 2014). Because the term endophyte is ambiguous, it is used to refer to fungi that during root tissue colonization of grasses and forbs (1) do not induce symptoms of disease and (2) do not form specialized fungal-plant interfaces for the exchange of resources like the ones observed in AMF (Aguilar-Trigueros et al. 2014). REF in grasslands occur in all major fungal phyla, but in comparison with AMF, there is less information on their influence on host physiology and their ecological role. However, there are several lines of evidence that indicate REF in *Ascomycota* may influence plant community structure. Firstly, REF in the *Ascomycota* are abundant and ubiquitous as revealed by community surveys using DNA sequencing methods in some cases being five times higher in terms of species richness compared to AMF among grassland species (Wehner et al. 2014). Secondly, they can have broad host ranges, while colonized plants species respond differently to even the same fungal genotype. For example, when multiple plant species were inoculated with the same strain of *Microdochium* sp. under the same conditions, some plant species showed increased biomass production, while others remained unaffected despite being also colonized, whereas a

strain of *Gibberella* sp. pathogenic on *Pinus radiata* has been found as an endophyte among neighbouring grasses without inducing disease (Sweet and Gordon 2012). In fact, it is increasingly acknowledged that fungal species in *Ascomycota* only described as pathogens actually live as asymptomatic endophytes on a larger set of hosts (Malcolm et al. 2013; Stergipoulos and Gordon 2014). Aguilar-Trigueros et al. (2014) designed an experimental microcosm to recreate conditions found in natural grassland in which 70% of vegetation is covered by *Festuca brevipila* and 47 other herbaceous species which have been frequently reported for dry grasslands in north-eastern Germany. The soil was inoculated with oat kernels that fungi (*Fusarium* and *Gibberella* species) were grown on. Some inoculated kernels were used to establish a control treatment for the experiment. In the presence of *Fusarium*, *F. brevipila* and *A. elongata* produced more shoot biomass in high sand treatment compared to the plants grown in the control low sand treatment (which had higher nutrient concentration compared to the high sand treatment). This effect may be due to fungal mineralization (saprotrophic) capabilities which are known to depend on availability of inorganic versus organic nutrient sources (Mayerhofer et al. 2013). They also found the soil parameters modify the community structure of REF in the grassland and its feedback on plant communities. The consequences of these differential plant growth responses to REF may result in interesting cases of indirect ecological interactions. For example, when some plant species supress the growth of competing neighbours by hosting fungal species detrimental to others, this has been referred to as 'apparent competition'.

Monocultures vs. mixtures also show that endophyte-endophyte interactions have important implications on host community structure. Wagg et al. (2011) also showed the competitive dominance and complementarity among three AMF species that were present in the soil of experimental microcosm depended on soil type.

## 4.2 Plant-Non-mycorrhizal Fungi Relationship

### 4.2.1 Contributions of Associated Rhizospheric Fungi to Plant

Non-mycorrhizal fungi have been identified from the roots of various terrestrial and epiphytic orchids, mostly during the attempt to find mycorrhizal fungi (Herrera et al. 2010). Unlike other mycorrhizal associations, in most cases orchid root fungi are not thought to be mycorrhizal with confirming bioassays. Although most of these reports only focused on identifying the fungi rather than exploring their potential effects on the host orchids, some investigations on root-associated fungi of other plant families revealed that endophytic fungi occurrence in roots have sometimes more greater abundance than mycorrhizal fungi. Moreover, there is accumulating evidence that both foliar and root-associated fungal endophytes provide underappreciated beneficial effects on host plants. There are possible ecological linkages, and interactions between above-ground and belowground fungal biota may occur and eventually influence the host phenotype. Non-mycorrhizal endophytic fungi constitute an important fungal consortium in *D. nobile* roots (Yuan et al. 2009).

Root-associated fungal communities showed that fungal alpha diversity is determined mainly by the microhabitat type, i.e. bulk sol, rhizosphere or root compartment. Furthermore the microhabitat type also affected the structure of fungal communities, i.e. the presence of taxa and their relative abundances. In addition to strong microhabitat effect, the soil geographical provenance was the second largest driver of fungal community structure. Soil fungal communities show strong biogeographical patterns shaped by local climatic and edaphic factors (Tedersoo et al. 2014; Talbot et al. 2014; Peay et al. 2016). The microbial activity in rhizosphere is under direct influence of plant roots, which release organic material, mainly as root exudates. The exudates serve as substrates for the indigenous microorganisms. Rhizospheric and non-rhizospheric populations could be discriminated on the basis of their ability to use specific organic compounds, to mobilize ferric iron and to reduce nitrogen oxides.

Microorganisms associated with plant roots both free and symbiotically living would help the host plant to adapt to stress conditions concerning water and mineral nutrition and soilborne pathogens. Non-mycorrhizal root endophytes are a heterogeneous group of fungi with possible beneficial associations with their host plants. Recent data from a primary successional subalpine ecosystem and a secondary successional temperate grassland ecosystem suggest greater abundance of non-mycorrhizal endophytes than mycorrhizal fungi. Two competing hypotheses are presented: (1) these fungi are parasitic and tap into the host photosynthate translocation during seasonal changes in host physiological activity; (2) these fungi are actively involved in controlling host photosynthate reallocation. Large proportion of the root and rhizosphere inhabiting fungi are common soil fungi and facultatively colonize host tissues (Jumpponen 2001).

P transfer to their hosts was considered a hallmark of mycorrhizal fungi, but the question of whether non-mycorrhizal species thrive due to the exploitation of alternative P-mining strategies is not well understood. Some studies on binary root-fungus interactions showed that two endophytes – the ascomycete *Colletotrichum tofieldiae* and the basidiomycete *Serendipita indica* (syn. *Piriformospora indica*) – are able to transfer P to their non-mycorrhizal host *Arabidopsis thaliana* promoting its growth under low P conditions (Almario et al. 2017).

The rhizosphere is also a battlefield where the complex rhizosphere community, both microflora and microfauna, interacts with soilborne pathogens and influences the outcome of pathogen infection. The growth or activity of soilborne pathogenic fungi, oomycetes, bacteria and/or nematodes can be inhibited by several beneficial rhizosphere microorganisms. The activity and effects of beneficial rhizosphere microorganisms on plant growth and health are well documented for fungi from *Deuteromycetes* (e.g. *Trichoderma*, *Gliocladium* and non-pathogenic *Fusarium oxysporum*). These beneficial fungi are also referred as biocontrol agents (Raaijmakers et al. 2009). Biocontrol microorganisms may adversely affect the population density, dynamics (temporal and spatial) and metabolic activities of soilborne pathogens via mainly three types of interactions, which are competition, antagonisms and hyperparasitism. In rhizosphere, competition takes place for space

at the root surface and for nutrients, noticeably those released as seed or root exudates. Competitive colonization of the rhizosphere and successful establishment in the root zone are prerequisites for effective biocontrol, regardless of the mechanism(s) involved. Competition can also take place for micronutrients, especially iron, that are essential for growth and activity of pathogen. Competition for iron and competition for carbon are documented as important modes of action for several biocontrol bacteria and fungi (Lemanceau et al. 1992; Alabouvette et al. 2006), with iron competition being particularly significant in calcareous soils where high pH leads to low iron solubility (Raaijmakers et al. 2009).

Production of extracellular lytic enzymes is quite common among antagonistic microorganisms. Extracellular lytic enzymes act in different ways; many of them can affect the cell wall of pathogens. In addition to competition and antagonism, direct biocontrol effects on soilborne plant pathogens can result from hyperparasitism. This is mainly documented for *Trichoderma* and *Gliocladium*, and it affects various fungal pathogens, such as *Rhizoctonia*, *Sclerotinia*, *Verticillium* and *Gaeumannomyces* (Harman et al. 2004). Hyperparasitism by *Trichoderma* involves secretion of chitinases and cellulases.

Almario et al. (2017) assessed the effect of root fungal microbiome of *A. alpina* and its contribution to plant P acquisition. In order to determine fungal microbiome of *A. alpina* roots (soil zone immediately surrounding the root), Illumina-based amplicon sequencing of the fungal taxonomical marker *ITS2* was used. Microbiome variability analysis showed that root fungal communities were more robust in response to changing environments and 15 fungal taxa comprised of one zygomycete (*Mortierella elongata*), one basidiomycete (Ceratobasidiaceae sp.), 13 ascomycete belonging to Helotiale (4 OTUs), Pleosporales (4 OTUs), The Hypocreales (3 OTUs), the Sordariales (1 OTU) an done unclassified order consistently detected. A fungal taxon belonging to *Helotiales* was detected in high abundance in roots of *A. alpina*.

Rhizosphere inhabiting microorganisms promote plant growth and protect plants from pathogen attack by a range of mechanisms (Lugtenberg and Kamilova 2009; Raaijmakers et al. 2009). These involve biofertilization, stimulation of root growth, rhizoremediation, control of abiotic stress and disease control. Most, if not all, rhizobacteria produce metabolites that inhibit the growth or activity of competing microorganisms. Also, rhizosphere fungi are prolific producers of antibiotic metabolites (Hoffmeister and Keller 2007). Especially *Trichoderma* species have received considerable attention for the production of antimicrobial compounds (Vyas and Mathus 2002; Harman et al. 2004; Elad et al. 2008; Druzhinina et al. 2011).

Next to the biocontrol activity of rhizosphere microorganisms, several can have a direct positive effect on plant growth and health. First, photostimulatory and biofertilizing microbes can promote plant health by making the plant ‘stronger’. Second, many rhizosphere microorganisms can induce a systemic response in the plant, resulting in the activation of plant defence mechanisms. This capacity has been identified in a wide range of bacteria, endophytes as well as saprophytic, hyperparasitic and arbuscular mycorrhizal fungi.

## 4.3 Lifestyles and Existence of Root-Associated Fungal Communities

Plant roots release a wide range of compounds that are involved in attracting beneficial organisms and forming mutualistic associations in the rhizosphere. Plant-released compounds like sugars and amino acids are potential fungal stimuli. Even less understood than the signalling between plants and mycorrhizae is the interaction of mycorrhizae with other soil microbes (Badri et al. 2009).

Non-mycorrhizal fungal species can be classified into several groups according to their lifestyles, i.e. ECM fungi, saprobes, parasites and latent pathogens. However, fungal lifestyles are not always stable traits. Some of the non-mycorrhizal endophytes are plant pathogens. For example, *Fusarium oxysporum* can cause plant wilt and root diseases. *Alternaria*, *Aspergillus*, *Chaetophoma* and *Trichoderma* have relationships with cotton plant disease. Latent pathogens in plants have been noticed from the 1950s. They may exist as endophytes and probably become pathogens during a later period of life, especially when plants are stressed.

The role of orchid non-mycorrhizal endophytes has rarely been addressed. In general, plant endophytes are thought to be the resources for bioactive compounds. For example, a *Trichoderma* species from Cupressaceae was shown to have antimicrobial properties. Screening bioactive compounds for disease treatment from higher plants has increased. Potential pharmaceutically important substances are abundant in orchids, and this to some extent may be a result of extreme diversity of non-mycorrhizal fungal metabolites. *Alternaria* sp. and *Fusarium oxysporum* isolated from orchids in Brazil showed strong inhibition to *Escherichia coli*. From the orchid *Anoectochilus setaceus*, an antibacterial nortriterpenoid helvolic acid was extracted from the endophytic taxon *Xylaria* sp. These orchid non-mycorrhizal endophytes may occur in other plants and possibly be involved in the production of bioactive compounds. Golgo et al. screened bioactive metabolites from *Hypocrea* spp. isolated from *Dillenia indica*. *Hypocrea* species have also been isolated from orchids such as *Wullschlaegelia aphylla* and *Himantoglossum adriaticum*. Xu et al. (2014) found that 160 metabolites isolated from *Pestalotiopsis* species had antitumor, antifungal or antimicrobial potential (Yuan et al. 2009).

Non-mycorrhizal *Fusarium* was reported to promote seed germination in *Cypripedium* and *Platanthera* orchids, even though the effect was relatively minor when compared to that of specific orchid *Rhizoctonia* mycorrhiza. Similarly, *Umbelopsis nana* isolated from *Cymbidium* spp. has a vigorous effect on development of *Cymbidium hybridum* enhancing K, Ca, Cu and Mn contents in symbiotic plantlets. Researchers detected fuel potential in volatile organic compounds isolated from *Phomopsis* sp. from orchid *Odontoglossum* sp.

Many saprobic species of *Agaricomycetes* (i.e. *Hydropus*, *Gymnopus*, *Marasmiellus*) and *Sordariomycetes* (i.e. *Clonostachys*, *Resinicium*) have been identified as orchid non-mycorrhizal endophytic fungi. Endophytes are important saprobic decomposers. The diversity of non-mycorrhizal endophytic fungi in orchids is higher in leaves than roots. Tao et al. found that there was overlap in the case of few endophytic in roots and leaves of *Bletilla ochracea*. They pointed out

that orchid leaves and roots had different endophyte associations and speculated that this was probably because organ texture provided different ecological habitats (air or below ground) with varying physiology and chemistry for the taxa.

Plants are colonized by an astounding number of microorganisms that can reach cell densities much greater than the number of plant cells. Also, the number of microbial genes in the rhizosphere outnumbers by far the number of plant genes. Rhizosphere, that is, the narrow zone surrounding and influenced by plant roots, is a hot spot for numerous organisms and is considered as one of the most complex ecosystems on Earth. These organisms which have been well studied for their beneficial effects on plant growth and health are nitrogen-fixing bacteria, mycorrhizal fungi, plant growth-promoting rhizobacteria, biocontrol microorganisms, mycoparasitic fungi and protozoa. A third group of microorganisms that can be found in the rhizosphere are the human pathogens. Understanding the processes that shape and drive the composition and dynamics of the rhizosphere microbiome is therefore an essential step not only to safeguard plant productivity but also to safeguard human health (Mendes et al. 2013).

Culture-independent approaches have shown that microbial diversity of soil and rhizosphere microbiomes is highly underestimated, and most studies have focused on the number and diversity of bacterial taxa rather than on other rhizosphere inhabitants. In addition to comprehensive phylogenetic analysis of the rhizosphere, there is need to beyond cataloguing microbial communities and to determine which microorganisms are active during the different developmental stages of plant/root growth.

## 4.4 Concluding Remarks

Understanding the effects of root-associated microbes in explaining plant community patterns represents a challenge in community ecology. Although typically overlooked, several lines of evidence point out that non-mycorrhizal, root endophytic fungi in the *Ascomycota* may have the potential to drive changes in plant community ecology given their ubiquitous presence, wide host ranges and plant species-specific fitness effects. Results indicate that plant responses to changes in the species identity of non-mycorrhizal fungal community species and their interactions can modify plant community structure (Trigueros and Rilig 2016).

The complexity of soil fungal communities challenges our ability to understand the effects of such interactions on plant performance and on ecosystem processes. Recent surveys show that roots interact with phylogenetically diverse groups of fungi (Tedersoo et al. 2009). Moreover, the effects of particular plant-fungal combinations depend on environmental conditions and on the host and fungal genotypes (Schulz and Boyle 2005).

Many ecosystem functions are determined by biotic root traits involving direct interactions with microorganisms, especially mycorrhizal fungi. But there is growing evidence that root traits have strong impacts on ecosystem processes via interactions with free-living microorganisms. Future studies need to explore how root traits

influence the soil community and its activities and how these impacts cascade to the soil processes on which the functioning of terrestrial systems depend (Bardgett et al. 2014). Recent advances in molecular methods and omic technologies provide an exciting opportunity to redefine the relationships between plants and the microbes in their rhizospheres and allow us to further underpin these interactions efficiently for agricultural benefit (Singh et al. 2004; Badri et al. 2009).

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# Bioactive Volatile Metabolites of *Trichoderma*: An overview

5

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## 5.1 Introduction

The agricultural crop loss occurred worldwide due to various biotic factors which can lead up to 40% economic loss (Oerke and Dehne 2004). To combat loss which occurs due to plant diseases and feeding growing human population without causing loss to ecosystem, alternative measures demand for sustainable approaches including the use of biocontrol agents/plant probiotic agents (Godfray et al. 2010; Mishra et al. 2015; Rasmann et al. 2017; Sharma et al. 2017a, b, c, d). The filamentous and saprophytic life cycle of *Trichoderma* have attracted considerable attention worldwide and can help in achieving sustainable agriculture growth. So far *T. harzianum*, *T. virens*, *T. viride*, *and T. saturnisporum* (Sharma and Shanmugam 2012, Sharma et al. 2017d; Sharma et al. 2018a) have been studied for their biocontrol attributes and commercial development of bioformulations against wide range of soilborne and airborne phytopathogens (Kubicek and Harman 1998; Harman et al. 2004; Lorito et al. 2010). Presently, *Trichoderma*-based bioformulations constitute over 60% of the registered biopesticides and are also effective for bio-management of insects (Jassim et al. 1990; Ganassi et al. 2007; Shakeri and Foster 2007; Verma et al. 2007; Bisen et al. 2015; Singh et al. 2016).

The molecular attributes of *Trichoderma* spp. related to its success as biocontrol agents include mycoparasitism (Weindling 1932; Howell and Stipanovic 1983; Verma et al. 2007; Bailey et al. 2009; Szabo et al. 2012; Sharma et al. 2018b), antibiosis (Howell 1998; Vos et al. 2015), competition for space and nutrients (Chet

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1987), promotion of plant growth, stimulation of lateral root development, degradation or detoxification of toxic compounds (Sharma et al. 2013), enhanced nutrient solubility and subsequent acquisitions of minerals through siderophores, organic acids and volatile compounds secretion (Altomare et al. 1999; Gravel et al. 2007; Bae et al. 2009; Contreras-Cornejo et al. 2009; Martinez-Medina et al. 2011; Vos et al. 2015), and induction of systemic resistance (Yedidia et al. 2001; Hoitink et al. 2006; Mathys et al. 2012). The recruitment of molecular arsenals by biocontrol agents is quite complex in nature and multistage regulated (Sharma et al. 2017a). For example, mycoparasitism by *Trichoderma* strain is largely executed through the extracellular secretion of lytic enzymes targeting cell wall degradation of host fungi (Sharma and Shanmugam 2012; Sharma et al. 2016; Sharma et al. 2017c). The role of different transcripts against various fungal plant pathogens has been investigated at transcripts and protein level (Sharma et al. 2013; Sharma et al. 2016; Sharma et al. 2017b) using deactivated autoclaved mycelium as simulated antagonism conditions. These conditions revealed the role of chitinases, glucanases, proteases, and other cell wall degrading enzymes as well as its transporters system in host-specific manner (Sharma et al. 2016; Sharma et al. 2017c). In a broader sense, the biocontrol mechanisms of *Trichoderma* share remarkable similarity to probiotics (Sharma et al. 2017b).

The production of secondary metabolites of volatile and nonvolatile nature is another hallmark of *Trichoderma* and considered to play significant and effective role in plant pathogen suppression and plant growth promotion (Bisen et al. 2016; Singh et al. 2017). The production of bioactive secondary metabolites of both volatile and nonvolatile nature by *T. album* and *T. harzianum* is known to inhibit the mycelial growth on *Botrytis fabae* (Barakat et al. 2014). Similarly, the antagonistic activity of *T. gamsii* YIM PH3001 against *P. notoginseng* is correlated to the production of VOCs such as dimethyl disulfide, dibenzofuran, methanethiol, and ketones. The *T. gamsii* YIM PH3001 also improved the seedling emergence and protected plants from soilborne disease in field conditions (Chen et al. 2016). The deactivated mycelium of *Fusarium oxysporum* is reported to upregulate the production of five and eight different VOCs of *T. harzianum* T-E5 (Zhang et al. 2014). The VOCs of *T. virens* Gv29.8, *T. atroviride* LU132, *T. asperellum* LU1370, and *T. atroviride* IMI206040 are well demonstrated for their ability to promote plant growth (Nieto-jacobo et al. 2017).

Similar to plants and bacteria, fungi are known to produce plethora of VOCs such as alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and their derivatives (Korpi et al. 2009). The nature, proportions, and concentrations of these VOCs are known to vary with species/strain and age of culture, substrate concentration, and interactions surrounding the environment (Sunesson et al. 1995; Wheatley et al. 1997; Wilkins et al. 2000). Starting from the discovery of first antifungal substance from *T. virens* in 1936 by Weindling and Emerson, a number of volatile and nonvolatile bioactive secondary metabolites from *Trichoderma* spp. such as anthraquinones (Luo et al. 2009), pyrones (Evidente et al. 2003), terpenes (Li et al. 2011;

Yamamoto et al. 2012), butenolides (Fukuda et al. 2012), alkaloids (Garo et al. 2003), isoharziandione (Mannina et al. 1997a, b; Warin et al. 2009), and 6-pentyl-α-pyrone have been characterized (Evidente et al. 2006). These bioactive metabolites such as isoharziandione are found to inhibit *Colletotrichum capsici* (Warin et al. 2009) and *S. rolfsii* (Mannina et al. 1997a, b), whereas 6-pentyl-α-pyrone were reported to inhibit *Pythium ulttimun* (Vinale et al. 2008) and *Armillaria mellea* (Tarus et al. 2003). 6-pentyl-α-pyrone has also been reported for its plant growth promotion ability (Dennis and Webster 1971a, b; Howell 2003). This book chapter highlights the biosynthesis and role of volatile bioactive secondary metabolites produced by *Trichoderma* spp.

## 5.2 Volatile Metabolites of *Trichoderma* spp.

The soil microbes are potential source of VOCs and play immense role in various interactions between biotic and abiotic factors of ecosystem (Bitas et al. 2013). At present, around 500 bacterial and fungal species have been explored for the production of different VOCs including alcohols, ketones, mono- and sesquiterpenes, esters, thioalcohols, lactones, and thioesters (http://bioinformatics.charite.de/mvoc/) (Splivallo et al. 2011; Kramer and Abraham 2012; Lemfack et al. 2013; Effmert et al. 2012; Lemfack et al. 2014). The beneficial *Trichoderma* strains in plant rhizosphere are known to produce a plethora of VOCs including alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and other derivatives which positively affect plant growth and reduce disease incidence (Ryu et al. 2003; Vespermann et al. 2007; Zhang et al. 2008; Korpi et al. 2009; Hung et al. 2012). The VOCs of fungi have been explored intensively for their role in signaling, agricultural and aroma in fermented foods (Chiron and Michelot 2005; Kues and Navarro-Gonzales 2009; Bennett et al. 2012), and antimicrobial activity (Strobel et al. 2001 2006). The VOCs of *Trichoderma* are gas-phase and carbon-based molecules of both low and high molecular weight origin. According to the Antibase database, over 370 different compounds of *Trichoderma* origin have been identified with importance in medicinal, agronomic, and ecological perspectives (Howell et al. 1993; Sivasithamparam and Ghisalberti 1998; Laatsch 2007; Reino et al. 2008). These VOCs of *Trichoderma* help in distributing long-lasting effects which inhibit other plant pathogens (Dennis and Webster 1970; Wheatley et al. 1997; Humphris et al. 2001; Bruce et al. 2004) and promote growth of plants (Hung et al. 2012). In recent studies, efforts have been made in understanding additional role of volatiles in multiple interactions under field conditions (Kai et al. 2009; Vespermann et al. 2007; Minerdi et al. 2009; Wenke et al. 2010; Blom et al. 2011; Junker and Tholl 2013; Naznin et al. 2013; D'Alessandro et al. 2014; Piechulla and Degenhardt 2014; Kottb et al. 2015; Chung et al. 2016). For example, the soil application of 2-butanone and 3-pentanol in cucumber seedlings led to reduced infestation of *M. persicae* aphids and increase in predatory coccinellids (Song and Ryu 2013).

## 5.3 Structure and Biosynthesis of Fungal Secondary Metabolites

The continuous studies on biocontrol attributes of *Trichoderma* spp. have led to the identification of several bioactive compounds (Moffatt et al. 1969; Collins and Halim 1972; Fujiwara et al. 1982; Almassi et al. 1991; Keswani et al. 2016). The different compounds produced by *Trichoderma* spp. include 6-pentyl-α-pyrone, antibiotics gliotoxin, viridin, gliovirin, glisoprenin, heptelidic acid, koninginins, anthraquinones, trichodermamides, peptaibols, polyketides, terpenoids, polypeptides, trichothecenes, trichodermaides, azaphilones, harzialactones, and metabolites derived from alpha-amino acids (Howell 1998; Vey et al. 2001; Reino et al. 2008; Keswani et al. 2014; Keswani 2015). These bioactive metabolites of biocontrol strains of *Trichoderma* are broadly grouped into volatile and nonvolatile compounds. The VOCs with their role as interspecies communication are also known as infochemicals or semi-ochemicals (Herrmann 2010). The volatile organic compounds (VOCs) are carbon-based molecules that readily enter the gas phase by vaporizing at 0.01 kPa (Pagans et al. 2006), hydrophobic in nature with low boiling point and polarity (Insam and Seewald 2010), and easily evaporate and diffuse to long distance in soil, air, and through porous materials (Wheatley 2002; Zogorski et al. 2006; Hung et al. 2012). They are chemically diverse in their structural compositions including main skeleton composed of hydrocarbons such as alkane, alkene, alcohol, amines, thiols, and terpenes (Korpi et al. 2009; Lemfack et al. 2013). The VOCs secreted by biocontrol strain of *Trichoderma* include hundreds of compounds such as 6-pentyl-α-pyrone, α-farnesene, calamenene, cadinene, β-cubeben, β-chamigrene, 1,2,3,4,5-pentamethyl-1,3-cyclopentadiene, α-muurolene, 2,2-dimethoxy-1,2-diphenyl-ethanone, limonene, β-bisabolene, benzoic acid, β-sesquiphellandrene,4-nitroso-,ethyl ester, farnesol, propanoic acid, and β-himachalene. Structurally, these diverse classes of VOCs belong to different hydrocarbons such as aldehydes, esters, ketones, aromatics, amines, thiols, and terpenes (Bruce et al. 2000; Vinale et al. 2008; Splivallo et al. 2011; Kramer and Abraham 2012; Lemfack et al. 2013) (Fig. 5.1a–c).

The biosynthesis of VOCs in fungi is underexplored area of research compared to plants. The VOCs are produced as side products from both the primary metabolism including synthesis of DNA, amino acids, and fatty acids, whereas secondary metabolism includes intermediates of the primary metabolism (Berry 1988; Korpi et al. 2009) and biotransformed products produced in central metabolism like terpenes (Kesselmeier and Staudt 1999; Dudareva et al. 2013; Lee et al. 2016). A brief description of the VOC s produced by *Trichoderma* is given below:

### 5.3.1 6-Pentyl-alpha-pyrone (6PP)

6PP, a compound with coconut-like odor, is one of the first volatile compounds characterized from *Trichoderma*. Initially explored in food industry (Collins and Halim 1972; Parker et al. 1999), it is now also studied for its role in plant growth

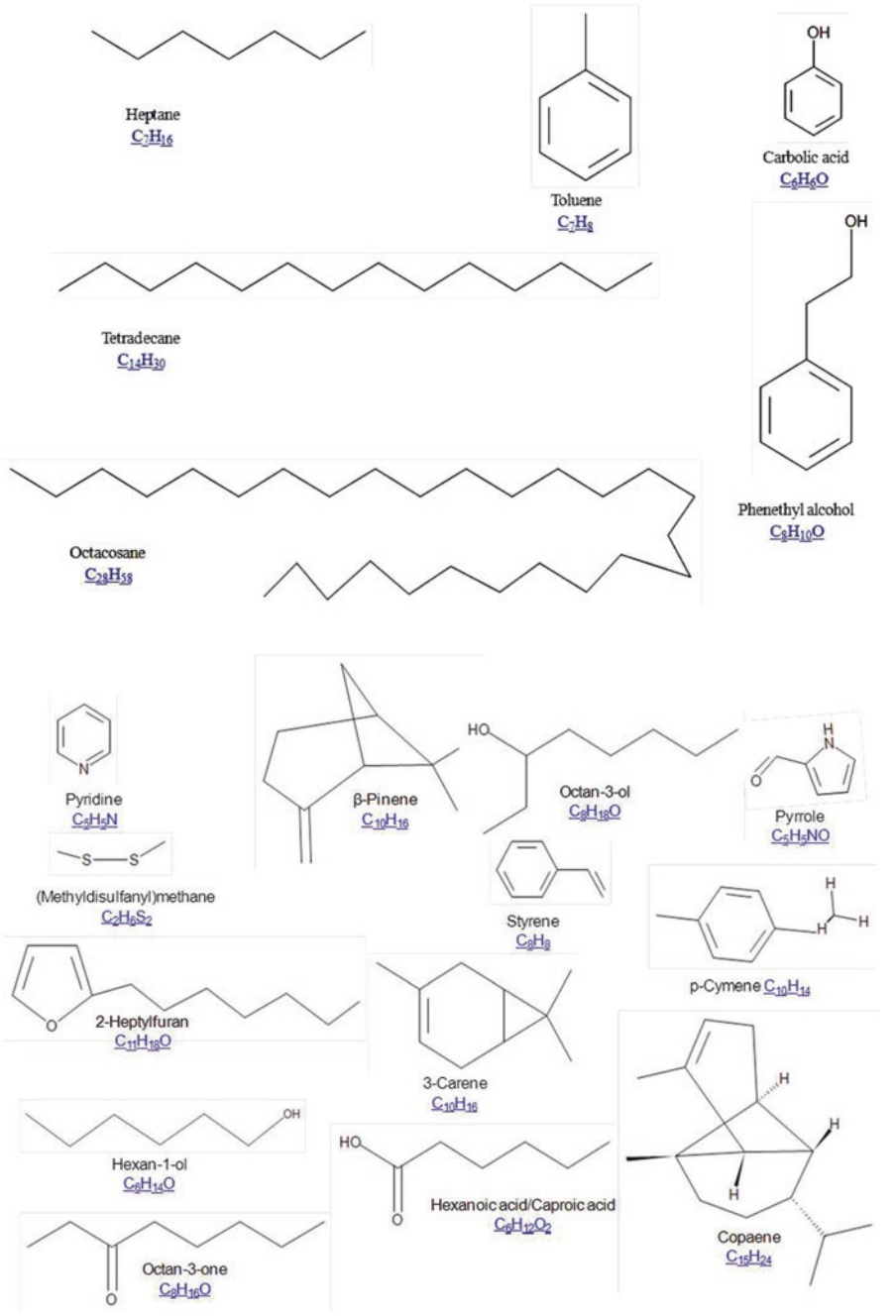


**Fig. 5.1** (**a**–**c**) Structure of volatile compounds produced by *Trichoderma* spp.

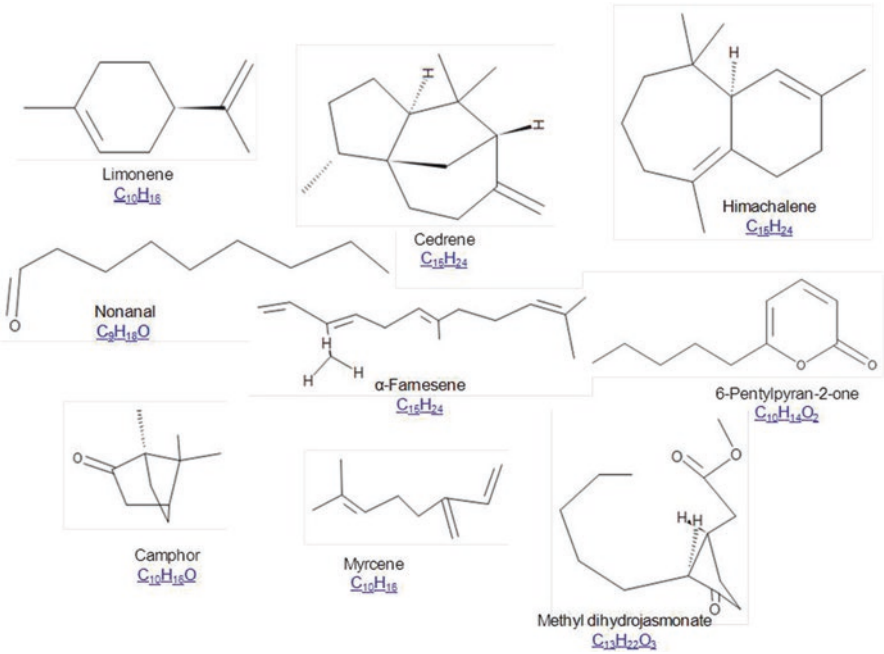


**Fig. 5.1** (continued)

promotion and plant disease suppression. Addition of 6PP (0.166–1 mg/l) to plant growth media or directly applying its solution to plant leaves is known to induce growth promotion and decrease disease symptoms (Vinale et al. 2008; Lee et al. 2016). Even though all the species of *Trichoderma* do not synthesize 6PP (Atanasova et al. 2013), still most of them are known to induce plant growth promotion (Kottb et al. 2015) which indicates that 6PP alone is not involved in its role (Nieto-jacobo et al. 2017). It is detected in *T. atroviride* IMI206040 (Reithner et al. 2005; Stoppacher et al. 2010), *T. citrinoviride*, *T. hamatum* (Jelen et al. 2014), *T. viride* (Collins and Halim 1972), *T. asperellum* (Wickel et al. 2013; Kottb et al. 2015), *T. harzianum* (Claydon et al. 1987), and *T. koningii* (Simon et al. 1988). The production of 6PP by *T. atroviride* is shown to enhance lateral root formation in *A. thaliana* (Garnica-Vergara et al. 2015; Nieto-jacobo et al. 2017).

The production of 6PP can be detected by TLC and HPLC analysis based on ethyl acetate extraction. For its detection, 12–14-day-old cell-free filtrate of *Trichoderma* previously grown in potato dextrose broth is harvested with three volume of ethyl acetate. The solvent is then dried and evaporated using Rotavapor at 35 °C. The dried crude residue is solubilized in 1 ml of ethyl acetate and analyzed by HPLC after filtration. For TLC analysis, 6PP was obtained by purification of crude extract by TLC eluted with dichloromethane/methanol in a 97:3 (v/v) ratio.

6PP is known to be synthesized from linoleic acid using reduction, β-oxidation, and isomerization process (Fig. 5.2). They can be built up by the catalytic activities

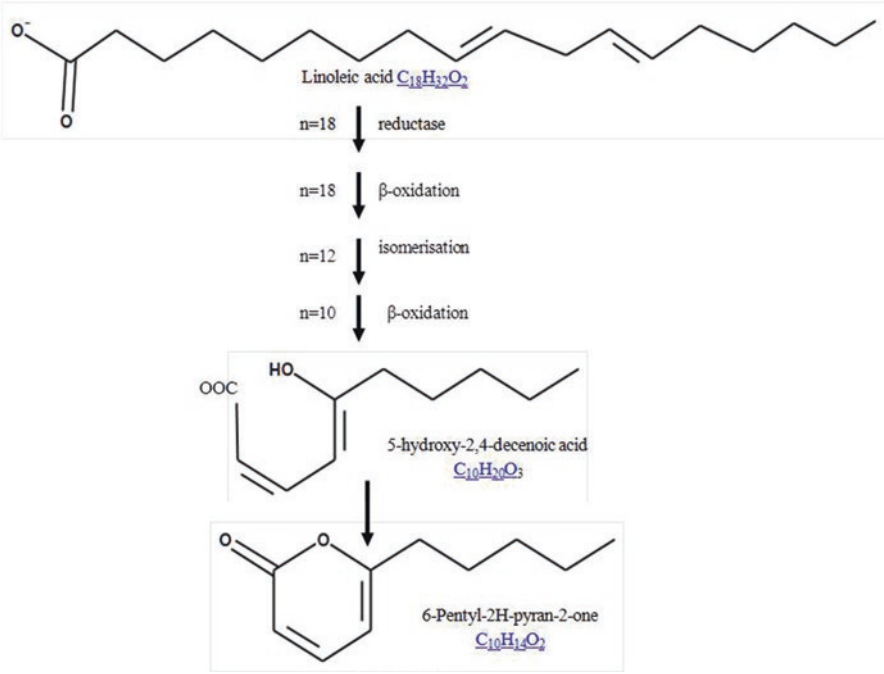


**Fig. 5.2** Hypothetical biosynthetic pathway of 6PP in *Trichoderma* spp.

of different polyketide synthase (PKS) systems and final ring formation yielding the pyrone moiety accomplished in different ways. Different mechanisms have been proposed for the biosynthesis of 6PP, and it is assumed that the route toward pyrone biosynthesis has been developed several times in evolution.

### 5.3.2 Hydrocarbons

The hydrocarbons such as alkanes, alcohols, aldehydes, and acids can be enzymatically synthesized from fatty acids via head-to-head condensation in prokaryotes (Sukovich et al. 2010) or by elongation-decarboxylation in majority of eukaryotes (Brown and Shanks 2012), and conversion of aldehyde to alcohol occurs with the loss of hydroxyl group. In *T. koningii* and *P. janthinellum*, the biocatalysis of decanoic and undecanoic fatty acids is known to occur under specific growth conditions and stored in cell membranes and lipid bodies (Chahal et al. 2014). A mixed fungal cell culture is reported to produce seven classes of lipids into intracellular and extracellular pools (Monreal et al. 2014; Monreal et al. 2016). The investigations led to the identification of variable long-chain primary alcohols with general formula R-OH, wherein R can be unbranched, unsubstituted, linear aliphatic group. The long-chain alcohols are reported to be phagodeterrent and avoid aphids from settling on treated leaves at low concentration 0.15 mM. Eight carbon volatiles

1-octen-3-ol, 3-octanone, 3-octanol, and 1-octen-3-one typical to mushroom (Fisher et al. 1978) are reported for attracting insects and ants and exhibiting fungicidal and fungistatic activity (Pinches 2007; Wilkes et al. 2003; Schirmer et al. 2010; Bernard et al. 2012).

### 5.3.3 Terpenes

Terpenoids are built up of five-carbon isoprene units and represent hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30), and tetraterpenes (C40) classes. Terpenes constitute one of the largest groups of secondary metabolites with over 40,000 structures in cosmopolitan distribution (Bohlmann and Keeling 2008). In actual, the basic building unit to all terpenes is isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In fungi and animals, IPP and DMAPP are synthesized via mevalonic acid (MVA) pathway (Fig. 5.3), whereas in algae and bacteria, it is synthesized by MEP pathway. In plants and some bacteria, both the pathways are used (Rohmer 1999; Walter et al. 2000; Grawert et al. 2011). The MVA pathway starts with the combination of three units of acetyl-coenzyme A to form a six-carbon MVA which is transformed to the five-carbon IPP through series of events such as phosphorylation, decarboxylation, and dehydration.

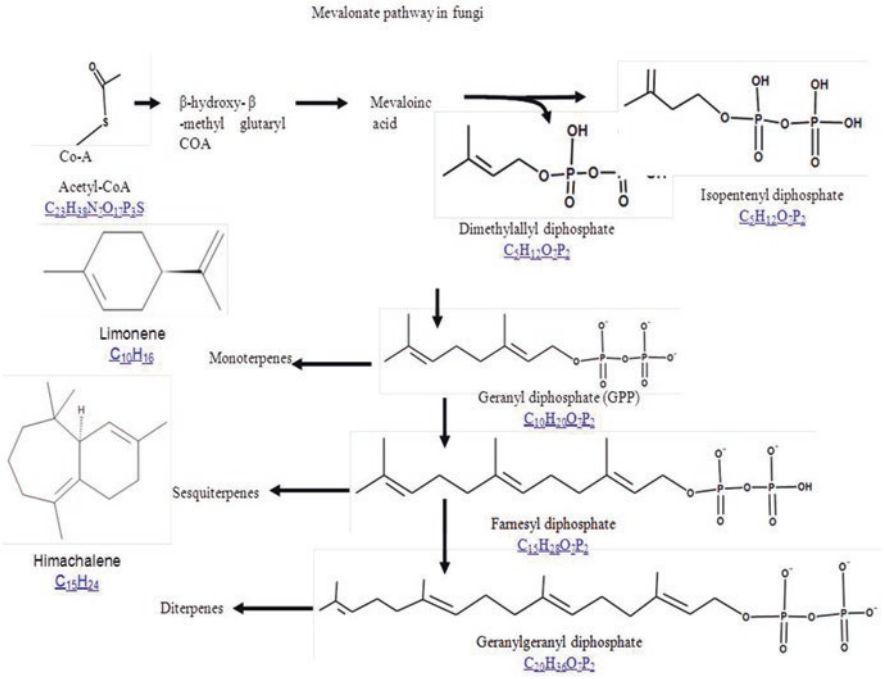


**Fig. 5.3** Terpene biosynthetic pathway in fungi

The isomerization of IPP can lead to the formation of DMAPP. All terpenes are linear or cyclic and saturated or unsaturated and can be modified in various ways. Different structures and properties of terpenoids are the results of modifications accomplished via enzymatic reactions such as changes to the oxidation state of a molecule by oxidation and reduction reactions, alkylation, decarboxylation, glycosylation, rearrangement, and cyclization reactions. Many of them are formed as a result of rearrangement reaction and cyclization reaction which are often carbocation driven (Hansson 2013). They are generated mostly from geranyl pyrophosphate, sesquiterpenes, and geranylgeranyl pyrophosphate through the action of terpene cyclase. Fungi are capable of producing a number of terpenes such as carotenoids, gibberellins, and trichothecenes. A large number of terpene cyclases have been characterized from fungi (Keller et al. 2005). A cosmid clone containing a cyclase gene was sequenced, and several full-length genes were identified as members of a putative secondary metabolism-related gene cluster. These genes included cytochrome P450 and terpene cyclase. The role of gene cluster was established using mutant generation harboring this cluster in *T. virens* and nonproducing strains *T. atroviride* and *T. reesei* followed by profiling of volatile compounds in generated mutants (Crutcher et al. 2013).

Terpenes of sesquiterpenes were identified from *T. virens* Gv29.8 along with β-elemene and ε-amorphene which were significantly overrepresented in the mixture, whereas VOCs reported from *T. asperellum* LU1370 were 1,3-octadiene,limonene, β-eudesmol, and valerianol (Nieto-jacobo et al. 2017). Terpenoids have many biological properties and are widely used as flavors, fragrances, pharmaceuticals, and food additives (Forster-Fromme and Jendrossek 2010; Dewick 2009).

## 5.4 Analysis of Volatile Compounds

The VOCs produced by *Trichoderma* spp. are either intermediate or end products of various metabolic pathways and belong to diverse classes such as alkanes, alkenes, alcohols, esters, terpenes, ketones, and lactones or C8 compounds (Schnurer et al. 1999; Korpi et al. 2009). The studies on these volatile compounds have suffered compared to other secondary metabolites due to lack of proper methods, techniques, and their dynamic production. The identification of VOCs is usually done by gas chromatographic (GC) or flame ionization detection (FID) (Elke et al. 1999) and mass spectrometry (MS)-based methods (Fig. 5.4). For analysis, microbial cultures are usually grown on standard PDA or NA medium or broth at 25 °C and 12 h light/12 h darkness for 4 days. For fungi, actively mycelial culture in liquid or solid media (Nemcovic et al. 2008) is grown in amber glass headspace vial containing a blue PTFE/silicone septum and then sealed. The vials are incubated at 25 °C for 24 h (Stoppacher et al. 2010). The background of PDA plates without the fungus can also be extracted and analyzed for the volatiles. The compounds representing VOCs can be detected by flame ionization detection (Elke et al. 1999) and mass spectrometry (Hynes et al. 2007). Structure characterization and confirmation of

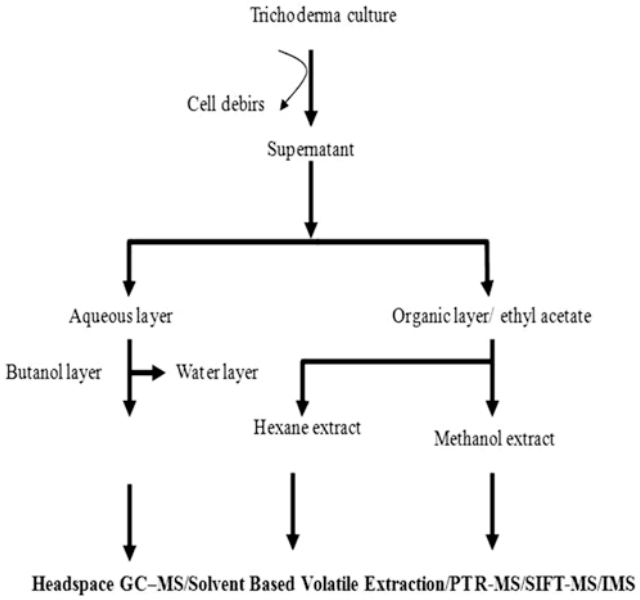


**Fig. 5.4** Schematic extraction of volatile compounds from *Trichoderma* spp.

identity are achieved by matching their mass spectra and linear retention indices using GC-MS solution v. 2.72 software with NIST 11 and Wiley 10 mass spectrum libraries (Oprean et al. 2001; Jelen 2003) or by using the software MassFinder4 with a specialized terpenoids library.

### 5.4.1 Headspace Gas Chromatography-Mass Spectrometry (HSGC-MS)

Due to high sensitivity and powerful separation, GC-MS is the main method for detecting fungal VOCs (Matysik et al. 2009). Another method of adsorbing and desorbing VOCs in culture headspace is solid-phase micro-extraction (SPME), where desorption occurs in the GC injector itself. SPME has become increasingly popular in recent years because it reduces preparation time by combining extraction, concentration, and introduction into one step while increasing sensitivity over other extraction methods. In alternate methods, solid-phase micro-extraction (SPME) volatiles from the headspace or from solution can be pre-concentrated prior to routine analysis onto a glass fiber. Additionally, Headspace-SPME-GC-MS can be automated for direct profiling of living fungal cultures (Stoppacher et al. 2010). Compounds are then identified using a library or database of mass spectra or by comparison of retention times and spectra with those of known standards.

For headspace volatile analysis, active culture of *Trichoderma* is grown glass flask (Stoppacher et al. 2010). Samples can be collected and concentrated using

headspace techniques such as closed-loop stripping analysis (static analysis) (Meruva et al. 2004) and dynamic headspace techniques (purge and trap) (Deetae et al. 2007; Qualley and Dudareva 2009). In static analysis, VOCs in samples are equilibrated with air in airtight container, and then a known volume of air is collected from that sample in a gastight syringe, for gas chromatography. In dynamic (purge and trap) headspace technique, purified air in known amount is passed over the sample, and then volatiles are concentrated onto an adsorbent trapping material such as graphite or an organic polymer. Alternatively, the air flow is recycled through the adsorbent trap known as closed-loop stripping. Volatiles can be removed over the adsorbent trap by elution with organic solvents (commonly with diethyl ether) and then heated with a stream of inert gas and transferred directly to the gas chromatograph (GC) and an autosampler for solid-phase micro-extraction (SPME). The desorption transfers all VOCs from the adsorbent trap onto the GC column thus provides better sensitivity and ability to analyze higher volatile compounds which will be difficult with organic solvent injection. For compound analysis, the compounds adsorbed onto the fiber after certain fixed time are desorped and inserted into the heated injection port of GC. SPME sampling usually occurs as an integrated process in real time although SPME fibers and desorption traps may be stored at low temperature (Rowan 2011).

### 5.4.2 Chromatography-Free Methods (PTR-MS/SIFT-MS/IMS)

The GC-MS-based techniques are time-consuming and need sample preparation, and chromatographic separation of metabolites requires a sufficiently low and stable temperature (30–40 °C) before introduction of the next sample. In proton-transfer-reaction mass spectrometry (PTR-MS), headspace air surrounding the sample is collected directly into the instrument where volatiles are ionized by protonated (charged) water molecules generated in a hollow cathode source. The protonated volatile compounds are then passed through a region by a quadrupole mass spectrometer. The other related technology such as ion flow tube mass spectrometry (SIFT-MS) generates ionized volatiles by interaction with a range of ions such as $H_3O^+$, $NO^+$, and $O_2^+$ with better opportunities for more selective ionization (Francis et al. 2007) for the resolution of compounds with same molecular mass (Lindinger and Jordan 1998). The PTR-MS/SIFT-MS has emerged as an alternative technology and offers real-time monitoring of volatiles, minimum sample preparation with maximum high sample throughput.

In addition, HPLC/LC-MS methods have been used for profiling of specific volatile classes like aldehyde lipid oxidation products and amines. The advent of liquid chromatography coupled to mass spectrometry (LC-MS) offers new possibilities in the analysis of volatile biosynthesis and the direct analysis of nonvolatile precursors that are frequently present in biological systems such as glycoside, glucuronide, sulfate, or phosphate derivatives (Beranek and Kubatova 2008). The availability of LC-MS can be helpful in routine metabolomic analysis of the volatile precursors, volatile biosynthesis, and their regulation in biological systems. Coupling SPME

sampling with LC-MS may also allow direct in vivo sampling and measurement of these compounds in different organisms.

### 5.4.3 Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

SIFT-MS is a rapid, broad-spectrum detection technique for traces of VOCs in moderately complex gas mixtures. SIFT-MS can quantify VOCs in real time from low part-per-billion (ppb) levels without pre-concentration (Senthilmohan et al. 2001). This technique has been used to study the VOCs produced by *Aspergillus*, *Candida*, *Mucor*, *Fusarium*, and *Cryptococcus* sp. (Scotter et al. 2005).

### 5.4.4 Proton Transfer Reaction Mass Spectrometry (PTR-MS)

PTR-MS ionizes VOCs through their reaction with $H_3O^+$, forming mostly molecules which can be detected by a standard quadrupole/multiplier mass analyzer (Lindinger and Jordan 1998). PTR-MS can be used to quantify fungal VOCs since it has fine detection capability and scale time response (Ezra et al. 2004). Additionally, analysis can be run in real time without sample preparation, derivatization, or concentration with the advantage of having sensitivity comparable to GC-MS. This technique is used to quantify the VOCs of *Muscodor albus* (Ezra et al. 2004).

### 5.4.5 The Electronic Nose or E-Nose

E-nose is a promising development for detecting fungal VOCs. Using arrays of electronic chemical sensors with appropriate pattern recognition systems, it can recognize simple or complex odors (Gardner and Bartlett 1992; Wilson and Baietto 2009). A typical E-nose relied on multisensor array, information collecting unit, pattern recognition software, and reference library. This technique can provide a qualitative overview of volatile compounds (Wilson and Baietto 2009, 2011).

### 5.4.6 Solvent-Based Volatile Extraction Method

The organic solvent-based extraction is generally better and gives a complete profile of metabolites including low molecular weight alcohols, hydroxyl acids, thiols, and flavor compounds such as acetoin (Zeppa et al. 1990; Keszler et al. 2000). But non-volatile compounds such as leaf waxes, triterpenes, triglycerides, and complex lipids can impede analysis. The solvent systems used for the optimized extraction of metabolites include pentane-ether mixtures and dichloromethane. The contaminating compounds such as lipids, pigments, and other hydrocarbons can be removed by simultaneous distillation-extraction (SDE) (Chaintreau 2001), vacuum micro distillation, or solvent-assisted flavor evaporation (SAFE) (Engel et al. 1999) or by

adsorption chromatography. The use of supercritical fluids (SCF) such as supercritical carbon dioxide, either pure or in the presence of modifiers, is an alternative to the organic solvent-based extraction. The polarity of these SCFs is comparable to pentane and has been used to extract volatiles from a wide range of plants (Pourmortazavi and Hajimirsadeghi 2007). While SCF extraction has the advantage of using totally volatile solvent, still these studies require specialized equipments (Pourmortazavi and Hajimirsadeghi 2007; Gressler et al. 2009).

## 5.5 Applications of *Trichoderma* Volatile Compounds

The VOCs produced by fungi have been intensively studied for their use as diagnostic agents as indicator for detecting contamination. The VOCs are implicated in "sick building syndrome" a controversial medical condition. The aromatic properties of these VOCs find applications in food fermentations and interkingdom signaling events (Chiron and Michelot 2005; Kues and Navarro-Gonzales 2009; Bennett et al. 2012). The VOCs of *Trichoderma* spp. are known to act as antibacterial and antifungal agents (Strobel et al. 2001, 2006). In agriculture, fungal VOCs have been used as part of biological control strategies to prevent the growth of plant pathogens and promoting plant growth. A number of VOCs have been reported from *Trichoderma* spp. which are beneficial to the plants (Wheatley et al. 1997; Van Loon et al. 1998; Stoppacher et al. 2010). In the food industry, the biological control through myco-fumigation is used to prevent postharvest fungal growth. The biotechnological potential of VOCs from *Trichoderma* is still underexplored. In recent studies, the role of these compounds in inducing systemic resistance through priming plants' immune response and nutrient acquisitions has been investigated (Van Wees et al. 2008). The soil application of 2-butanone and 3-pentanol in cucumber seedlings has been reported to reduce aphid *M. persicae* infestation and increase in predatory coccinellids (Song and Ryu 2013).

### 5.5.1 Antimicrobial Activity

The VOCs including nonanal, N-decanol, cyclohexanol, ethyl-1-hexanol, benzothiazole, and dimethyl trisulfide are identified for their inhibitory role (Fernando et al. 2005). Fungal endophytes are known to produce volatile mixtures having strong antibacterial effects (Strobel et al. 2001; Strobel 2006) which indicate the role of several VOCs in synergistic mode for antimicrobial activity. The VOCs of *Trichoderma* are known for their action against pathogenic fungi (Nemcovic et al. 2008; Vinale et al. 2008) and have potential for being used as biocontrol agent in agriculture. The GC-MS analysis of *T. viride* VOCs identified 51 metabolites among which isobutyl alcohol, isopentyl alcohol, and 3-methylbutanal are most prevalent and inhibit wood-decaying basidiomycetes and plant pathogens (Dennis and Webster 1970; Wheatley et al. 1997; Humphris et al. 2001; Bruce et al. 2004). The prominent headspace volatile identified as 6-pentyl-α-pyrone (6PP) from *T.*

*asperellum* showed significant reduction of disease symptoms in *Arabidopsis* when infected with *Alternaria brassicicola* and *Botrytis cinerea*. The volatile bioactive metabolites are also known to inhibit growth of fungal mycelium, spore germination, and pigmentation of plant pathogenic fungi. The VOCs of the endophyte *M. albus* can be used to control soilborne diseases caused by *Rhizoctonia solani* and *Phytophthora capsici* (Mercier and Manker 2005). Some VOCs are known to stimulate or enhance soilborne biocontrol agents (Wheatley 2002). The volatiles emitted by *T. atroviride* are known to increase the expression of a primary biocontrol gene of *Pseudomonas fluorescens* (Lutz et al. 2004).

### 5.5.2 Nutrient Acquisitions

In saline soil and other parts of the world, $Fe^{2+}$ deficiency is a major limiting nutrient. The manipulation of iron homeostatic mechanisms by microbial VOCs is a feature conserved among different root-associated mutualists, ranging from bacteria to fungi (Wintermans et al. 2017). The numerous root-associated beneficial microbes such as *Trichoderma* play important role in nutrient uptake and are highly effective in promoting plant growth and resistance to both abiotic and biotic stresses (Zhao et al. 2014). Induction of Fe uptake-related genes by microbial volatiles has been previously demonstrated for VOCs of bacterial origin. VOCs released by the plant growth-promoting rhizobacterium *Bacillus subtilis* GB03 and the ISR-inducing rhizobacterium *Pseudomonas simiae* WCS417 are found to trigger the expression of Fe uptake-related genes in *Arabidopsis* roots, leading to elevated endogenous Fe levels in the plant (Zamioudis et al. 2015). The VOCs of *T. asperellum* and *T. harzianum* are known to trigger MYB72 expression and $Fe^{2+}$ uptake in *Arabidopsis* roots. The volatile compounds of *Trichoderma* origin also enhanced resistance through priming of jasmonic acid-dependent defense against *Botrytis cinerea*. The VOCs of *Trichoderma* are reported for eliciting Fe deficiency responses and shoot immunity in tomato which suggest that the phenomenon worked across plant species. The VOCs of *Trichoderma* were able to trigger local readjustment of Fe homeostasis in roots through systemic elicitation of ISR by priming of jasmonic acid-dependent pathway (Zhao et al. 2014).

### 5.5.3 Induction of Conidiation

The VOCs produced fungal species that are known to exhibit a cross-species action both at intra- and interspecific level. The ability to influence their own development and other fungi is one of the interesting features of several fungi. The molecular mechanisms of the VOCs in fungal development are largely unknown, but the physiological significance and the stimulatory effect on conidiation may be associated to their role as inter-colony communication and warning signals under unfavorable conditions. The switching from vegetative growth to formation of conidia is marked by enhanced production of secondary metabolites (Calvo et al. 1999). The

production of secondary metabolites of volatile nature such as 3-octanol, 1-octen-3-ol, and 3-octanone by *Trichoderma* during conidia formation clearly depicts the role of these metabolites in conidiation. The fungal isolates are capable of inducing conidia formation under dark conditions, and the amount is reported to vary with the concentration of each VOC. The signaling events are assumed to take place at cytoplasmic membrane level which leads to membrane potential and permeability (Chitarra et al. 2005). The compounds such as 1-octen-3-ol are found to be effective at 0.1 mM concentration, whereas at higher concentration of 500 mM, 3-octanone is found to induce highest levels of conidia formation (Nemčovič et al. 2008). The sporulating *T. viride* is reported to produce over 50 VOCs including isobutyl, isopentyl alcohols, and 3-methylbutanal.

### 5.5.4 Plant Growth Promotion

The role of volatile compounds can be realized from the fact that species of *Trichoderma* are able to stimulate *Arabidopsis thaliana* growth, enhanced lateral root formation, early-flowering and fruit development phenotypes in absence of any direct physical contact (Hung et al. 2013; Lee et al. 2016). Plants grown in the presence of fungal VOCs emitted by different *Trichoderma* spp. exhibited a range of effects. Exposure to the VOCs produced by these strains led to an increase in plant biomass (37.1 to 41.6%) and chlorophyll content (82.5 to 89.3%) in a strain and species-specific way. The VOCs of *T. pseudokoningii* (CBS 130756) showed highest *Arabidopsis* growth promotion. Similarly, tomatoes exposed to VOCs from *T. viride* BBA 70239 showed a significant increase in plant biomass (>9%) and significant development of lateral roots depending on the duration of the volatile exposure. VOCs produced by both *T. aggressivum* and *T. pseudokoningii* were able to enhance the *Arabidopsis* growth. The continuous exposure to VOCs of *Bacillus*, a plant growth-promoting rhizobacterium, is reported to trigger plant growth and development which signifies the importance of volatile exposure in plant growth development (Xie et al. 2009; Bailly and Weisskopf 2012; Lee et al. 2015). Similar effects are also reported in lettuce (Minerdi et al. 2009). The VOCs from bacteria and *F. oxysporum* in combination enhanced the growth promotion; however VOCs of fungal origin alone were not able to enhance plant growth (Hung et al. 2012).

Experiments conducted using grafts of fungal volatile compounds preexposed and nonexposed *Arabidopsis* seedlings established that these compounds in roots were able to transduce plant immunity through unknown ISR pathways to leaves systematically (Zhao et al. 2014). GC-MS analysis of VOCs from *Trichoderma* strains identified over 141 unique compounds including sesquiterpenes, diterpenes, and tetraterpenes which are not reported earlier. The nature of volatiles produced by actively growing fungi influences the outcome of interactions. Compounds such as 6-pentyl-2H-pyran-2-one were not common to all promising and bio-stimulatory strains and instead have higher number of complex terpenes which may be involved for variation in growth accelerated by different *Trichoderma* strains (Lee et al. 2016).

### 5.5.5 Biofuels

The terpenes representing VOC such as monoterpene derivative 1,8-cineole have potential to be explored as fuel additive similar to VOCs released by *Hypoxylon* sp. (Tomsheck et al. 2010). Fungal species are known to produce various biofuel substrates including alkane and alkene such as ethane, propane, ethylene, and propylene (Ladygina et al. 2006), while others can produce terpenes and isoprenoids which may be explored for fuels (Grigoriev et al. 2011). In summary, fungi are an excellent platform for exploiting biosynthetic routes to hydrocarbon biofuels or its precursors (Grigoriev et al. 2011).

## 5.6 Conclusion

*Trichoderma* spp. are already explored as bio-fungicides to agricultural soils to enhance crop productivity. The research on bioactive volatile compounds of *Trichoderma* is challenging, emerging, and frontier area of research. The emergence of latest techniques has already played vital role in the identification of several classes of volatile compounds. The VOCs have the ability to suppress plant diseases and promotion of plant growth and productivity through overlapping mode of action including induced systemic resistance, antibiosis, and enhanced nutrient efficiency. Presently, the coupling of modern omics technologies can help in the identification of volatile compounds and bioprospection of vast untapped potential of volatile compounds in agriculture and mining the promises for new products for agricultural exploitation and will begin a new era in fundamental biology.

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# 6 Phytopathogen Biomass as Inducer of Antifungal Compounds by *Trichoderma asperellum* Under Solid-State Fermentation

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## 6.1 Introduction

*Trichoderma* is a prominent and well-studied biocontrol agent, due to its capabilities to control and kill several phytopathogen pests, such as *Phytophthora tropicalis*, *Phytophthora palmivora*, *Alternaria solani*, *Bipolaris oryzae*, *Pyricularia oryzae*, and *Sclerotinia sclerotiorum*, among others (Prabhakaran et al. 2015; Singh et al. 2016, 2017; Sriwati et al. 2015). The competence of space and nutrients, mycoparasitism, and the production of antibiotics are the main mechanisms of *Trichoderma* for the biocontrol of pests (Ghorbanpour et al. 2018; Jeleń et al. 2014; Keswani et al. 2014). The term antibiosis is related to secretion of chemicals with biological activity, such as cell wall-degrading enzymes, siderophores, chelating iron, and volatile and nonvolatile metabolites (El-Debaiky 2017; Mutawila et al. 2016; Keswani 2015). It is well known that *Trichoderma* has the ability to produce different secondary metabolites, such as alcohols, ketones, alkanes, furans, and mono- and sesquiterpenes, in order to inhibit the growth of phytopathogens (including fungi, bacteria, yeast) and also promote plant growth (Hu et al. 2017; Jeleń et al. 2014). The production and release of secondary metabolites from fungi are activated by the presence of an organism that represents a threat to its survival (Sun et al. 2016). The production of antibiotic compounds depends on several factors, such as fungal growth phase and nutritional, biological, and environmental

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conditions, and they could be induced or activated by other organisms when they are invading the space in the soil, competing for available nutrients, among others (Arinbasarova et al. 2017; Vinale et al. 2016). There are several reports focusing on the identification of antibiotic compounds produced by biological control agents (BCAs), particularly by *Trichoderma* spp. Most of them are reported compounds effective against various phytopathogens (Angel et al. 2016; Yamazaki et al. 2016). The majority of the reports about the production of antibiotic compounds are focused on the use of liquid cultures; however, filamentous fungi seem to be more adapted to solid environments (Shakeri and Foster 2007; Viniegra-González 2014). In literature it is possible to find a lot of information about solid-state fermentation and fungi in order to produce all kinds of enzymes, antioxidants, bioactive compounds, and biomass, among others (El-Gendy et al. 2017; Elegbede and Lateef 2017; Mohamed et al. 2016). Therefore, the objective of the present study was focused on the evaluation of the phytopathogen biomass of *Phytophthora capsici* and *Colletotrichum gloeosporioides* as inducers of antifungal metabolites from *Trichoderma asperellum* through solid-state fermentation conditions.

## 6.2 Materials and Methods

### 6.2.1 Chemicals and Reagents

HPLC grade acetonitrile and acetic acid were purchased from Sigma-Aldrich. Ultrapure water (Milli-Q) was generated by the Millipore System (Bedford, USA). SPE cartridges and Oasis MAX 96-well plate 30 lm (30 mg) were obtained from Waters (Milford, MA, USA).

### 6.2.2 Microorganism and Culture Conditions

The strains of *Trichoderma asperellum* and *Phytophthora capsici* were kindly proportioned by the Agricultural Parasitology Department of the UAAAN (Universidad Autónoma Agraria Antonio Narro, Saltillo, México). *Colletotrichum gloeosporioides* was proportioned by the Food Research Department of the UAdeC (Universidad Autónoma de Coahuila). Fungal strains were cultivated and preserved in a milk-glycerol 8.5% solution. Potato dextrose agar (PDA) was used to reactivate all fungi strains. The incubation was done at 28 °C during 5 days; then the preservation was at ±4 °C.

### 6.2.3 Phytopathogen Biomass Production

A cornmeal medium (17 g/L) was used to produce phytopathogen biomass. This medium was maintained under shaking for 1 h at 58 °C. Then, it was filtrated and sterilized (15 min at 115 °C). The inoculation of phytopathogens was as follows: *C.*

*gloeosporioides* ($1 \times 10^6$ spores/mL) and *P. capsici* (10 PDA plugs from a culture of 7 days old). The incubation was at 28 °C during 7 days under shaking (200 rpm).

### 6.2.4 Substrates

The corncob was proportioned by the Mexican Institute of Maize, UAAAN Coahuila, México. The material was dried, ground, fractioned (300–1680 μm), and stored under low moisture conditions for further evaluation. This material was used as a substrate on SSF without any pretreatment.

### 6.2.5 Culture Conditions

Polyethylene bags were used as a bioreactor in this study. The corncob (30 g) was mixed with phytopathogen biomass (3%). The biomass from each phytopathogen was evaluated in a separate experimental procedure. *T. asperellum* was inoculated at $1 \times 10^7$ spores $g^{-1}$ of substrate adjusting the relative moisture at 50%. The fermentation was incubated for 5 days at 24 °C. Three different solid-state fermentations were done: (1) substrate mixed with biomass of *C. gloeosporioides*, (2) substrate mixed with biomass of *P. capsici*, and (3) substrate without phytopathogen biomass. Not fermented corncob was used as a control.

### 6.2.6 Samples Extraction

Water, ethanol, and toluene were the solvents evaluated to recover the metabolites released. The fermented material (20 gm) was eluted with 40 mL of each solvent using a plastic column (60 mL). Toluene extract was concentrated by evaporation at 35 °C, and then it was dissolved in 5 ml of ethanol (Vinale et al. 2009). All samples were passed through a Millipore® nylon membrane (0.45 μm) and then injected in a vial (2 mL).

### 6.2.7 Antifungal Assays

The crude extracts obtained in the last section were tested against *C. gloeosporioides* and *P. capsici* to evaluate their antibiotic properties. Pathogen plugs (5 mm diameter) from growing edges of colonies were placed at the center of Petri plates containing PDA (Vinale et al. 2006). The crude extract (10 μL) was applied on the top of each plug. Ethanol, toluene, and water were applied alone as a solvent control. The growth of the phytopathogens on PDA without application of solvent or extract was used as a control. The results were presented as a percentage of inhibition growth. A bifactorial arrangement (3 × 2) was made to the antifungal determination. The analysis of variance and means comparison (Tukey) were done in all cases. All treatments were done in triplicate.

### 6.2.8 Extracts Fractionation

Only the extracts (ethanolic and aqueous) from the fermentation supplemented with *P. capsici* biomass showed biological activity and therefore were fractioned. The ionic polymeric resin Amberlite XAD16® was used to perform the fractionation of the extracts. This resin was packed into a glass column (200 mL) and then it was filled with 40 mL of the extract. The aqueous extract was eluted first with distilled water (aqueous fraction); then, absolute ethanol was used to recover the compounds adsorbed in the resin (ethanolic fraction). The ethanolic extract was eluted first with absolute ethanol (ethanolic fraction); then, absolute methanol was used to recover the compounds adsorbed in the resin (methanolic fraction) (Ruiz-Martínez et al. 2011). At the end of the fractionation, nine samples were obtained.

### 6.2.9 LC-ESI-MS Analysis

The analyses by reversed-phase high-performance liquid chromatography were performed on a Varian HPLC system including an autosampler (Varian ProStar 410, USA), a ternary pump (Varian ProStar 230I, USA), and a PDA detector (Varian ProStar 330, USA). A liquid chromatograph ion trap mass spectrometer (Varian 500-MS IT Mass Spectrometer, USA) equipped with an electrospray ion source also was used. Samples (5 μL) were injected onto a Denali C18 column (150 mm × 2.1 mm, 3 μm, Grace, USA). The oven temperature was maintained at 30 °C. The eluents were formic acid (0.2%, v/v; solvent A) and acetonitrile (solvent B). The following gradient was applied: initial, 3% B; 0–5 min, 9% B linear; 5–15 min, 16% B linear; and 15–45 min, 50% B linear. The column was then washed and reconditioned. The flow rate was maintained at 0.2 mL/min, and elution was monitored at 245, 280, 320, and 550 nm. The whole effluent (0.2 mL/min) was injected into the source of the mass spectrometer, without splitting. All MS experiments were carried out in the negative mode $[M\text{-}H]^-$. Nitrogen was used as nebulizing gas and helium as damping gas. The ion source parameters were spray voltage 5.0 kV; capillary voltage and temperature were 90.0 V and 350 °C, respectively. Data were collected and processed using MS Workstation software (V 6.9). Samples were firstly analyzed in full scan mode acquired in the m/z range 50–2000. MS/MS analyses were performed on a series of selected precursor ions.

## 6.3 Results

### 6.3.1 Antifungal Assays

The antifungal activity of the toluene, ethanol, and water extracts from the SSF by *T.* asperellum is presented in Table 6.1. The extracts obtained from the SSF added with *P. capsici* biomass showed important effects on the reduction of *P. capsici* growth rate compared with the control (no extract added). On the other hand, any

**Table 6.1** Inhibition of phytopathogen growth using crude extracts from SSF by *T. asperellum*

| Biomass as inducer in SSF | Solvent | *P. capsici* | *C. gloeosporioides* |
|---|---|---|---|
| *C. gloeosporioides* | Toluene | – | <0.5 |
| | Ethanol | – | <0.5 |
| | Water | – | <0.5 |
| *P. capsici* | Toluene | 1.33 ± 0.62 | – |
| | Ethanol | 9.62 ± 2.65 | – |
| | Water | 6.11 ± 0.78 | – |

extract obtained from the SSF added with *C. gloeosporioides* biomass showed important values of activity against the growth rate of *C. gloeosporioides*. The growth of phytopathogen strain *P. capsici* was reduced by the three extracts evaluated. The activity showed by toluene extract resulted in a very low value. However, the best results were shown by the ethanol and the water extract, with values of 9.6 and 6.1%, respectively.

### 6.3.2 HPLC Analyses

The three extracts with antifungal activities were analyzed by high-pressure liquid chromatography (HPLC), in order to identify important signals and its retention times. The water extract showed two signals differenced and more intense than the controls. The first peak was observed at 9.0 min and the second one at 18.9 min of retention time (RT) (Fig. 6.1). In the ethanolic extracts, it was possible to observe one signal in the chromatogram, different than the controls. This compound was observed with high intensity at 18.7 of RT (Fig. 6.2). The toluene extract showed exactly the same profile than the controls (Fig. 6.3).

### 6.3.3 LC-ESI-MS Analysis

The LC-ESI-MS analysis revealed the presence of six compounds from the extracts of the SSF by *T. asperellum*. The major compounds detected correspond to an unknown compound (1) and dihydroxybergamotene (2), with a molecular mass of $[M + H]^-$ (*m/z* 478) and $[M + H]^-$ (*m/z* 260), respectively. In addition, other four compounds were detected: viridepyronone (3), koninginin D (4), acetyltetrahydroxyanthraquinone (5), and virone or gliotoxin (**6**) (Table 6.2). All molecular masses obtained in the present study were compared with microbial bioactive metabolites reported in literature.

## 6.4 Discussion

In the present study, the induction of bioactive compounds through a solid-state fermentation by *T. asperellum* was observed. Several studies have been focused on the production of antifungal metabolites, such as Jeerapong et al. (2015), who

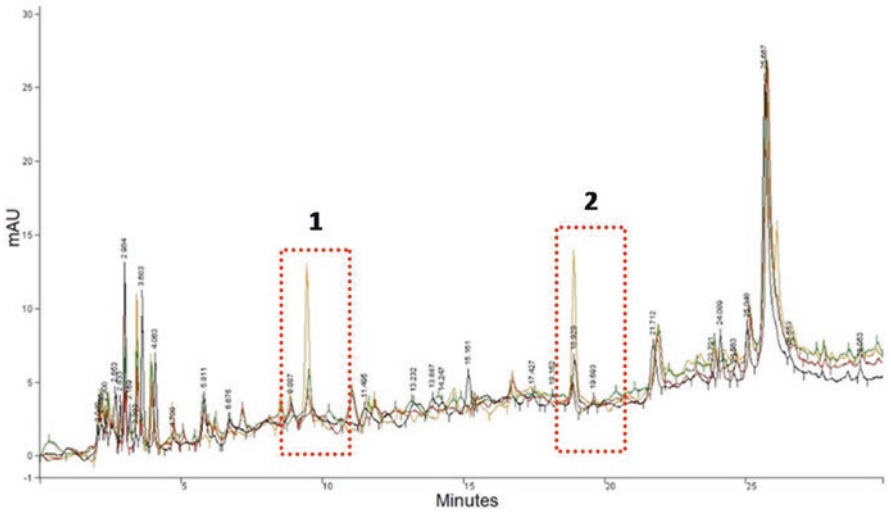


**Fig. 6.1** Chromatogram of the aqueous extract from the SSF by *T. asperellum* at 254 nm

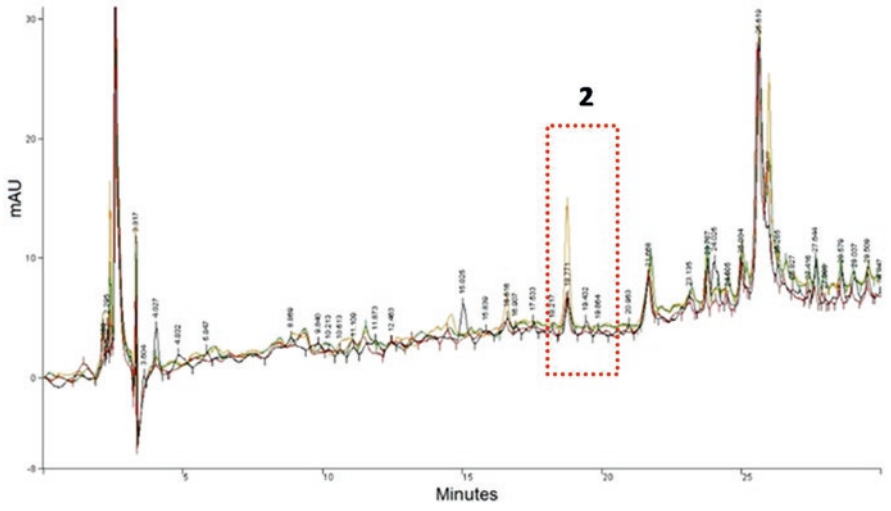


**Fig. 6.2** Chromatogram of the ethanolic extract from the SSF by *T. asperellum* at 254 nm

reported the extraction of metabolites from a culture of *T. harzianum* F031 in a submerged fermentation (PDB) showing a reduction of 76.6% growth rate on *C. gloeosporioides* by an agar dilution assay. Also, several metabolites obtained from a liquid culture of *T. harzianum* have been reported as inhibitors of the growth rate of *Fusarium oxysporum* (Saravanakumar et al. 2016).

In the present study, dry biomass (evaluating *P. capsici* and *C. gloeosporioides*) was added in the fermentation process in order to evaluate a possible induction of

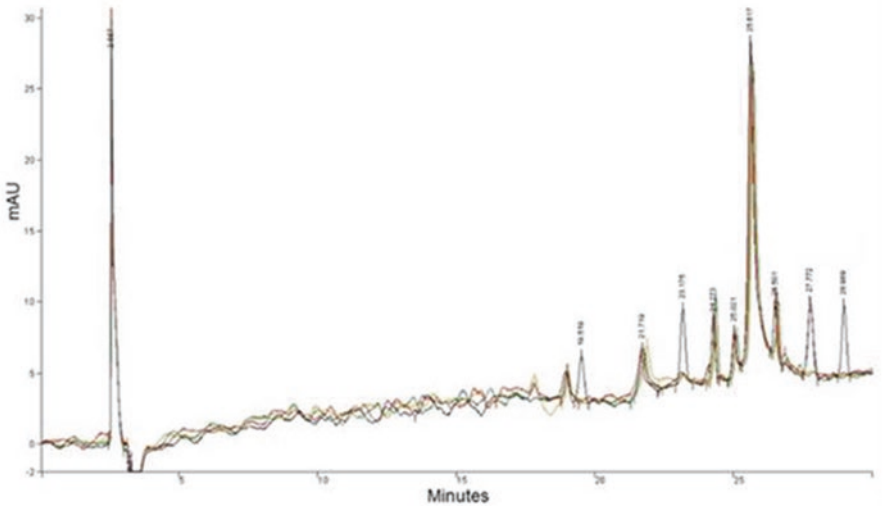


**Fig. 6.3** Chromatogram of the toluene extract from the SSF by *T. asperellum* at 254 nm

specific antifungal compounds. A low percentage of inhibition was achieved by the extracts from the SSF with *P. capsici* biomass as inducer, and on the other hand, negligible activity was detected by the extracts from the SSF with *C. gloeosporioides* biomass as an inducer. It has been reported that the behavior of *Trichoderma* with other microorganisms who represent a competence in a microenvironment. Normally, several signals are sent and perceived between the microorganisms involved (Albuquerque and Casadevall 2012; Hogan 2006). The share of this information causes a response on each microorganism secreting enzymes or specific secondary metabolites. Low inhibition activity could be due to the use of inert phytopathogen biomass as inducer (no signaling). Therefore, these results suggest the necessity of *Trichoderma* to secrete its compounds as a reaction to the presence of a living organism, as it happened with the induction of several metabolites by the effect of a co-cultivation of *Trichoderma* sp. and *Acinetobacter johnsonii* (Zhang et al. 2017).

The fractionation of the crude extracts indicates the high polarity of the compounds released by *Trichoderma* and its potential role in phytopathogen inhibition. Metabolites 1 and 2 were detected in the chromatogram of the water extract showing a phytopathogen inhibition of 6.11%. However, in the ethanolic extract, only metabolite 2 was detected resulting in a phytopathogen inhibition of 9.6%. This result suggests that metabolite 2 could be more potent to act as antifungal compound working alone than in a synergy with compound 1.

Most of the bioactive metabolites from fungi reported in the literature have been obtained in liquid culture without any process of specific induction (Li et al. 2016). Those metabolites are commonly secreted by the fungal strain evaluated in each investigation. It is possible to find *Trichoderma* bioactive compounds with capacity to inhibit the growth of phytopathogenic microorganisms and also working as a

**Table 6.2** Literature comparison of the compounds detected in the extracts obtained from SSF with phytopathogen biomass

| Id. | $[M-H]^-$ (*m/z*) | RT (min) | Proposed compound | Author |
|---|---|---|---|---|
| 1 | 478 | 9 | – | No data reported |
| 2 | 260 | 18.929 | Dihydroxybergamotene | Zhang et al. (2009) |
| 3 | 180 | – | Viridepyronone | Evidente et al. (2003) |
| 4 | 298 | – | Koninginin D | Kang et al. (2011) |
| 5 | 312 | – | Acetyltetrahydroxyanthraquinone | Betina et al. (1986) |
| 6 | 326 | – | Virone | Blight and Grove (1986) and Wafaa and Mohamed (2002) |

plant growth promoting, such as harzianic acid (Vinale et al. 2013), azaphilone (Mevers et al. 2016), harzianolide (Mazzei et al. 2016), T39 butenolide (Keswani et al. 2017), and harzianopyridone (Ahluwalia et al. 2015), among others, mainly produced by using potato dextrose broth (PDB) (Saravanakumar et al. 2018). Several microorganisms have been reported as sensitive to the reduction of their growth, such as *Gaeumannomyces graminis*, *Rhizoctonia solani*, *Pythium ultimum*, *P. irregular*, and *Sclerotinia sclerotiorum*, due to the high antibiotic activity of the metabolites produced by *Trichoderma* spp. (Vinale et al. 2006, 2009). Frequently, the culture of *T. harzianum* in PDB is used by several authors to the production of bioactive metabolites (Shakeri and Foster 2007; Shentu et al. 2013).

Six metabolites obtained in the present study have been already reported by other authors, such as viridepyronone (3) reported by Evidente et al. (2003), showing antifungal inhibition against *Sclerotium rolfsii*. Koninginin D (4) was identified by (Kang et al. 2011), from the culture of *T. koningii* on malt extract agar (MEA), affecting the growth of several fungal phytopathogens (Zhou et al. 2014). The family of anthraquinones, particularly the acetyl-tetra-hydroxy-anthraquinone (5) shows a low effect *G. graminis*. However, good results are reported reducing the gray mold severity caused by *B. cinerea* (Vinale et al. 2008). Blight and Grove (1986) reported the production of virone (6) as the major metabolite by *G. virens*.

In the present study, it is reported the identification of a molecular mass (*m/z* 478) corresponding to a compound (1) not reported yet. The second compound denominated dihydroxybergamotene (2) was identified by Zhang et al. (2009), using a fungal strain of *Acremonium* sp. reporting anti-inflammatory as the main activity of this metabolite.

## 6.5 Conclusion

Six compounds were identified in the extracts obtained from a SSF with *T. asperellum* and the use of phytopathogen biomass as inducer under SSF culture conditions. Under the present conditions, two major compounds were detected, an unknown compound (1) and dihydroxybergamotene (2), respectively. Both compounds were obtained with water and ethanol suggesting the high polarity of them and the facilities to further extractions and applications. Biomass of *C. gloeosporioides* as inducer on the SSF did not show any effect on phytopathogen inhibition. Biomass of *P. capsici* as inducer on the SSF showed little effect on phytopathogen inhibition. The results suggest a further research focused on the possibilities to increase the quantity of phytopathogen biomass (more than 3%) expecting major induction of antifungal compounds and also the possibility to enhance the induction of metabolites evaluating co-culture conditions under SSF.

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# 7 Bioactive Secondary Metabolites of *Trichoderma* spp. for Efficient Management of Phytopathogens

Laith Khalil Tawfeeq Al-Ani

## 7.1 Introduction

*Trichoderma* has an amazing genome that enables the species to confront various biotic and abiotic stresses, allowing *Trichoderma* spp. to survive globally in different agro-climatic regions. Fungi produced very dangerous secondary metabolites such as mycotoxins (Attitalla et al. 2010a, b; Nor Azliza et al. 2014) and very useful secondary metabolites (Singh et al. 2016, 2017). *Trichoderma* as a biocontrol agent is showing a high efficacy such as PGPR and non-pathogenic Fusarium with other methods in controlling the plant pathogens and pests ((Al-Ani 2006; Al-Ani and Salleh 2010; Mohammed et al. 2011, 2012, 2013, 2014; Al-Ani and Al-Ani 2011; Al-Ani et al. 2012; Al-Ani et al. 2013a, b; AL-Ani 2017a,b; Al-Ani and Albaayit 2018a, b; Al-Ani et al. 2018; AL-Ani 2018a, b; Al-Ani 2019a, b, c, d, e; Al-Ani et al. 2019). *Trichoderma* antagonizes all phytopathogens through either direct or indirect confrontation (Al-Ani 2018a). Direct physical contact between antagonistic fungi is known as mycoparasitism; *T. virens* attacks *Rhizoctonia solani* through mycoparasitism (Guzmán-Guzmán et al. 2017). However, indirect methods include (1) competition, (2) the production of antibiotics, (3) the secretion of bioactive secondary metabolites (4), and (5) the induction of the host defense system. This chapter focuses on the role of secondary metabolites of *Trichoderma* spp. (SMTs) for managing a broad spectrum of seed and soil-borne phytopathogens (Singh et al. 2016, 2017). The biocontrol strains of *Trichoderma* detected the production of SMT affecting by the internal cAMP level that producing chitinase and mycoparasitism-associated coiling (Zeilinger and Omann 2007).

SMTs are an active area of research as they provide an eco-friendly alternative to synthetic pesticides (Wu et al. 2017). *Trichoderma* produces several SMTs that have

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(1) antifungal, (2) antibacterial, (3) antiviral, (4) insecticidal, (5) nematicidal, and (6) herbicidal properties; the SMTs also have properties that (7) induce the host defense system and (8) are beneficial for plant growth (Keswani et al. 2015a, b).

Shentu et al. (2014) found that trichodermin, an active metabolite of *T. brevicompactum*, was able to inhibit various plant pathogens. Fungistatic or fungicidal compounds are produced by some species of *Trichoderma*, e.g., *T. virens* (Druzhinina et al. 2011). *Trichoderma* produces several terpenes, which have activity against bacteria (Hermosa et al. 2014). *Trichoderma* also produces some antifeedant compounds against aphids (Ganassi et al. 2007). Further, 6-pentyl-2H-pyran-2-one (6-PP), a volatile organic compound of *Trichoderma* spp., showed significant activity against nematodes (Yang et al. 2012). Kuang et al. (2016) demonstrated the role of SMTs in weed control. Also, some SMTs can induce host defense responses against phytopathogens (Zeilinger et al. 2016). Mukherjee et al. (2013) demonstrated the importance of SMTs in promoting plant growth.

An array of chemically diverse SMTs have shown activity against a diverse group of phytopathogens (Barakat et al. 2014). The modes of action of SMTs against phytopathogenic fungi, bacteria, nematodes, viruses, weeds, and insects are as follows:

(A) For fungi:
1. SMTs affect the germination of spores, elongation of hyphae, and mycelial growth.
2. SMTs cause degradation and malformation of the hyphae and spores.
3. SMTs affect sporulation.
4. SMTs affect the production of enzymes and secondary metabolites.
5. SMTs cause cytotoxicity by producing toxins.
6. SMTs suppress the formation of sexual structures.
7. SMTs cause starvation in competing microorganisms by chelating useful nutrients.
8. SMTs decrease the virulence level by hampering mycotoxin synthesis.

(B) For antagonistic plant bacteria:
1. SMTs prevent binary fission.
2. SMTs cause degradation of the cell wall.
3. SMTs cause cytotoxicity by producing toxins.
4. SMTs affect the production of intercellular and extracellular enzymes and metabolites.
5. SMTs cause starvation by chelating useful nutrients.
6. SMTs decrease the virulence level by suppressing toxin synthesis.

(C) For nematodes:
1. SMTs cause cytotoxicity by producing toxins.
2. SMTs affect egg hatching.
3. SMTs cause juvenile motility.
4. SMTs affect egg productivity.
5. SMTs affect the production of intercellular and extracellular enzymes and metabolites.

6. SMTs cause starvation by chelating useful nutrients.
7. SMTs decrease the virulence level.

(D) For plant viruses:
1. SMTs inactivate virus particles.
2. SMTs decrease the virulence level.

(E) For weeds:
1. SMTs cause cytotoxicity by producing phytotoxins.
2. SMTs cause essential micronutrient deficiency.
3. SMTs cause vascular wilt.
4. SMTs cause blight in leaves.
5. SMTs cause rot in leaves, stems, and roots.
6. SMTs affect the emergence of seeds.

(F) For insects, may be the mode of action of SMT, as following:
1. It causes cytotoxicity by producing the mycotoxins.
2. It affects the mechanisms of metabolism.

Indirect mechanisms include interactions between SMTs and plants. SMTs are able to induce host defense and resistance responses, such as induced systemic resistance and systemic acquired resistance, in the host (Bisen et al. 2015, 2016). Thus, SMTs have the potential to replace traditional synthetic pesticides. Hence, the rapid detection of several classes of potential SMTs for the management of phytopathogens is the need of the hour (Keswani et al. 2013, 2014, 2016).

## 7.2 Applications of Secondary Metabolites from Various *Trichoderma* spp.

SMTs are low-molecular-weight organic compounds that are not essential for growth and reproduction. However, SMTs have found the following applications in the reduction of crop losses:

### 7.2.1 Antifungal

Many SMTs have been reported to possess antifungal activities. Trichorzianine A IIIc was isolated from *T. harzianum* and showed high in vitro antifungal potential against three phytopathogens (Bodo et al. 1985). Three octaketide compounds secreted by *T. harzianum* showed high inhibition against the wheat pathogen *Gaeumannomyces graminis* var. *tritici* (Ghisalberti and Rowland 1993). Hanianum A, a trichothecene compound, was isolated from a culture extract of *T. harzianum* that contained a (Z,E,E)-2,4,6-octatriendioic acid esterified on the 4p hydroxyl group of trichodermol (Corley et al. 1994). Trichorzianines A1 and B1 (peptaibols) were isolated from *T. harzianum* (Schirmböck et al. 1994). Two classes of peptaibols, two asperelines (A and E), and five trichotoxins (T5D2, T5E, T5F, T5G, and 1717A) were isolated from *T.* asperellum TR356 (Brito et al. 2014). Harzianins HC, an antifungal peptide, was isolated from *T. harzianum* (Rebuffat et al. 1995).

Further, crude metabolites from *T. harzianum* (MTCC 2050) could inhibit some soil-borne pathogens, such as *Sclerotium rolfsii*, *R. solani*, and *Fusarium oxysporum* (Choudary et al. 2007). Two strains of *T. harzianum* showed significant in vitro control of the pathogen *F. moniliforme* by secreting the compound 6-pentyl-α-pyrone (El-Hasan et al. 2008). Viridiofungin A from the T23 strain of *T. harzianum* affected the germination of (1) the conidia of *Verticillium dahliae*, (2) the sporangia of *Phytophthora infestans*, and (3) the sclerotia of *Sclerotinia sclerotiorum* (El-Hasan et al. 2009). The production of T39-butenolide, harzianolide, T22-azaphilone, harzianic acid, and harzianopyridone from *T. harzianum* showed significant inhibition of some major fungal phytopathogens (Vinale et al. 2006, 2009a, b, 2014; Ahluwalia et al. 2015). Also, by using gas chromatography-mass spectroscopy GC-MS/MS, Dubey et al. (2011) detected many antifungal compounds, including 6-nonylene alcohol, massoia lactone, methyl cyclopentane, methyl cyclohexane, N-methyl pyrollidine, dermadin, ketotriol, koningin-A, 3-methyl-heptadecanol,2-methylheptadecanol,palmiticacid,and3-(2′-hydroxypropyl)-4-(hexa-2′-4-dineyl)-2-(5H)-furanone, and 3-(propenone)-4-(hexa-2′-4′-dineyl)-2-(5H)-furanone from *T. harzianum* IARI P4. A new compound, trichoharzianol, from *T. harzianum* showed significant antifungal potential against *Colletotrichum gloeosporioides* (Jeerapong et al. 2015).

Lignoren, a new compound, was isolated from *T. lignorum* HKI 0257 (Berg et al. 2004). Viridin, an antifungal compound isolated from *T. koningii*, *T. viride*, and *T. virens*, is active against several plant fungal pathogens, including *S. rolfsii*, *R. solani*, and *Pythium* sp. (Singh et al. 2005; Mukherjee et al. 2007). Fifth compounds, (1) acorane-type sesquiterpene, (2) 2b-hydroxytrichoacorenol (A), (3) a bisabolane-type sesquiterpene, (4) trichoderic acid (B), and (5)?, with three known compounds, cyclonerodiol (1), cyclonerodiol oxide (2), and sorbicillin (3), isolated from *Trichoderma* sp., were shown to have antifungal activities (Wu et al. 2011). Trichokonin VI from *T. pseudokoningii* SMF2 caused apoptotic cell death in the cells of *F. oxysporum* (Shi et al. 2012). *T. virens* IARI P3 and *T. viride* IARI P1 and IARI P2 also produced antifungal compounds (Dubey et al. 2011). Cytosporone S, isolated from *Trichoderma* sp. FKI-6626, also had potent antifungal activity (Ishii et al. 2013).

In addition, an antifungal compound, 6-PP, was produced by several species of *Trichoderma,* including *T. hamatum*, *T. citrinoviride*, *T. viridescens*, *T. atroviride*, and *T. viride* (Jeleń et al. 2014). *T. brevicompactum* secretes the active antifungal compound trichodermin, which inhibited three plant fungal pathogens, viz., *Botrytis cinerea, Colletotrichum lindemuthianum,* and *R. solani* (Shentu et al. 2014, 2015). The culture filtrate (CF) of *Trichoderma* H921 suppressed spore germination and appressorium formation in *Magnaporthe oryzae,* suggesting the existence of some antifungal compounds in the filtrate (Nguyen et al. 2016). Also, two *Trichoderma* isolates, *T. atroviride/petersenii* (Korea Agricultural Culture Collection 40, 557) and *T. virens* (KACC 40929), inhibited *Phytophthora capsici* (KACC 40157), *P. drechsleri* (KACC 40463), *P. infestans* (KACC 43071), *P. cactorum* (KACC, 40166), *P. melonis* (KACC 40197), *P. sojae* (KACC 40412), and *P. nicotianae* (KACC 44717)

(Bae et al. 2016). Three antifungal tricyclic polyketide compounds were produced by *T. koningiopsis* QA-3 (Shi et al. 2017). Further, 6-pentyl-α-pyrone from *T. asperellum* T23 and *T. harzianum* T16 suppressed perithecium formation and ascospore discharge in *F. graminearum* (El-Hasan et al. 2017; Marques et al. 2018).

### 7.2.2 Antibacterial

*T. harzianum* and *T. longibrachiatum* secreted 6-n-pentyl-α-pyrone, which showed high inhibition against both gram-positive and gram-negative bacteria (Tarus et al. 2003). *T. asperellum* produced an antibacterial compound, trichotoxin (Chutrakul et al. 2008). Five antibacterial compounds from *T. longibrachiatum* were able to inhibit the growth of three pathogenic bacteria, viz., *Escherichia coli*, *Staphylococcus albus*, and *Shigella sonnei* (Wu et al. 2011; Shi et al. 2017). A new antibacterial compound, cytosporone S, was produced by *Trichoderma* sp. FKI-6626 (Ishii et al. 2013). Trichokonins from *T. pseudokoningii* SMF2 were able to control a gram-negative bacterium, *Pectobacterium carotovorum*, that caused soft rot in Chinese cabbage (Li et al. 2014). *T. harzianum* produced bioactive compounds that showed high efficacy in inhibiting plant bacterial pathogens such as *Xanthomonas campestris, Clavibacter michiganensis*, *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus* (Anwar and Iqbal 2017).

### 7.2.3 Antiviral

Selim et al. (2012) reported the role of secondary metabolites that were isolated from endophytic fungi for the control of viruses. Some antiviral compounds were isolated from *T. atroviride* (Fukami et al. 2000; Omura et al. 2001). Trichokonin, an antiviral peptaibol from *T. pseudokoningii* SMF2, inhibits the lesion, and decreases the average lesion diameter of tobacco mosaic virus (TMV) infection in tobacco (Luo et al. 2010). Two compounds of *Trichoderma*, trichodermin and trichoderminol, which are new secondary metabolites, are very effective against plant viruses. Lee et al. (2014) extracted trichodermin and trichoderminol from *T. albolutescens*. Interestingly, both these metabolites were tricothecene-based compounds and could manage plant viruses such as cucumber mosaic virus, pepper mottle virus (PepMoV), TMV, watermelon mosaic virus2, zucchini green mottle mosaic virus, melon necrotic spot carmovirus, turnip mosaic virus, tomato spotted wilt virus, zucchini yellow mosaic virus, pepper mild mottle virus, cymbidium mosaic virus, lily symptomless virus, odontoglossum ringspot virus, strawberry mottle virus, watermelon mosaic virus, potato leafroll virus, lily mottle virus, tomato ringspot virus, potato virus Y, tobacco ringspot virus, cactus X virus, broad bean wilt virus , and cucumber green mottle mosaic virus (Lee et al. 2017). Trichodermin and trichoderminol from *T. albolutescens* were effective in protecting tobacco and pepper from infection with PepMoV (Ryu et al. 2017).

### 7.2.4 Nematicidal

Plant parasitic nematodes can be controlled by the use of some SMTs from *Trichoderma* spp. Indeed, SMTs from some species of *Trichoderma* have been used instead of synthetic pesticides to kill plant parasitic nematodes. *T. harzianum* and *Trichoderma* spp. secreted some compounds having nematicidal activities against *Meloidogyne javanica* (Nitao et al. 1999; Sharon et al. 2001). SMT of *T. harzianum* caused high mortality in *M. incognita* nematodes. Also, SMT of *T. harzianum* reduced the survival of *M. incognita* in soil without the existence of a tomato (A host plant), which was shown the toxicity of SMT for both of J2 and eggs (Dababat 2007). A trichodermin compound identified by spectroscopic data in 15 *Trichoderma* strains showed strong nematicidal activity (Yang et al. 2010). The YMF 1.00416 strain of *Trichoderma* sp. showed great efficacy in killing three species of nematodes; namely, *Panagrellus redivivus*, *Caenorhabditis elegans*, and *Bursaphelenchus xylophilus*. The YMF 1.00416 strain secreted a nematicidal compound, 6-PP, and also produced two other compounds, one new compound, 1β-vinylcyclopentane-1α,3α-diol, and one known compound, 4-(2-hydroxyethyl)phenol (Yang et al. 2012). Zhou et al. (2014) detected three new compounds, isolated from *T. neokongii 8722*, that have nematicidal activity. Two novel compounds, koninginins L and M, produced from *T. neokongii* 8662, were found to be nematicidal (Lang et al. 2015).

### 7.2.5 Herbicidal

Weeds affect the production of economic crops and cause great damage to agriculture and animal grazing land. Many synthetic pesticides have been used to eradicate weeds and this has led to pollution of the ecosystem. Several strains of *Trichoderma* produce multiple SMTs that can be used instead of synthetic herbicides. The herbicidal compound such as viridiol, produced by *T. virens*, that was phytotoxic for weeds strongly (Jones and Hancock 1987; Héraux et al. 2005). Viridiol produced by *T. virens* grown on special media containing composted chicken manure decreased the seedling growth of the weed *Amaranthus retroflexus* L. (redroot pigweed) (Héraux et al. 2005). The CF of *T. harzianum*, *T. reesei*, and *T. pseudokoningii* has been sprayed as a herbicide on wild oat (*Avena fatua* L.), which is a wheat weed; the shoots and roots of wild oat were significantly diminished (Javaid and Ali 2011a). The treatment of *Rumex dentatus* L. (a wheat weed), by different methods, with the CF of some *Trichoderma* spp., affected the growth of this weed's roots and shoots. A foliar spray with the CF of four *Trichoderma* spp., namely, *T. harzianum*, *T. pseudokoningii*, *T. reesei*, and *T. viride,* reduced the biomass of the roots and shoots of *Rumex dentatus* L., while the CF of three *Trichoderma* spp., namely, *T. pseudokoningii*, *T. reesei*, and *T. viride*, decreased different parameters of seedling growth for wheat (Javaid and Ali 2011b). Treatment with a detached leaf injection bioassays containing two fractions (3 mg/mL$^{-1}$) of (1) *n*-hexane fractions from *T. viride, T. pseudokoningii*, and *T. reesei*, and (2) ethyl acetate fractions of *T. harzianum* and *T. pseudokoningii* had a toxic action on *Rumex dentatus* L. weed, indicating the

herbicidal activity of *Trichoderma* (Javaid and Ali 2011b). The CF from four *Trichoderma* spp., namely, *T. pseudokoningii*, *T. harzianum, T. viride,* and *T. reesei*, prepared in M-1-D medium, reduced the shoots and roots of parthenium weed (Javaid et al. 2013). The CF of *T. longibrachiatum* strain Tr673 inhibited shoot/root growth and seed germination in three species of weeds, namely, purslane (*Portulaca oleracea*), *Amaranthus retroflexus* L., and barnyard grass (*Echinochloa crus-galli*). The CF of strain Tr673 grown on PDB (Potato dextrose broth) + 0.4% sodium glutamate was more effective as a herbicide on weeds than the CF of the same strain grown on PDB only (Kuang et al. 2016).

### 7.2.6 Insecticidal

Many endophytic fungi produce toxic compounds that have a strong effect on insects. Some *Trichoderma* spp. secrete several SMTs that cause mortality in insects. Some SMTs of *T. viride* affected the activity of the mosquito *Culex quinquefasciatus* by more attracting for the gravid female mosquitoes to oviposition and huge in the percentage of egg rafts laid in the test solution (Geetha et al. 2003). The peptaibols produced by two strains of *T. harzianum* (101, 645 and 206, 040) are insecticidal and can be used for direct treatment on insect cuticles, together with some enzymes, or by addition to larval food (Shakeri and Foster 2007).

## 7.3 Role of Secondary Metabolites of *Trichoderma* in the Control of Plant Pathogens

*Trichoderma* has already been registered as biological control agent and it is used against many plant pathogens worldwide. The potential of *Trichoderma* for the control of various plant pathogens depends on multiple factors, such as mycoparasitism, competition, and the secretion of SMTs. *Trichoderma* does not depend on all these factors simultaneously for its actions against plant pathogens, as the factors differ according to the kind of plant pathogen. In this section, we note the capacity of *Trichoderma* SMTs to stimulate plant defenses and resistance, and the role of SMTs in controlling the *Fusarium* pathogen of plants is outlined as an example.

### 7.3.1 Stimulation of Plant Defenses and Resistance by the Secondary Metabolites of *Trichoderma*

SMTs can stimulate the defenses of plants and their resistance to plant pathogens. The 6PP compound of many *Trichoderma* strains, such as T22, T39 and A6, and P1, acts on plant defenses by detecting pathogenesis-related proteins; this compound is considered to act as an auxin inducer (Vinale et al. 2008). The trichokonin compound of *Trichoderma* strain SMF2 induced resistance to TMV by increasing the production of reactive oxygen species and phenolic compounds in tobacco (Luo

et al. 2010). The CF of *T. asperellum* SKT-1 induced resistance in *Arabidopsis thaliana* Col-0 against the bacteria *Pseudomonas syringae* pv. tomato (Pst) DC3000 (Yoshioka et al. 2011). A novel role for a harzianolide compound of *T. harzianum* strain SQR-T037 was shown in enhancing defenses in the tomato plant (Cai et al. 2013). The isoharzianic acid (iso-HA) compound of HA, a new HA compound, inhibited the mycelial growth of two plant pathogens, *Sclerotinia sclerotiorum* and *Rhizoctonia solani,* and stimulated systemic resistance to *B. cinerea* (Vinale et al. 2014). The volatile compound 6PP stimulated plant defenses in *A. thaliana* against *B. cinerea* and *Alternaria brassicicola* (Kottb et al. 2015).

### 7.3.2 *Fusarium oxysporum*

*Fusarium* spp., as plant pathogens, attack almost all plants worldwide and can cause great damage to the economy. The *Fusarium* genus is very difficult to control, because:

(1) The pathogen's survival in soil and plant residue is achieved by producing dormant spores such as chlamydospores, and some strains produce sclerotia.
(2) New strains are occurring that are resistant to synthetic fungicides.

Therefore, we need to change the control method by using biopesticides as alternatives to chemical fungicides. The use of *Trichoderma* SMTs is the best method to reduce spreading damage caused by *Fusarium* pathogens in agriculture. The 6-n-pentyl-2H-pyran-2-one (6-PAP) compound from strain IMI 288012 of *T. harzianum* completely inhibited *F. oxysporum* f. sp. *lycopersici* after 2 days (Scarselletti and Faull 1994). *F. oxysporum* was inhibited by the crude media extracted from *T. harzianum* strain MTCC 2050 (Choudary et al. 2007). Isolates of *T. viride* (Tv-1) reduced the hyphal growth of *F. oxysporum* by 41.88% (Amin et al. 2010). The growth of *F. oxysporum* was inhibited by 53.99% by volatile compounds produced from *T. viride* (Tapwal et al. 2011). The volatile compounds of two species of *Trichoderma*, namely, *T. harzianum* T1 s and *T. viride* (TvPDs), inhibited the growth of *F. oxysporum*, at a range of 25.97–40.91%, in date palm soil, tested in vitro (Perveen and Bokhari 2012).

Indeed, different strains of *Trichoderma*, namely, *T. harzianum* (Th-5 and Th-7), *T. viride* (Tv-2 and Tv-18), and *T. koningii* (Tk-9), showed high inhibition of *F. oxysporum* f. sp. *lentis* (FOL), which causes fusarium wilt of lentil, at a range between 51.1% and 83.3% with the use of liquid CF (Sharfuddin and Mohanka 2012). Local isolates of *Trichoderma*, namely, *T. viride* (T35) and *T. koningiopsis* (T18), produced volatile and non-volatile compounds that were very effective against *F. oxysporum* f. sp. *phaseoli* (FOP), which causes fusarium wilt of bean (Oloo 2013). Also, *T. brevicompactum* inhibited the mycelial growth of *F. oxysporum* through producing a trichodermin compound (Shentu et al. 2013). The fungicidal activities of CF from *T. hamatum* strain IMI388876 showed high efficacy in the growth inhibition of *F. oxysporum* f. sp. *lentis* (El-Hassan et al. 2013). Two species of *Fusarium*, namely, *F. oxysporum* f.sp. *cepae* and *F. proliferatum,* attack onion bulbs and cause great

damage, but two strains of *T. harzianum* (Th. and T100) and *T. haematum* (T.haem.) produced volatile and non-volatile compounds that acted very effectively against these two onion pathogens (Ghanbarzadeh et al. 2014).

Further, some SMTs can reduce the occurrence of secondary infection by plant pathogens. *T. harzianum* SQR-T037 significantly inhibited the growth of *F. oxysporum* f. sp. *niveum* both in vitro and in vivo by producing volatile and non-volatile compounds (Raza et al. 2013). Secondary metabolites of *T. asperellum* inhibited the sporulation and the conidia germination of *F. oxysporum* (Daniel et al. 2014). The decrease of inoculation by plant pathogens in the environment is a very interesting step that leads to success in the biological control process. The volatile compounds of *Trichoderma* can reduce seed diseases. Carvalho et al. (2014), in Brazil, detected some isolates of *T. harzianum* that produced volatile compounds that were very effective against *F. oxysporum* f. sp. *Phaseoli*, which attacks the seeds of the common bean (*Phaseolus vulgaris* L.)

Surprisingly, *T. harzianum* T-E5 produced several volatile organic compounds that suppressed fusarium wilt in cucumbers infected with the plant pathogen *F. oxysporum* f. sp. *cucumerinum* (FOC) (Zhang et al. 2014). Volatile and non-volatile compounds from *T. virens*, *T. pseudokoningii*, *T. atroviride*, and *T. koningii* showed high efficacy for reducing the growth of *F. oxysporum* f. sp. *lycopersici* (Reddy et al. 2014). A harzianopyridone compound inhibited *F. oxysporum* growth by more than 90% (Ahluwalia et al. 2015). The growth of *F. oxysporum* was suppressed by volatile and non-volatile compounds produced by two species of *Trichoderma*, *T. viride* and *T. harzianum* (Tapwal et al. 2011). The ZJSX5003strain of *T. asperellum* produced peptaibols and secondary metabolites that were effective in reducing (by 71%) corn stalk rot disease in maize caused by *F. graminearum* (Li et al. 2016). The isolates $T_{22}$, $T_9$, and $T_6$ of *T. harzianum* showed high efficacy against *F. oxysporum* f. sp. *radicis-cucumerinum* (Javid et al. 2016). Two local isolates, Tv-9 and TNAU of *T. viride*, inhibited the mycelial growth of *F. oxysporum* f. sp. *Cepae*, which causes wilt in crossandra. These two isolates, Tv-9 and TNAU (Local isolate), produced 17 compounds, comprising: (1) 3-hexanol, 2-methyl, (2) furan, 2,3-dihydro-4-(1-methylpropyl)-s-(CAS), (3) 1,1-dibutoxy-2-propanone, (4) benzene ethanol, (5) 1,3-benzenediol, 5 methyl (CAS), (6) N-methyl-pyrrolidine, (7) 2-isopropanol-4 methoxy pyrimidine, (8) 3-cyclohexene-1-amine, 6-(chlorophenyl)-2,5-diphenyl, (9) cyclooctenone, dimer (CAS), (10) octodecanoic acid, (11) hexadecanoic acid, methyl (CAS), (12) quinoline, 2-sec-butyl, (13) 1,2-benzenedicarboxylic acid, dibutylester (CAS), (14) 8–11-octodecanoic acid, methylester (CAS), (15) cholic acid, (16) cis-2-pheny-l,3- dioxalone-4-methyl octaden-9, 12, 15-trienoate, and (17) isochiapin A, that were detected by GC-MS analysis (Mallaiah et al. 2016).

In addition, Nagamani et al. (2017) detected many isolates of *Trichoderma* spp. that produced very toxic volatile and non-volatile compounds against *F. oxysporum* f. sp. *ciceri* (FOC). Volatile compounds produced from *T. asperellum* (ATPU 1 and KNPG 3) and *T. harzianum* (ATPP 6) inhibited the mycelial growth of FOC at ranges of 83.5–86.7%. Also, non-volatile compounds secreted from *T. viride* (KNN 2), *T. harzianum* (ATPP 6), and *T. longibrachiatum* (KR 4) at a concentration of 20%, inhibited the mycelial growth of FOC at a range of 83.3–95.0%. Several

isolates of *Trichoderma* spp. were very effective against *F. oxysporum* f sp. *melogenae* (FOM), which causes fusarium wilt of eggplant. The mycelial growth of FOM was inhibited, at a range of 77.77–81.11%, by non-volatile compounds produced from seven *Trichoderma* spp., namely, *T. harzianum*, *T. reesei*, *T. koningii*, *T. viride*, *T. virens*, *T. atroviride*, and *T. pseudokoningii*, while *T. harzianum* and *T. viride* produced toxic volatile compounds that inhibited FOM at a range of 48.88–54.44% (Cherkupally et al. 2017). The transcription coactivator MBF1 was found to play an important role in the production of volatile compounds in *T. harzianum* T34 strain that acted against *Fusarium oxysporum* f. sp. *lycopersici* race 2 (FO) (Rubio et al. 2017). Jeleń et al. (2014) found that eight species of *Trichoderma* that produced the 6-PAP compound, with 40 volatile compounds, were able to reduce the growth of seven *Fusarium* species, namely, *F. avenaceum* (KF 2818), *F. cerealis* (KF 1157), *F. culmorum* (KF 846 and KF 350), *F. graminearum* (KF 2870), *F. proliferatum* (KF 925), and *F. subglutinans* (KF 506). A special mix of *T. harzianum*, *T. viride*, and cow manure was very effective in inhibiting *F. oxysporum* f. sp. *lycopersici* in vivo, suggesting that the production of volatile and non-volatile compounds acted together with other mechanisms (Moosa et al. 2017). Three *Trichoderma* spp., *T. spirale*, *T. harzianum*, and *T. brevicompactum*, produced non-volatile metabolites that inhibited the mycelial growth of *F. oxysporum* (Marques et al. 2018).

Interestingly, many new volatile and non-volatile compounds of very significant species of *Trichoderma,* such as *T. atroviride*, *T. harzianum*, *T. koningii*, *T. viride*, and *T. virens*, are now being detected by solid phase microextraction and GC-MS. Some SMTs affect plant fungal pathogens by suppressing mycotoxin synthesis. El-Hasan et al. (2008) detected a 6-pentyl-alphapyrone compound from two strains of *T. harzianum* that played a role in degrading fusaric acid synthesis in *F. moniliforme*. Chakraborty and Chatterjee (2008) found that *T. harzianum*, *T. viride*, *T. lignorum*, and *T. hamatum* inhibited the growth of *F. solani*, which causes fusarium wilt of eggplant, by 100%, by the production of non-volatile compounds; however, the growth inhibition generated by volatile compounds ranged from 55.92 to 78.22%. Four isolates of *Trichoderma*, namely, *T. harzianum* (Tveg1 and TL5), *T. parareesei* (T26), and *T. koningii* (TR102), produced around 30 volatile compounds that were active against *F. oxysporum* f. sp. *cubense* tropical race 4 (*Foc*TR4), detected by GC-MS analysis, and these isolates showed high efficacy in inhibiting *Foc*TR4 both in vitro and in vivo (Al-Ani et al. 2013b; Al-Ani 2017b).

## 7.4 The Beneficial Role of Secondary Metabolites of *Trichoderma* in Plant Growth

The SMTs of *Trichoderma* are very effective in the control of plant pathogens, as well as in enhancing plant growth. Several biocontrol isolates of *Trichoderma*, namely, *T. harzianum* strains T22, T39, and A6, and *T. atroviride* strain P1, were found to improve plant growth after the stems of tomato plants were treated with some SMTs, including a 6PP compound, and harzianolide (Vinale et al. 2008; Cai et al. 2013). The 6PP compound was considered to be auxin-like (Vinale et al.

2008). Harzianic acid (HA) of *T. harzianum* at a low concentration improved plant growth (Vinale et al. 2009b). The biocontrol isolate $TVC_3$ of *Trichoderma* produced very interesting results in increasing the plant vigor of chilli by volatile and non-volatile metabolites that it produced (Muthukumar et al. 2011). A new compound, iso-HA of *T. harzianum* HA, increased the germination of tomato seeds (Vinale et al. 2014). *T. virens* and *T. atroviride* produced several volatile and non-volatile compounds, e.g., indole-3-ethanol, indole-3-carboxaldehyde, auxin indole-3-acetic acid (IAA), and 6-PP, that increased plant growth (Garnica-Vergara et al. 2016).

The biofertilizer role played by the SMTs of some biocontrol *Trichoderma* isolates needs to be elucidated by analytic methods. Lee (2015), by using GC-MS analysis, showed that the *T. atroviride* GJS 01–209 strain produced 26 compounds and these compounds increased *Arabidopsis* vigor, i.e., its seed germination, chlorophyll content, plant biomass, development of lateral roots, and plant fresh shoot weight. Of note, image analysis is being used to study the effects of SMTs on plants and to show plant responses to the SMTs. Garnica-Vergara et al. (2016), using microscopy and confocal imaging, showed that 6-PP of *T. atroviride* enhanced root development in *A. thaliana*. Nieto-Jacobo et al. (2017) determined some analytic parameters for estimating the efficacy of three strains of *Trichoderma*, namely, *T. atroviride* IMI206040, *T. virens* Gv29.8, and *T.* sp. "*atroviride* B" LU132, in producing volatile compounds such as auxins and IAA. These three strains of *Trichoderma* improved the plant vigor of *A. thaliana* by (1) increasing the plant biomass of shoots and roots, (2) increasing chlorophyll content, and (3) increasing root production (Nieto-Jacobo et al. 2017).

Of interest, *T. harzianum* in a special formulation enhanced the indexes of plant vigor, such as seed germination, seedling mean ?, root length, dry matter production, and shoot length, for many plants, including cotton, black gram, chilli, tomato, and sunflower (Balakrishnan et al. 2017). Twelve isolates of *T. harzianum* produced IAA that enhanced quinoa grain yield and the growth of radish and lettuce plants (Ortuño et al. 2017). *T. viride* secreted SMTs that improved seed germination and also improved root length and seedling shoots in *Triticum aestivum* and *Sorghum vulgarae* (More and Gachande 2017). The plant vigor of banana, in terms of chlorophyll content, plant length, leaf number, and plant mass, was increased after treatment with four isolates of *Trichoderma* spp. (TL5, Tveg1, T26, and TR102), although the plants were infected with *Foc*TR4, one of the very dangerous strains of fusarium wilt (Al-Ani 2017b). *T. harzianum* improved the growth of tomato by supplying beneficial nutrients for the root system and increasing the tomato tolerance for drought (Alwhibi et al. 2017).

## 7.5 Conclusion

Secondary metabolites of *Trichoderma* are playing a large role in the successful control of many plant pathogens, as SMTs can be substituted for synthetic or chemical pesticides. The species of *Trichoderma* that secrete SMTs which are effective against different species of plant pathogens, pests, and weeds can be

categorized into three groups according to their importance. *T. harzianum* is the most interesting species in inhibiting different plant pathogens, pests, and weeds. The second most important group of species consists of *T. atroviride*, *T. koningii*, *T. koningiopsis*, *T. virens*, and *T. viride*. The third group includes *T. lignorum*, *T. pseudokoningii*, *T. hamatum*, *T. citrinoviride*, *T. viridescens*, *T. brevicompactum*, *T. asperellum*, *T. albolutescens*, *T. reesei*, and *T. longibrachiatum*. Many species of *Trichoderma* produce many important SMTs, including trichorzianines, harzianins, peptaibols, 6-pentyl-α-pyrone, viridiofungin A, harzianopyridone, harzianic acid, and trichoharzianol. These SMTs very effective in inhibiting the mycelial growth of *F. oxysporum* and other plant fungal pathogens, and some of these compounds are able to stimulate the defenses and resistance of plants, with the enhancement of plant growth. Finally, in addition to their effects on plant pathogens, SMTs can reduce the effects of weeds and insects that attack plants and cause great damage in agriculture.

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# Part II

# Bacterial PGPRs

# 8 Secondary Metabolites of the Plant Growth Promoting Model Rhizobacterium *Bacillus velezensis FZB42* Are Involved in Direct Suppression of Plant Pathogens and in Stimulation of Plant-Induced Systemic Resistance

Rainer Borriss, Huijun Wu, and Xuewen Gao

## 8.1 Introduction

Biocontrol effects exerted by antagonistic acting bacilli are due to different mechanisms; besides direct antibiosis and competition by secretion of a spectrum of secondary metabolites in the rhizosphere, the beneficial action on the host-plant microbiome (Erlacher et al. 2014) and stimulation of plant-induced systemic resistance (ISR) (Dornboos et al. 2012) are of similar importance. ISR is induced by a range of secondary metabolites, which are called "elicitors." Different signaling pathways, such as jasmonic acid (JA), ethylene (ET), and salicylic acid (SA), are activated to trigger plant resistance. Keeping this in mind, the focus of this review is directed to the characterization of antimicrobial compounds synthesized by the biocontrol bacterium FZB42 and their beneficial action on plant health.

The group of plant-associated, endospore-forming rhizobacteria, previously known as *Bacillus amyloliquefaciens* subsp. *plantarum* (Borriss et al. 2011) and nowadays reclassified as being *B. velezensis* (Dunlap et al. 2016), are able to enhance yield of crop plants (plant growth promotion function) and to suppress plant pathogens (biocontrol activity) (Borriss 2011). Representatives of this group

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of bacteria are increasingly applied in sustainable agriculture in order to replace, at least in part, chemical pesticides and fertilizers. Taxonomically they belong to a group we have recently designated as "*B. amyloliquefaciens* operational group" (Fan et al. 2017). Besides *B. velezensis*, also *B. amyloliquefaciens*, known for its ability to produce extracellular enzymes with industrial importance (amylases, glucanases, and proteases), and *B. siamensis*, mainly occurring in Asian food, are members of this operational group, which is distinct from *B. subtilis*. FZB42 (=BGSC 10A6, DSM23117), the prototype of Gram-positive bacteria with phytostimulatory and biocontrol action, has been genome sequenced in 2007 (Chen et al. 2007) and is subject of intensive research. Since its isolation from beet rhizosphere (Krebs et al. 1998), more than 200 articles dealing with FZB42 have been published (http://amylowiki.top/reference.php).

## 8.2 Special Features of the FZB42 Genome

The 3918-kb FZB42 genome, containing an estimated 3695 protein-coding sequences (CDS), lacks extended phage insertions, which occur ubiquitously in the related *Bacillus subtilis* 168 genome, which is recently considered as being also a plant-associated bacterium (Wipat and Harwood 1999; Borriss et al. 2018). Many genes, essential for a plant-associated lifestyle, are shared between *B. subtilis* 168 and FZB42 as well. Spectacular examples are YfmS, a chemotaxis sensory transducer recognizing a still unknown substrate, is involved in the colonization of *Arabidopsis thaliana* roots (Allard-Massicotte et al. 2017) and BlrA (formerly YtvA), a blue light receptor related to plant phototropins (Borriss et al. 2018).

FZB42 secretes different hydrolases, enabling them to use external cellulosic and hemicellulosic substrates present in plant cell walls. Microbe-associated hydrolytic enzymes digesting plant cell wall structures, resulting in free oligosaccharides, have been shown to act as elicitors of plant defense (Ebel and Scheel 1997). Some genes encoding for extracellular hydrolases, such as *amyE* (α-amylase), *eglS* (endo-1,4-ß-glucanase), and *xynA* (xylanase), were found in the plant-associated representatives of the "*B. amyloliquefaciens* operational group" but not in their soil-associated counterparts (Borriss et al. 2011; Zhang et al. 2016). Similarly, an operon with *xylA*, involved in xylose degradation (EC 5.3.1.5); *xynP*, encoding an oligosaccharide transporter; *xynB*, encoding 1,4-β-xylan xylosidase (EC 3.2.1.37); and *xylR*, encoding the xylose operon repressor, are present in *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 but missing in the *B. amyloliquefaciens* DSM7$^{T}$ genome (Rückert et al. 2011).

Three unique genes encoding enzymes involved in hexuronate degradation were found in *B. velezensis*: *kdgK1*, (2-dehydro-3-deoxygluconokinase EC:2.7.1.45), *kdgA* (2-dehydro-3-deoxyphosphogluconate aldolase, EC:4.3.1.1.16), and LacI-like transcription regulator *kdgR*. The three genes are part of a six-gene *kdgKAR* operon and located within a cluster of ten genes flanked by two rho-independent transcription terminators. Inside of the ten-gene cluster, three independent transcription units exist: besides the six-gene *kdgKAR* operon, a probably monocistronic *exuT* gene

with sugar phosphate transporter function and a three-gene *yndGHJ* operon with unknown function (He et al. 2012). Besides *yjmD*, a gene with putative galactitol-1-phosphate dehydrogenase function and, also present in *B. subtilis*, two genes encoding enzymes involved in D-mannonate metabolism are part of the six-gene transcription unit: the mannonate dehydratase UxuA, EC 4.2.1.8, and *uxuB* encodes mannonate oxidoreductase (EC 1.1.1.131). In addition, a second operon containing the genes *uxaC*, *uxaB*, and *uxaA* encoding enzymes for degrading and isomerizing of different hexuronates to D-altronate and D-fructuronate occurs remote from the ten-gene cluster. Since 6-phosphogluconate dehydratase converting 6-phosphogluconate to KDPG is lacking in *B. velezensis*, we assume that D-mannonate oxidoreductase, UxuB, catalyzes the NAD-dependent interconversion of D-mannonate and D-fructuronate. YjmE/UxuA dehydrates then mannonate to 2-keto-3-deoxygluconate, KDG, which is phosphorylated to 2-keto-3-deoxy-6-phosphogluconate, KDPG, by KDG kinase. This metabolic route is part of a derivative pathway of aldohexuronates in *E. coli* K12 in which UxuA, KdgK, and KdgA are involved (Portalier et al. 1980). Thus, the complete biochemical pathway from galacturonate to KDG is present in *B. velezensis* (He et al. 2012), but no gene encoding D-glucuronate isomerase was detected, suggesting that *B. velezensis* is not able to metabolize D-glucuronate. *B. subtilis yjmD*, *yjmE (uxuA)*, *yjmF (uxuB)*, and *yjmG (exuT)* displayed high similarity (75–83%) to the corresponding genes in the *B. velezensis* ten-gene cluster.

After a recent literature search, we found 576 genes involved in plant-bacteria interaction (http://amylowiki.top/interaction.php).

## 8.3 Structure of Gene Clusters Involved in Synthesis of Secondary Metabolites in FZB42

The FZB42 genome reveals a huge potential to produce secondary metabolites, including the polyketides bacillaene, macrolactin, and difficidin (Chen et al. 2006; Schneider et al. 2007) and the lipopeptides surfactin, bacillomycin D, and fengycin (Koumoutsi et al. 2004). In total, the FZB42 genome harbors 13 gene clusters involved in non-ribosomal and ribosomal synthesis of secondary metabolites with putative antimicrobial action. In two of them, in the nrs gene cluster and in the type III polyketide gene cluster, their products are not identified till now (Table 8.1). Similar to *B. subtilis* $168^T$, the genome of the non-plant-associated soil bacterium *B. amyloliquefaciens* $DSM7^T$ harbors a significantly lower number of gene clusters involved in non-ribosomal synthesis of secondary metabolites than strain $FZB42^T$ (Table 8.1). Polyketides and lipopeptides comprise two families of natural products biosynthesized in a similar fashion by multimodular enzymes acting in assembly line arrays. The monomeric building blocks are organic acids or amino acids, respectively (Walsh 2004). Synthesis of lipopeptides and polyketides is depending on Sfp, a PPTase that transfers 4′-phosphopantetheine from coenzyme A to the carrier proteins of nascent peptide or polyketide chains. In *B. subtilis*-type strain $168^T$, there is a frame shift mutation within the *sfp* gene hindering non-ribosomal

**Table 8.1** Presence of genes and gene clusters encoding for secondary metabolites in *B. velezensis* FZB42, *B. amyloliquefaciens* DSM7$^T$, and *B. subtilis* 168$^T$

| Gene cluster | Size | metabolite | FZB42 NC_009725.1 | FZB42 genome | DSM7 NC_014551.1 | BS168 NC_000964.3 | MIBiG accession |
|---|---|---|---|---|---|---|---|
| Sfp-dependent non-ribosomal synthesis of lipopeptides (NRPS) | | | | | | | |
| *srfABCD, aat,ycxC, ycxD,sfp,yczE* | 29.1 kb | Surfactin | 342,618–374,584 | Core genome | 333,123–362,173 | 376,967–408,887 | BGC0000433 |
| *bmyCBAD* | 39.7 kb | Bacillomycin D | 1,871,171–1,908,422 | Core genome | 1,968,514–2008850[a] | – | BGC0001090 |
| *fenABCDE* | 48.1 kb | Fengycin | 1,921,411–1,969,477 | G15: 1939781–1,967,431 | 2,017,516–2040900[b] | 1,949,681–2,002,351 | BGC0001095 |
| *nrsABCDEF* | 15.0 kb | Orphan | 2,885,927–2,868,410 | G22: 2868278–2,887,889 | – | – | – |
| Sfp-dependent non-ribosomal synthesis of Bacteriocin-Nrps | | | | | | | |
| *dhbABCDEF* | 27.2 kb | Bacillibactin | 3,019,044–3,038,453 | Core genome | 3,053,649–3,066,379 | 3,278,324–3,297,919 | BGC0001185 |
| Sfp-dependent non-ribosomal synthesis of polyketides (Transatpks-Nrps type I) | | | | | | | |
| *mlnABCDEFGHI* | 52.2 kb | Macrolactin | 1,391,841–1,444,003 | G13: 1402380–1,445,564 | – | – | BGC0000181 |
| *baeBCDE,acpK,baeGHIJLMNRS* | 71.1 kb | Bacillaene | 1,700,344–1,772,787 | Core genome | 1,785,330–1,856,436 | 1,782,712–1,859,783 | BGC0001089 |
| *dfnAYXBCDEFGHIJKLM* | 69.5 kb | Difficidin | 2,276,742–2,346,266 | G19: 2276734–2,347,685 | – | – | BGC0000176 |
| Type III polyketide synthesis | | | | | | | |
| *bpsAB* | 1.6 kb | Triketide pyrone | 2,122,078–2,123,684 | Core genome | 2,189,857–2,191,463 | 2,316,446–2,318,053 | – |
| Sfp-independent non-ribosomal synthesis | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *bacABCDE,ywfG* | 7.3 kb | Bacilysin | 3,593,876–3,601,174 | Core genome | 3,654,159–3,660,055 | 3,867,492–3,874,150 | BGC0001184 |
| Ribosomal synthesis of modified peptides (RiPP) | | | | | | | |
| *pznFKGHIAJCDBEL* | 9.96 kb | Plantazolicin | 726,469–736,360 | GI 6: 724191–740,699 | – | – | BGC0000569 |
| *acnBACDEF* | 4.2 kb | Amylocyclicin | 3,044,505–3,048,679 | Core genome | 3,076,887–3,081,038 | – | BGC0000616 |
| *lci* | 0.3 kb | Antibacterial peptide | 310,858–311,142 | Core genome | 1,296,288–1,296,563 | – | – |
| Immunity, but no synthesis genes | | | | | | | |
| *mrsK2R2FGE* (partial) | 4.82 kb | Mersacidin | 3,769,734–3,774,552 | Core genome | – | – | BGC0000527 |
| *bceBASR* (partial) | 4.49 kb | Bacitracin | 2,856,835–2,861,322 | Core genome | – | – | BGC0000310 |
| *spaKREF*(partial) | 4.29 kb | Subtilin | 3,210,423–3,214,712 | Core genome | – | – | BGC0000559 |

Genomic islands (GIs) in FZB42 were identified by SeqWord and M-GCAT (Rückert et al. 2011). The MIBiG accession numbers (Medema et al. 2015) are indicated

[a]DSM7$^T$ contains the gene cluster for synthesis of iturin A (BGC0001098), which is closely related to *bacillomycin* D

[b]The gene cluster for non-ribosomal synthesis of *fengycin* is only present in part in the genome of DSM7$^T$

synthesis of surfactin, fengycin, and bacillaene in this domesticated laboratory strain (Borriss et al. 2018). Around 8.5% of the whole genomic capacity of FZB42 is devoted to non-ribosomal synthesis of these both families of secondary metabolites (Chen et al. 2009b) (Fig. 8.1).

### 8.3.1 Type I and Type III Polyketides

Polyketides are an important class of secondary metabolites, which are synthesized through decarboxylative condensation of carboxylic acids by polyketide synthases (PKSs). PKSs are a giant assembly of multifunctional polypeptides, each consisting

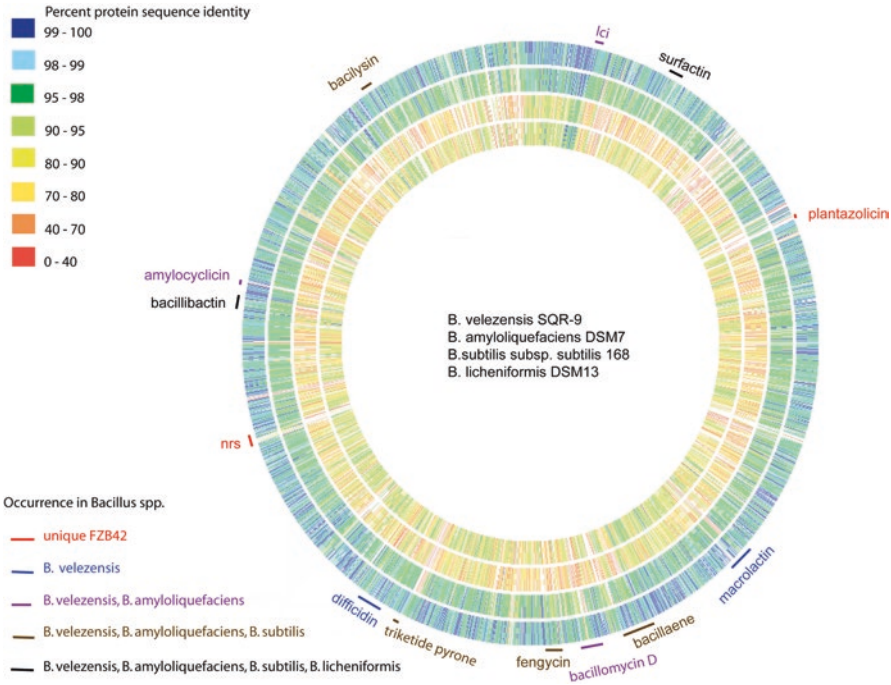


**Fig. 8.1** Genome comparison of FZB42 with *B. velezensis*, *B. amyloliqufaciens*, *B. subtilis*, and *B. licheniformis*. The whole genomes of *B. velezensis* SQR-9 (outside circle), *B. amyloliquefaciens* DSM7$^T$ (2nd circle), *Bacillus subtilis* 168$^T$ (3th circle), and *B. licheniformis* DSM13$^T$ (inner circle) were aligned with FZB42$^T$ using the RAST server (Aziz et al. 2008). The color code indicates % similarity of single gene products. Thirteen sites (genes or gene clusters) involved in synthesis of antimicrobial compounds were identified within the genome of FZB42 (compare also Table 8.1). The gene clusters responsible for non-ribosomal synthesis of the polyketides macrolactin and difficidin are unique in *B. velezensis*. The gene cluster for synthesis of bacillomycin D/iturin A and amylocyclicin and the gene for synthesis of the antimicrobial peptide Lci occur also *in B. amyloliquefaciens*. The gene clusters for non-ribosomal synthesis of bacillaene, fengycin, and the hypothetical tripeptide pyrone occur *in B. velezensis*, *B. amyloliquefaciens*, and *B. subtilis*. (The figure has been redrawn after Fig. 1 in Chowdhury et al. 2015b).

of a series of catalytic domains. Essential domains for chain elongation are ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP). In bacilli, e.g., FZB42, a special class of PKSs that lack the cognate AT domain and require a discrete AT enzyme acting iteratively *in trans* (trans-AT), was detected (Shen 2003). Unfortunately, structural instability of these polyketides excluded until now their use as antibacterial agents.

Besides type I PKS, also genes encoding type III polyketide synthases are present in the genome of FZB42. By contrast to type I PKSs, the type III PKSs do catalyze the priming, extension, and cyclization reactions iteratively to form a huge array of different polyketide products (Yu et al. 2012). In *Bacillus subtilis*, gene products of bspA-bspB operon were functionally characterized and found to be involved in synthesis of triketide pyrones. The type III PKS BspA is responsible for the synthesis of alkylpyrones and BspB is a methyltransferase that acts on the alkylpyrones to yield alkylpyrone methyl ethers (Nakano et al. 2009). However, their biological role needs further elucidation. Orthologs of *bspA* and *bspB* are present in FZB42 and DSM7$^T$ (Table 8.1).

### 8.3.2 NRPS

Another important class of secondary metabolites, also non-ribosomally synthesized by giant multifunctional enzymes (peptide synthetases, NRPS), is formed by lipopeptides. Similar to PKS, three catalytic domains are involved in each elongation cycle: (1) the A-domain (adenylation domain) selects its cognate amino acid; (2) the PCP domain (peptidyl-carrier domain) is equipped with a PPan prosthetic group to which the adenylated amino acid substrate is transferred and bound as thioester; and (3) the condensation domain (C-domain) catalyzes formation of a new peptide bond (Duitman et al. 1999).

Nearly 10% of the FZB42 genome is devoted to synthesizing antimicrobial compounds by pathways either involving or not involving ribosomes. Notably, the gene clusters involved in non-ribosomal synthesis of the antifungal lipopeptide bacillomycin D and the antibacterial polyketides difficidin and macrolactin are absent in DSM7$^T$ and other representatives of *B. amyloliquefaciens* suggesting that synthesis of these secondary metabolites might be important for the plant-associated lifestyle. Instead of the bacillomycin D synthesis genes, the gene cluster for synthesis of iturin A is present within the DSM7$^T$ genome. Notably, the genes involved in synthesis of fengycin are only fragmentary present in DSM7$^T$ (Table 8.1). It has been shown experimentally that DSM7$^T$ is unable to produce fengycin (Borriss et al. 2011).

Five out of a total of 13 gene clusters are located within variable regions of the FZB42 chromosome (Table 8.1), suggesting that they might be acquired via horizontal gene transfer. Except the fengycin gene cluster (see above), all others (bacillomycin D, macrolactin, difficidin, plantazolicin, and the orphan *nrsA-F* gene cluster) were without counterpart in DSM7$^T$ and *B. subtilis* 168$^T$.

### 8.3.2.1 Lipopeptides

The lipopeptides of *Bacillus* are small metabolites that contain a cyclic structure formed by 7–10 amino acids (including 2–4 D-amino acids) and a beta-hydroxy fatty acid with 13–19 C atoms (Zhao et al. 2017). They can be classified into four main families: the surfactins, the iturins, the fengycins or plipastatins, and the kurstakins (Jacques 2011). Lipopeptides could act by direct antibiosis against fungi and bacteria but were also found to stimulate ISR (Ongena et al. 2007). *B. velezensis* SQR9 mutants deficient in surfactin, bacillomycin, and fengycin synthesis were found impaired in triggering induced systemic resistance in *Arabidopsis* plantlets against plant pathogens *P. syringae* pv. tomato (Pst DC3000) and *Botrytis cinerea* (Wu et al. 2018).

#### Surfactin

Surfactin is a heptapeptide with an LLDLLDL chiral sequence linked by a ß-hydroxy fatty acid consisting of 13–15 carbon atoms to form a cyclic lactone ring structure. Surfactin is surface active (biotenside) and acts hemolytic, antiviral, and antibacterial by altering membrane integrity (Peypoux et al. 1999). The biological role of surfactin is thought as supporting colonization of surfaces and acquisition of nutrients through their surface-wetting and detergent properties. Similar to *B. subtilis* (Kovacs et al. 2017), FZB42 is capable of sliding on surfaces, dependent on the presence of surfactin. Mutants of *B. amyloliquefaciens*, blocked in surfactin biosynthesis, were shown to be impaired in biofilm formation (Chen et al. 2007).

Besides direct antagonism of phytopathogens, surfactin could also interact with plant cells as determinant for turning on an immune response through the stimulation of the induced systemic resistance pathway (Chowdhury et al. 2015a, b). Surfactins were detected in the root environment in much higher relative amounts, which are representing more than 90% of the whole LP production, and their synthesis is rapidly progressing during early biofilm formation. Syntheses of iturin and fengycin were also detected but found delayed until the end of the aggressive phase of colonization (Nihimborere et al. 2012; Debois et al. 2014). Earlier experiments performed with FZB42 colonizing duckweed (*Lemna minor*) plantlets corroborated that surfactin is the most prominent compound which could be detected by MALDI-TOF-MS in the plant-bacteria system (Idris et al. 2007). Mutant strains of FZB42, devoid in synthesis of surfactin (CH1, CH5), were found impaired in triggering of JA/ET-dependent ISR in lettuce plants, when challenged with plant pathogen *R. solani* (Chowdhury et al. 2015a). The lower expression of the JA/ET-inducible plant defensin factor (PDF1.2) in mutant strain CH5 (Δsfp) compared to CH1 (Δsrf) suggests that secondary metabolites other than surfactin might be involved in triggering plant response.

Gray leaf spot disease caused by *Magnaporthe oryzae* is a serious disease in perennial ryegrass (*Lolium perenne*). A mutant strain of FZB42 (AK3) only able to produce surfactin but no other lipopeptides (Bacillomycin D, fengycin) was shown to induce systemic resistance (ISR). A similar effect as in live cells was obtained in root-drench application of solid-phase extraction (SPE)-enriched surfactin.

Treatment led to reduced disease incidence and severity on perennial ryegrass. ISR defense response was characterized by enhanced hydrogen peroxide ($H_2O_2$), elevated cell wall/apoplastic peroxidase activity, and deposition of callose and phenolic/polyphenolic compounds underneath the fungal appressoria in naïve leaves. Moreover, a hypersensitive response (HR)-type reaction and enhanced expression of *LpPrx* (Prx, peroxidase), *LpOXO4* (OXO, oxalate oxidase), *LpPAL* (PAL, phenylalanine ammonia lyase), *LpLOXa* (LOX, lipoxygenase), *LpTHb* (putative defensin), and *LpDEFa* (DEFa, putative defensin) in perennial ryegrass were associated with SPE-enriched surfactin and live AK3 cell treatments, acting as a second layer of defense when preinvasive defense responses failed (Rahman et al. 2015). Surprisingly there are *B. velezensis* strains descibed which could positively affect plant growth and health although they were found impaired in synthesis of surfactin (He et al. 2012).

### Bacillomycin D

Members of the iturin family are iturins A, C, D, and E; bacillomycins D, F, and L; bacillopeptin; and mycosubtilin. They contain one ß-amino fatty acid and seven α-amino acids (Chen et al. 2009b). The peptide moiety of the iturin lipopeptides contains a tyrosine in the D-configuration at the second amino acid position and two additional D-amino acids at positions 3 and 6. While the majority of *B. velezensis* strains were found to contain a gene cluster encoding bacillomycin D, strain CAU B946 was found to synthesize iturin A which is reflected by its *ituA* operon located at the same site as the bmyD gene cluster in FZB42 (Blom et al. 2012). The same is true for the type strain of *B. amyloliquefaciens* DSM7$^T$ (Borriss et al. 2011). Transcription of the bacillomycin D gene cluster is directly controlled by global regulator DegU. A transmembrane protein of unknown function, YczE, is also necessary for synthesis of bacillomycin D (Koumoutsi et al. 2007).

The members of the iturin family exhibit strong fungicidal activity, and bacillomycin D has been identified as the main antifungal activity directed against fungal plant pathogens in *B. velezensis* strains FZB42 and C06. Mycelium growth and spore germination are suppressed in *Fusarium oxysporum, Rhizoctonia solani*, and *Monilinia fructicola* (Koumoutsi et al. 2004; Chowdhury et al. 2013). Purified iturin A suppressed the *Fusarium* yellows at tatsoi by soil amendment at relatively low concentration (0.47 mg/L soil) (Yokota and Hayakawa 2015). Recently, bacillomycin D was proven to show strong fungicidal activity against *Fusarium graminearum*. Bacillomycin D caused morphological changes in the plasma 60 membrane and cell wall of *F. graminearum*, induced accumulation of reactive oxygen species, and ultimately caused cell death in *F. graminearum*. Interestingly, when challenged by bacillomycin D, deoxynivalenol production, gene expression, mitogen-activated protein kinases phosphorylation, and pathogenicity of *F. graminearum* were significantly altered. Similar as in other cyclic lipopeptides, bacillomycin triggers ISR against plant pathogens (Wu et al. 2018).

**Fengycin**

Fengycin (synonymous to plipastatin) is a cyclic lipo-decapeptide containing a ß-hydroxy fatty acid with a side change of 16–19 carbon atoms. Four D-amino acids and one non-proteinogenic ornithine residue have been identified in the peptide portion of fengycin. Fengycin is active against filamentous fungi and is known for inhibiting phospholipase $A_2$. Similar to bacillomycin D, toxicity against pathogenic fungi relies mainly on their membrane permeabilization properties. Due to its high productivity in synthesizing fengycin, biocontrol exerted by strain C06 relies rather on fengycin than on bacillomycin D (Liu et al. 2011). Fengycin is known for triggering induced systemic resistance in *B. velezensis* (Wu et al. 2018).

## 8.3.3 Type I Polyketides

### 8.3.3.1 Bacillaene

The *pks* genes encode the enzymatic mega-complex that synthesizes bacillaene (Chen et al. 2006; Straight et al. 2007). The majority of *pks* genes appear to be organized as a giant operon (>74 kb from *pksC-pksR*). Bacillaene is, due to its molecular structure, a highly unstable inhibitor of prokaryotic protein synthesis and does have no effects on eukaryotic organisms (Patel et al. 1995). NMR studies of partially purified extracts from *B. subtilis* revealed bacillaene as an open-chain, unsaturated enamine acid with an extended polyene system (Butcher et al. 2007). Features of bacillaene synthesis, the archetype of trans-AT PKS, were uncovered, and bacillaene B bearing a glucosyl moiety was identified as the final product of the *bae* pathway (Moldenhauer et al. 2007, 2010).

Regulation of bacillaene synthesis has been extensively investigated in *B. subtilis*. A deletion of the *pks* operon in *B. subtilis* was found to induce prodiginine production by *Streptomyces coelicolor* (Straight et al. 2007). Expression of the *pks* genes in liquid culture requires the master regulator of development, Spo0A, through its repression of AbrB and the stationary phase regulator, CodY, which regulates metabolism in response to nutrient status and can bind to multiple sites in the bacillaene operon (Belitzky and Sonenshine 2013). Deletions of *degU*, *comA*, and *scoC* had moderate effects, disrupting the timing and level of *pks* gene expression (Vargas-Bautista et al. 2014). Interestingly, the polyketide bacillaene, produced in *B. subtilis* NCIB3610, functions as a significant defense protecting *Bacillus* cells from predation by *Myxococcus xanthus* (Müller et al. 2014).

### 8.3.3.2 Difficidin

Difficidin and oxydifficidin were identified as products of the *dfn* gene cluster in FZB42$^T$ (Chen et al. 2006). Difficidin has been shown to inhibit protein biosynthesis (Zweerink and Edison 1987), but the exact molecular target remains unknown. The polyketides are highly unsaturated 22-membered macrocyclic polyene lactone phosphate esters (Wilson et al. 1987) and are by far the most effective antibacterial compounds produced by FZB42$^T$. Difficidin is the most effective antibacterial compound produced by FZB42$^T$. Notably, difficidin is efficient in suppressing plant

pathogenic bacterium *Erwinia amylovora,* which causes fire blight disease at orchard trees (Chen et al. 2009a). In addition, difficidin produced by FZB42 was efficient in suppressing rice pathogens *Xanthomonas oryzae.* Together with bacilysin (see below), difficidin caused downregulated expression of genes involved in *Xanthomonas* virulence, cell division, and protein and cell wall synthesis (Wu et al. 2015). Analyses using fluorescence, scanning electron, and transmission electron microscopy revealed difficidin and bacilysin caused changes in the cell wall and structure of *Xanthomonas*. Biological control experiments on rice plants demonstrated the ability of difficidin and bacilysin to suppress economically damaging rice diseases such as bacterial blight and bacterial leaf streak.

#### 8.3.3.3 Macrolactin

Macrolactins are the biosynthesis product of the *mln* gene cluster in FZB42$^T$ and were characterized as an inhibitor of peptide deformylase (Yoo et al. 2006). Macrolactins, originally detected in an unclassified deep-sea bacterium, contain three separate diene structure elements in a 24-membered lactone ring (Gustafson et al. 1989). 7-O-malonyl macrolactin induces disruptions of cell division, thereby inhibiting the growth of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* (Romero-Tabarez et al. 2006). In the culture fluid of FZB42$^T$, four macrolactins were identified – macrolactins A and D as well as 7-O-malonyl and 7-O-succinyl macrolactin (Schneider et al. 2007). By contrast to other polyketides, macrolactin triggers ISR in *Arabidopsis* plantlets against *P. syringae* pv. tomato (Pst DC3000) and *Botrytis cinerea* (Wu et al. 2018).

### 8.3.4 Bacilysin

Like difficidin, the dipeptide bacilysin was found as also being involved in suppression of *Erwinia amylovora*. Bacilysin [L-alanyl-[2,3-epoxycyclohexanone-4]-L-alanine] contains L-alanine residue at the N-terminus and non-proteinogenic L-anticapsin, at the C-terminus. The peptide bond with L-alanine proceeds with a non-ribosomal mode catalyzed by amino acid ligase DhbE. Bacilysin is active in a wide range of bacteria and against the yeast, *Candida albicans,* due to the anticapsin moiety, which becomes released after uptake into susceptible cells and blocks glucosamine synthetase, an essential enzyme of cell wall biosynthesis. By contrast to the lipopeptides and polyketides mentioned above, bacilysin synthesis is not dependent on the Sfp PP-transferase. A mutant strain CH3, with a disruption of the *sfp* gene and unable to produce any polyketide or lipopeptide, was still able to synthesize bacilysin and to suppress *E. amylovora,* the causative agent of fire blight at orchard trees (Chen et al. 2009b). More recent experiments demonstrated that bacilysin is efficient in suppressing *Microcystis aeruginosa*, the main causative agent of cyanobacterial bloom in lakes (Wu et al. 2014a), and *Xanthomonas oryzae*, the causative agent of bacterial rice blight and bacterial leaf streak on rice (Wu et al. 2015).

The study of Wu et al. (2014a) is of special interest, since they described carefully the molecular effects exerted by FZB42 on cyanobacteria, especially on

*Microcystis aeruginosa*, the causative agent of harmful algal blooms in lakes and rivers. The authors could show that the suppressing effect was due to bacilysin. In a mutant strain disrupted in the *bacB* bacilysin synthesis gene, the suppressing effect on *Microcystis* growth was found abolished, but this was restored when bacilysin synthesis was complemented. Bacilysin caused apparent changes in the algal cell wall and cell organelle membranes, and this resulted in cell lysis. Bacilysin addition led to downregulating of genes involved in peptidoglycan synthesis, photosynthesis, microcystin synthesis, and cell division in *M. aeruginosa*.

In order to enhance bacilysin synthesis in FZB42, a genetic approach using the powerful Cre-Lox system was applied. Replacement of the native bacilysin promoter by constitutive promoters P*repB* and P*spac* was achieved. These strains contained two antibiotic resistance genes, and markerless strains were constructed by deleting the chloramphenicol resistance cassette and promoter region bordered by two *lox* sites (*lox*71 and *lox*66) using Cre recombinase expressed from the temperature-sensitive vector pLOSS-cre. The vector-encoded spectinomycin resistance gene was removed by high-temperature (50 °C) treatment. The engineered strains produced up to 173.4% and 320.1% more bacilysin than wild type, respectively. Bacilysin overproduction was accompanied by enhancement of the antagonistic activities against *Staphylococcus aureus* (an indicator of bacilysin) and C*lavibacter michiganense* subsp. *sepedonicum* (the causative agent of potato ring rot). Both the size and degree of ring rot-associated necrotic tubers were decreased compared with the wild-type strain, which confirmed the protective effects and biocontrol potential of these genetically engineered strains (Wu et al. 2014b).

### 8.3.5 Bacteriocins

Besides the secondary metabolites (lipopeptides and polyketides), which are synthesized independently from ribosomes, bacteriocins are ribosomally synthesized and present a class of posttranslationally modified peptide antibiotics (Schnell et al. 1988). Together with peptides without antibiotic activity, they are generally termed RiPPs (ribosomally synthesized and posttranslationally modified peptides). RiPP precursor peptides are usually bipartite, being composed of an *N*-terminal leader and *C*-terminal core regions. RiPP precursor peptides can undergo extensive enzymatic tailoring, yielding structurally and functionally diverse products, and their biosynthetic logic makes them attractive bioengineering targets (Burkhart et al. 2015). According to our current knowledge about their biosynthesis, more than 20 distinct compound classes can be distinguished (Arnison et al. 2013). In recent years, two RiPPs with antibacterial activity (bacteriocins) were identified in FZB42 (Scholz et al. 2011, 2014).

#### 8.3.5.1 Plantazolicin

Plantazolicin (PZN) was predicted by bioinformatics to be an excreted metabolite from FZB42 (Lee et al. 2008). An antibacterial substance still produced by FZB42 mutant, deficient in the Sfp-dependent synthesis of lipopeptides and polyketides

and in the Sfp-independent bacilysin synthesis, was identified as being the searched compound together with the gene cluster responsible for its biosynthesis. This cluster encodes a small precursor peptide that is posttranslationally modified to contain thiazole and oxazole heterocycles. These rings are derived from Cys and Ser/Thr residues through the action of a trimeric "BCD" synthetase complex, which consists of a cyclodehydratase (C), a dehydrogenase (B), and a docking Protein (D) (Scholz et al. 2011). Cyclodehydration was shown to precede dehydrogenation in vivo as hypothesized from earlier work on microcin B17 and azol(in)e-containing cyanobactins (Molohon et al. 2011). PZN A and B structures have been resolved unveiling a hitherto unusual number of thiazoles and oxazoles formed from a linear 14mer precursor peptide (Kalyon et al. 2011). PZN A has striking antimicrobial selectivity for *Bacillus anthracis (Sterne)*, the causative agent of anthrax (Molohon et al. 2011), and is efficient against plant pathogenic nematodes (Liu et al. 2013), while precursor molecule PZNB is inactive (Kalyon et al. 2011).

Biosynthetic *pzn* genes are located in a variable part of the genome within a genomic island, together with unique genes involved in the restriction and modification of DNA. They are transcribed into two polycistronic mRNAs (*pznFKGHI* and *pznJCDBEL*) and a monocistronic mRNA for *pznA* as revealed by reverse transcriptase PCR (RT-PCR) (Scholz et al. 2011).

Recently, PZN was described as a selective small molecule antibiotic toward *B. anthracis*. Its mode of action was first examined by gene expression profiling, which yielded an expression signature distinct from broader-spectrum antibiotics. It ruled out that the bacterial membrane is the most probable target of PZN. Remarkably, PZN localizes to the cell envelope in a species-selective manner and is associated with rapid and potent membrane depolarization. Thereby PZN interacts synergistically with the negatively charged phospholipid, cardiolipin (CL), suggesting that PZN causes transient weaknesses specifically in the *B. anthracis* cell membrane (Molohon et al. 2016).

#### 8.3.5.2 Amylocyclicin

The head-to-tail cyclized bacteriocin amylocyclicin was firstly described in *B. amyloliquefaciens* FZB42 (Scholz et al. 2014). Circular bacteriocins are non-lanthionine-containing bacteriocins with broad-spectrum antimicrobial activity, including against common food-borne pathogens, such as *Clostridium* and *Listeria* spp. The positively charged patches on the surface of the structures are thought to be the driving force behind the initial attraction to and subsequent insertion into the negatively charged phospholipid layer of the target cell membrane (van Belkum et al. 2011). Transposon mutagenesis and subsequent site-specific mutagenesis combined with matrix-assisted laser desorption time of flight mass spectroscopy revealed that a cluster of six genes covering 4490 bp was responsible for the production, posttranslational maturation including cleavage and cyclization, and export of the highly hydrophobic compound (Scholz et al. 2014). Amylocyclicin was highly efficient against Gram-positive bacteria, especially against a *sigW* mutant of *B. subtilis* (Y2) (Butcher and Helmann 2006). An orthologous gene cluster was also detected in *B. amyloliquefaciens* DSM7$^T$ (Table 8.1).

#### 8.3.5.3 Mersacidin

Mersacidin, a representative of globular type B lantipeptides, is not synthesized in FZB42, but parts of the mersacidin gene cluster are still remnant in the chromosome (Table 8.1) allowing immunity against this compound. MIC determinations of HIL Y-85 (25 mg/l) and FZB42$^T$ (25 mg/l) demonstrated that FZB42$^T$ was at least as resistant to mersacidin as the producer strain. Interestingly, mersacidin was first detected in *Bacillus* sp. HIL Y-85 (Chatterjee et al. 1992), a strain which was shown later as closely related to FZB42 (Herzner et al. 2011). Another plant-associated *Bacillus* strain, *B. velezensis* Y2, is also able to synthesize mersacidin (He et al. 2012). It was possible to reconstitute synthesis of heterologous mersacidin in FZB42$^T$ by introducing the respective biosynthetic genes cloned from HIL Y-85 (Herzner et al. 2011).

Another representative of the type B lantibiotics, *amylolysin* from *B. velezensis* GA1, was recently described. Similar as mersacidin, it is active on an array of Gram-positive bacteria, including *Listeria* spp. and methicillin-resistant *S. aureus* by interacting with the membrane lipid II (Arguelles Arias et al. 2013).

#### 8.3.5.4 Subtilin

By contrast to mersacidin, subtilin is a representative of the type A lantipeptides. Type A lantibiotics (21–38 amino acid residues) exhibit a more linear secondary structure and kill Gram-positive target cells by forming voltage-dependent pores into the cytoplasmic membrane but are inactive to Gram-negative bacteria. Their inactivity against Gram-negative bacteria results from their relatively large size (approximately 1800–4600 Da) which prevents them from penetrating the outer membrane of the Gram-negative cell wall (Stein 2005). Subtilin was the first lantibiotic isolated from *B. subtilis*. As in the case of mersacidin, only the immunity genes are present in FZB42, while biosynthesis and modification genes are missing. However, a corresponding gene cluster involved in synthesis of the lantibiotic-like peptide ericin was found in plant-associated *Bacillus* sp. A1/3 (Stein et al. 2002). We characterized strain A1/3 as a member of the *B. amyloliquefaciens plantarum* group (Borriss et al. 2011), nowadays *B. velezensis*, and therefore, ericin can be considered as an early example of a lantibiotic produced by plant-associated bacilli.

#### 8.3.5.5 Antimicrobial Peptide Lci

Lci was reported as an antimicrobial peptide synthesized by a *B. subtilis* strain with strong antimicrobial activity against plant pathogens, e.g., *Xanthomonas campestris* pv. *oryzae* and *Pseudomonas solanacearum* PE1. Its solution structure has a novel topology, containing a four-strand antiparallel β-sheet as the dominant secondary structure (Gong et al. 2011). The gene is not present in the *B. subtilis* 168 genome but was detected in FZB42 and *B. amyloliquefaciens* DSM7$^T$ (Table 8.1).

### 8.3.6 Volatiles

A blend of volatile organic compounds (VOCs) is released by several PGPR *Bacillus* strains, including FZB42$^T$ (Borriss 2011, Tahir et al. 2017a). These are low molecular weight, gaseous, metabolic compounds, which are emitted from bacterial cells having no physical contact to their target cells. The volatiles 3-hydroxy-2-butanone (acetoin) and 2,3 butandiol are triggering enhanced plant growth, control plant pathogens, and induce systemic resistance (Ryu et al. 2003). To synthesize 2,3-butanediol, pyruvate is firstly converted into acetolactate by acetolactate synthase (AlsS) under conditions of low pH and oxygen starvation. The next step of this alternative pathway of pyruvate catabolism, conversion of acetolactate to acetoin, is catalyzed by acetolactate decarboxylase (AlsD). The final step, from acetoin to 2,3-butandiol, is catalyzed by the *bdh*A gene product, acetoin reductase/2,3-butanediol dehydrogenase (Nicholson 2008). The FZB42$^T$ genome contains all the three genes encoding this pathway. FZB42$^T$ mutant strains, incapable of producing volatiles due to knockout mutations introduced into the *als*S and *als*D genes, are unable to support growth of *Arabidopsis* seedlings (Borriss 2011).

Besides plant growth promotion, volatiles act against plant pathogens by inducing systemic resistance in plants; in addition direct inhibitory effect of VoCs against plant pathogenic fungi was reported (Tahir et al. 2017b). Thirteen VOCs produced by FZB42 were identified using gas chromatography-mass spectrometry analysis (Table 8.2). Benzaldehyde, 1,2-benzisothiazol-3(2 H)-one, and 1,3-butadiene significantly inhibited the colony size, cell viability, and motility of *Ralstonia*

**Table 8.2** VOC profile of *Bacillus velezensis* FZB42

| Volatile compound (VOC) | Abbreviation | Inhibition[a] |
|---|---|---|
| Silanediol, dimethyl | SDD | – |
| 1,2-Benzisothiazol-3(2H)-one | 1,2-BIT | +++ |
| Benzeneacetamide | BAM | ++ |
| Oxime-, methoxy-phenyl | OMP | NT |
| (1R)-2,6,6 Trimetyhlbicyclo[3–1.1] hept-2-ene | TMB | + |
| Benzoic acid,- formyl - dimethoxy -,8,8 - dimethoxyoct - 2 - yl | BA | + |
| Benzaldehyde | BDH | +++ |
| Sulfurous acid, cyclohexyl-methyl isobutyl ester | SCE | – |
| 6-Tridecen, 2,2,4,10,12,12-hexamethyl-7-(3,5,5-trimrthylhexyl) | 6-THT | NT |
| 2-Undecanethiol, 2-methyl | 2-UT,2-M | – |
| Dodecane, 1-fluoro | DCF | ++ |
| Dodecane | DCN | ++ |
| Phenol, 2-(1,1-dimethylethyl)-6-methyl | PH | – |

According to Tahir et al. (2017a)

[a] Inhibition of *Ralstonia solanacearum*

*solanacearum*, the causative agent of bacterial wilt in a wide variety of potential host plants (Tahir et al. 2017a). Severe morphological and ultrastructural changes in cells of *R. solanacearum* were registered. Furthermore, VOCs downregulated transcription of type III (T3SS) and type IV secretion (T4SS) system, extracellular polysaccharides (*eps*), and chemotaxis-related genes (*motA, fliT*), which are major contributors to pathogenicity, resulting in decreased wilt disease. The VOCs significantly upregulated the expression of genes related to wilt resistance and pathogen defense. Transcription of tobacco resistance gene RRS1 was enhanced in the presence of VOCs. Overexpression of plant defense genes *EDS1* and *NPR1* suggests the involvement of salicylic acid (SA) pathway in induction of systemic resistance (Tahir et al. 2017a).

A recent analysis performed with FZB42 volatiles revealed that signal pathways involved in plant systemic resistance were positively affected. JA response (VSP1 and PDF1.2) and SA response genes (PR1 and FMO1) were triggered either in the leaves or roots of *Arabidopsis* plantlets after incubation with the volatiles. Noteworthy, defense against nematodes were elicited by volatiles in *Arabidopsis* roots (Hao et al. 2016).

Our present knowledge about the complex network of biocontrol actions exerted by FZB42 within a tripartite model system consisting of the plant (e.g., lettuce), the pathogen (*R. solani*), and the beneficial bacterium (FZB42) is tentatively summarized in Fig. 8.2.

## 8.4 Outlook

Most of the biocontrol agents currently in use are based on living microbes. Representatives of the *B. subtilis* species complex, including *B. amyloliquefaciens*, *B. subtilis*, and *B. pumilus*, are increasingly used for commercial production of biofungicides (Borriss 2016). Most of them are stabilized liquid suspensions or dried formulations prepared from durable endospores. They are developed for seed coating, soil, or leave application. Unfortunately, it is very unlikely that concentration of *Bacillus*-synthesized CLPs (iturins and fengycins) within the plant rhizosphere reaches levels sufficient for antibiosis (Debois et al. 2014). A possibility for circumventing this problem are bioformulations consisting of both *Bacillus* spores and concentrated culture supernatants with antimicrobial metabolites. However, only a few bioformulations currently on the market, such as SERENADE$^{(R)}$ prepared from *B. subtilis* QST713 and Double Nickel 55 prepared from *B. amyloliquefaciens* D747, contain together with living spores antimicrobial compounds, such as cyclic lipopeptides (iturins, fengycin). Unfortunately, also in these products only the number of spores is declared as active ingredient of the biofungicide. In contrast to chemical fungicides, there is no indicative about metabolites and their concentration, excluding an exact treatment of pathogen-infected plant parts. I recommend indicating a fixed concentration of the active principle for suppressing the target pathogen on the label of the biocontrol product. This would allow comparison of chemical and biological pesticides (Borriss 2015). To the best of my knowledge, no bioformulations containing exclusively antimicrobial metabolites are commercially

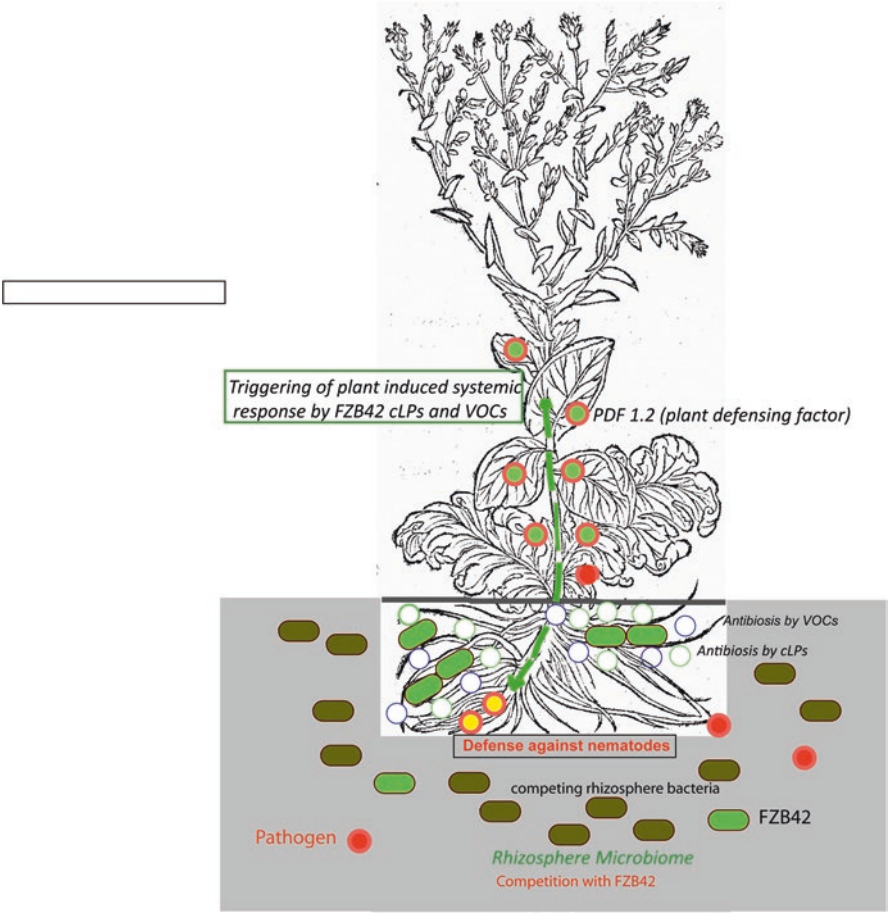


**Fig. 8.2** Biological control exerted by FZB42. The cartoon illustrates our present picture about the complex interactions between a beneficial Gram-positive bacterium (FZB42, light green), a plant pathogen (*R. solani,* symbolized by red-filled circles), and plant (lettuce, *Lactuca sativa*). FZB42 colonizes the root surface and is able to produce cyclic lipopeptides (green circles) and VOCs (blue circles). Direct antibiosis and competition for nutrients (e.g., iron) suppress growth of bacterial and fungal plant pathogens in the rhizosphere. However, these effects seem to be of minor importance, since the composition of the root microbiome is not markedly affected by inoculation with FZB42 (Erlacher et al. 2014). Due to production of *Bacillus*-signaling molecules (cLPs and VOCs) and in simultaneous presence of the pathogen, the plant defensing factor 1.2 (PDF1.2) as indicated by the green-filled red circles is dramatically enhanced and mediates defense response against plant pathogens (Chowdhury et al. 2015a). VOCs have shown to trigger defense against nematodes within plant root tissues (Hao et al. 2016). The picture of the *lettuce* plant ("*Lactuca crispa*") was taken from Bock 1552, p. 258. (Adapted after Fig. 5 in Chowdhury et al. 2015b)

available, although companies like ABiTEP performed extended large-scale trials with concentrated and stabilized *Bacillus* supernatants in order to suppress plant pathogens. Concerning biosafety issues, no representatives of the *B. subtilis* species complex and of the genus *Paenibacillus* spp. have been listed as risk group in "The

Approved List of biological agents" (2013). However, *B. cereus* and *B. anthracis* were listed in human pathogen hazard group 3, excluding their use as biocontrol agents in agriculture.

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# Pyrroloquinoline quinone (PQQ): Role in Plant-Microbe Interactions

9

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## 9.1 Introduction

Pyrroloquinoline quinone (PQQ) (Fig. 9.1) is synthesized by bacteria during the stationary phase of their growth. It is heat stable and soluble in water, and was first discovered in methylotrophic bacteria (Salisbury et al. 1979). PQQ, among its various functions, serves as a cofactor and belongs to the family of cofactors of the o-quinone type, which is comprised of other four cofactors well characterized: tryptophan tryptophyl quinone, lysine tyrosyl quinone, cysteine tryptophyl quinone, and topaquinone (Stites et al. 1999).

The enzymes involved in the above types of cofactors have been designated as quinoproteins (Anthony and Gosh 1998; Duine 1999; Matsushita et al. 2002; Miyasaki et al. 2006; Ikemoto et al. 2012). PQQ is the only cofactor in this family that is non-covalently bound to enzymes, such as glucose dehydrogenases, methanol dehydrogenase, sorbitol dehydrogenase, and glycerol dehydrogenase, which are involved in the oxidation of sugars, alcohols, and amines. PQQ is also bound to glutamate decarboxylase and lactate dehydrogenases (Knowles et al. 1987; Duine 1999; Anthony and Gosh 1998; De Biase et al. 1991; Akagawa et al. 2016). PQQ may also activate protein kinase, which is involved in signal transduction (Khairnar et al. 2007; Rajpurohit et al. 2013). Matsumura et al. (2014) reported that, in the basidiomycete *Coprinopsis cinerea*, PQQ may also activate a new type of quinoprotein with a signal peptide for extracellular secretion and a domain for adsorption on cellulose, besides the PQQ-dependent sugar dehydrogenase and cytochrome domains.

PQQ has been found in prokaryotes and eukaryotes, i.e., in both higher and lower organisms (Misra et al. 2012). In food, this molecule is found in quantities ranging

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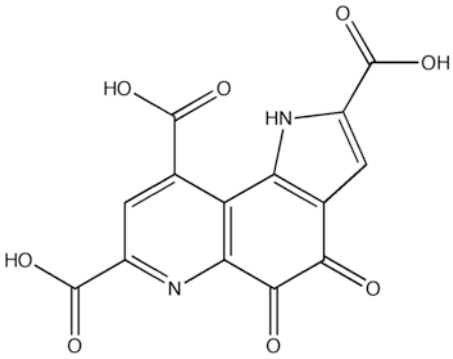


**Fig. 9.1** Pyrroloquinoline quinone (PQQ) structure

from 0.7 to 7.0 ng/g (or ml), depending on whether the food is solid or liquid (Noji et al. 2007). Although plants and animals do not produce PQQ themselves, PQQ is present in plant and animal tissues in nanogram-to-gram ranges (Kumazawa et al. 1992, 1995).

The presence of PQQ in food could be related to the production of the cofactor by bacteria present in the food (Stites et al. 1999; Rucker et al. 2009). The existence of PQQ in all these organisms, even if they do not produce it, is relevant, because it may be involved in health, fertility, neonatal development, energy metabolism, and probiotic properties in mammals (Bauerly et al. 2011), in addition to being able to act as a powerful antioxidant in animals and humans (He et al. 2003).

Although PQQ can be present in different organisms and function in them, only some bacteria synthesize it. It has been estimated, by the in silico analysis of approximately 126 bacterial species (mainly gram-negative), that these species contain the genes necessary for the synthesis of this cofactor (Shen et al. 2012; Klinman and Bonnot 2014).

Five genes are required in the synthesis of PQQ, although the number, and their position and nomenclature, vary among genuses and species, and even among strains (Schnider et al. 1995; Choi et al. 2008, Klinman and Bonnot 2014). Some bacterial species (mostly gram-negatives), contain the genes *pqqA*, *pqqB*, *pqqC*, *pqqD*, and *pqqE* organized in an operon; on the other hand, *pqq*F is usually separated from the rest of the genes (Shen et al. 2012; Klinman and Bonnot 2014).

The PQQ synthesis pathway has not been completely elucidated and is not yet fully understood. Bioinformatic studies have elucidated the characteristics of participating proteins, allowing a vision of how PQQ is synthesized. It is now known that the peptide precursor for the synthesis of PQQ (PqqA), is relatively conserved in size, although this may differ between genuses and species, varying between 23 and 39 amino acids (Puehringer et al. 2008).

PqqA contains a region conserving approximately half of its amino acid sequence, which corresponds to Glu-X-X-X-Tir (Stites et al. 1999; Choi et al. 2008; Puehringer et al. 2008). Through mutagenesis studies, it was found that this peptide is essential

for the synthesis of PQQ in most bacteria, although this is not the case for *Methylobacterium extorquens* AM1, because, in *pqq*A mutants, although the production of PQQ continues, the concentration is low in comparison with wild-type strain, and, there was no another copy of the pqqA gene (Toyama and Lidstrom 1998). This finding suggests that, in the mutant strain, there would be a peptide similar in length to that in the wild type, or at least a conserved region of PqqA, and thus there would be slight synthesis in the mutant strain. On the other hand, in *Methylokorus* sp. MP 688, the synthesis of PqqA is increased in the stationary phase under conditions of acidic pH and 50% dissolved oxygen (Ge et al. 2013). PqqB is a necessary protein for the synthesis of this cofactor, which consists of approximately 300 amino acids and is located within the family of metallo-beta-lactamases, as shown by bioinformatic analysis (Puehringer et al. 2008; Shen et al. 2012). In addition, PqqC may catalyze the terminal step in the biosynthesis of PQQ (Magnusson et al. 2004; Puehringer et al. 2008), facilitating the oxidation and cycling of PQQ, as well as accelerating its catalysis in the presence of molecular oxygen (Magnusson et al. 2004). Furthermore, this protein has been proposed as a phylogenetic marker, at least for the genus *Pseudomonas* (Meyer et al. 2011).

PqqD is a chaperone of the family of RiPP chaperone proteins, which consists of approximately 90 amino acid residues (Evans RL III et al. 2016; Puehringer et al. 2008), and can bind the precursor peptide PqqA (perhaps in a hydroxylated state) and provide it to the PqqE enzyme (Evans RL III et al. 2016, Tsai et al. 2009; Wecksler et al. 2010).

Barr et al. (2016) showed that, in the presence of the peptide chaperone PqqD, PqqE is a radical S-adenosylmethionine (SAM) protein that catalyzes the carbon-carbon bond formation between a glutamate and tyrosine side chain within the small peptide substrate PqqA. As a result of linkage of the Cγ of glutamate and Cϵ of tyrosine by PqqE, these two residues are hypothesized to be cleaved from PqqA by PqqF (Wei et al. 2016).

Various conditions can encourage or undermine the production of PQQ. One of these conditions is related to the carbon source available for the growth of bacteria such as *Acinetobacter calcoaceticus*, *Pseudomonas putida*, and *P. stutzeri,* in which the production of PQQ is favored in the presence of ethanol and methanol, but not in the presence of glucose, succinate, and quinate (Van Kleef and Duine 1989). In contrast, *P. aeruginosa pqq* operon was induced upon aerobic growth on ethanol, 1- propanol, 1,2-propanediol, and 1-butanol, however on glycerol, succinate and acetate, transcription was low (Gliese et al. 2010). In some methylotrophic bacteria, the presence of trace elements, such as calcium, zinc, manganese, and copper, can promote the production of PQQ, at low cell density. Otherwise, in the presence of iron, at high cell density, the output of PQQ is deficient (Urakami et al. 1992). Some ions are relevant in the binding of quinoproteins. For example, many studies confirm that $Ca^{2+}$ and $Mg^{2+}$ ions are involved in the binding of PQQ to dehydrogenases (Anthony and Gosh 1998; Asteriani and Duine 1998).

## 9.2 Functions, Mechanisms, and/or Effects of PQQ in Bacteria and Plants

Some of the mechanisms and attributes by which plant growth-promoting bacteria (PGPB) act, including those that allow them to compete with ? and maintain and promote the growth of plants, are influenced by PQQ or its synthesis genes, either directly or indirectly (Table 9.1).

### 9.2.1 PQQ as a Plant Growth Promoter

It is now clear that there are several mechanisms by which bacteria stimulate the growth of plants, with PQQ being a molecule that has a direct part in this process. A member of the Rhizobiaceae family, *Rhizobium tropici* CIAT 899, can establish nitrogen-fixing symbiosis with a wide range of legume hosts and synthesize an inactive apo-glucose dehydrogenase (GDH), which requires the presence of PQQ to be activated. Inoculation experiments in *Phaseolus vulgaris* L. beans, when PQQ was added at a concentration of 10 nM, significantly increased shoot and root weight, N and P contents, nodule weight, and acetylene reducing activities compared with plants where PQQ was not added. Further, the synthesis of gluconic acid and 2-keto-gluconic acids, and the solubilization of phosphates, were different in *Rhizobium tropici* CIAT 899 when exogenous PQQ was added, showing that PQQ produced an advantage in the promotion of plant growth (Cho et al. 2003).

Plant growth promotion has been associated with the production of PQQ, as evidenced by a significant increase in the fresh weight of cucumber (*Cucumis sativus*) seedlings when synthetic PQQ was added (5–1000 nM), thereby confirming that PQQ is a plant growth promotion factor. *Pseudomonas fluorescens* B16 is a bacterium that may promote plant growth in the tomato (*Solanum lycopersicum*), among others, and random mutations have identified the possible genes responsible for this phenotype. Phenotype generated by mutation of the *pqq H* gene resulted in a loss of ability to promote growth. In addition, it was demonstrated that pqq H gene acts as a transcriptional regulator that acts on pqq genes, which presented homology with TetR family of transcriptional repressors (Choi et al. 2008). An experimental study has suggested that PQQ acts as an antioxidant in plants, as shown by the treatment of cucumber leaf discs with PQQ and wild-type B16 resulting in the scavenging of reactive oxygen species (ROS) and hydrogen peroxide (Choi et al. 2008). Different species of *Pseudomonas,* isolated from the rhizosphere of peas, were confirmed as being phosphate-solubilizing bacteria, as shown by an increase in the total weight of the plant (Oteino et al. 2015).

*Rahnella aquatilis* HX2, which was isolated from soybean rhizosphere (Kim et al. 1997) can promote maize growth. The *pqqA* and *pqqB* mutants showed an adverse effect on its growth-promoting activities, such as a decrease in the length, as well as a decrease in the dry and fresh weight of maize plants (Li et al. 2014). In *Pseudomonas aeruginosa* CMG860, *pqq*A-d and *pqq*E mutants obtained with acridine orange, showed a change in their capacity to promote growth in bean plants (a

**Table 9.1** Functions, mechanisms or effects of pyrroloquinoline quinone PQQ

| Function | Mechanisms and effects | References |
|---|---|---|
| PQQ as a plant growth promoter | Higher plant weight, higher fresh weight and dry weight of seedlings, increased nitrogen fixation and phosphorus solubilization | Oteino et al. (2015), Naveed et al. (2015), Li et al. (2014), Ahmed and Shahab (2010), Choi et al. (2008), Cho et al. (2003), and Kim et al. (1997) |
| | Increased nodulation | |
| Phosphate solubilization | Phosphorus biofertilizers, secretion of gluconic acid as a phosphate-solubilizing agent | Rodríguez et al. (2000, 2006), Farhat et al. (2013), Patel et al. (2015), Wagh et al. (2014), Stella and Halimi (2015), and Anzuay et al. (2017) |
| PQQ and biocontrol | Bacterial and fungal biocontrol regulation | Han et al. (2008), Kim et al. (2003), Li et al. (2014), Guo et al. (2009), and Kremmydas et al. (2013) |
| PQQ and systemic resistance in plants | Induction of systemic resistance in plants | Han et al. (2008) |
| PQQ and bacterial mutualism | PQQ acts as an exogenous molecule that activates apo-quinoproteins in non-PQQ-synthesizing bacteria | Van Schie et al. (1984), Hommes et al. (1984), Groen et al. (1986), and Shimao et al. (1984) |
| PQQ and synthesis of antimicrobials | Regulation of antimicrobial synthesis | Schnider et al. (1995), Xu et al. (2014), and Arakawa et al. (2005) |
| PQQ and oxidative stress | Antioxidant in the cyclic redox system | Khairnar et al. (2003), Misra et al. (2004), Rucker et al. (2009), Paz et al. (1990), Ouchi et al. (2009), and Choi et al. (2008) |
| | Stimulates catalase and superoxide dismutase activity | |
| PQQ involved in swarming and chemotaxis | PQQ as a chemoattractant. Biosynthesis of pqq genes is modulated according to swarming motility | Tremblay and Déziel (2010), Van Schie et al. (1985), Matsushita et al. (1997), and De Jonge et al. (1996) |
| PQQ in signal transduction and UV and γ radiation stress resistance | PQQ as an activator of protein kinases and an antioxidant that prevents damage to proteins and DNA caused by UV and γ radiation | Khairnar et al. (2007) and Rajpurohit et al. (2013) |
| PQQ as a growth factor | PQQ as a cofactor in different quinoproteins with different metabolic activities | Ameyama et al. (1984), Shimao et al. (1984), and Trček et al. (2006, 2007) |

*PQQ* Pirrolequinolinequinone, *UV* Ultraviolet, *γ* gamma radiation

22–25% reduction), even though they still produced indole acetic acid, a known phytoregulator that promotes plant growth (Ahmed and Shahab 2010).

Regarding P. *fluorescens* QAU67 and P. *putida*, QAU90 have been demonstrated by using in vitro tests that they can synthesize GDH and PQQ when they are inoculated in the roots, and both had played a crucial role for their growth-promoting

effects in lettuce. On the other hand, in vivo test with crops such as rice, bean, and tomato showed a significant increase in the following parameters: plant height, fresh and dry weight (Naveed et al. 2015).

### 9.2.2 PQQ and Phosphate-Solubilizing Capacity

Phosphorus is the second most crucial nutrient after nitrogen in heterotrophic bacteria (Mills et al. 2008). It is also required by plants for carrying out processes such as photosynthesis, and for transduction and respiration signals, among others (Khan et al. 2010). Phosphorus is mostly present in insoluble complexes, or linked to organic compounds such as phytates, which cannot be assimilated by plants (Sharma et al. 2013). Among the mechanisms by which plants may have access to phosphorus in the soil are those where phosphate-solubilizing microorganisms are involved. These microorganisms, mainly bacteria, can produce organic acids and can synthesize phosphatases to solubilize phosphates (Rodríguez et al. 2006).

Numerous studies have been conducted seeking to implement the solubilization of phosphates in bacteria that are unable to do so for themselves; this has been achieved through the cloning of genes involved in the synthesis of PQQ. The PQQ synthase of *Erwinia herbicola*, which was cloned in *Burkholderia cepacia* S-16 and *Pseudomonas* sp., resulted in a solubilizing phosphate bacterium (Rodríguez et al. 2000). The PQQ biosynthesis genes (*pqqBCDE*) and the *gdh* gene belonging to *Serratia marcescens* were cloned in *Escherichia coli*, and it was observed that, regardless of whether these genes are together or separated, they provide the capacity to solubilize phosphates (Farhat et al. 2013). Of note, there are genetically manipulated growth-promoting bacteria, which, despite having enzymes such as glucose dehydrogenase, are unable to use the enzyme because they do not have the PQQ genes. However, in *Rhizobium leguminosarum,* in which the PQQ genes were cloned from *P. fluorescens* B16, the genes provided the capacity to solubilize phosphate for the bacteria (Patel et al. 2015).

In *Herbaspirillum seropedicae* Z67, the *pqq* genes belonging to *P. fluorescens* and *Acinetobacter calcoaceticus* were cloned, conferring on *H. seropedicae* the capacity to produce PQQ and to solubilize phosphate (Wagh et al. 2014). It has been reported that, owing to their production of PQQ and gluconic acid, various bacteria, such as *Klebsiella* sp., *Enterobacter* sp., and *Pseudomonas* sp., have the capacity to solubilize insoluble phosphates ($Ca_3(PO_4)_2$, $FePO_4$, or $AlPO_4$) (Stella and Halimi 2015). Therefore, these bacteria are considered as potential candidates for use as P-biofertilizers for peanut and maize (Anzuay et al. 2017).

It has been determined that the expression of PQQ synthesis genes is stimulated when bacteria (*P. putida* KT2440) are grown on glucose as the sole carbon source, and with low amounts of soluble phosphate; the levels of expression of the *pqqF* and *pqqB* genes reflect the levels of PQQ synthesized. Multiple studies suggest that one or both of these genes may serve to modulate PQQ levels according to growth conditions (An et al. 2016).

### 9.2.3 PQQ and Biocontrol

As a continuous and natural process, biocontrol is conceptualized as a balancing force that allows the maintaining of an ecosystem in good condition. Biocontrol is a characteristic of PGPB, and the mechanisms by which these types of bacteria exert biocontrol on pathogens are diverse. One of the indirect mechanisms to achieve this biocontrol seems to be the production of PQQ, and even the synthesis of glucose dehydrogenase is dependent on PQQ.

The *pqq*A mutants of *Enterobacter intermedium*, a biocontrol bacterium that acts on the pathogenic rice fungus, *Magnaporthe grisea*, lose the ability to biocontrol this fungus, in addition to losing the ability to produce gluconic acid, with the loss of capacity to solubilize insoluble phosphates. However, gluconic acid only cannot eliminate this phytopathogenic fungus. It is well known that *E. intermedium* 60-2G produces 3-methylpropanoic acid, an antibiotic with antifungal activity, perhaps PQQ is required for the synthesis of this antifungal agent. This suggests that PQQ is indirectly involved in biocontrol of pathogenic fungus *Magnaporthe grisea* (Han et al. 2008; Kim et al. 2003).

*Rahnella aquatilis* HX2, as a biocontrol agent of grapevine crown gall, also has the capacity to suppress the crown gall in sunflowers caused by *Agrobacterium viti*. Pqq and gdh mutants of *R. equatilis* caused the loss of biocontrol of *A. vitis*. This phenotype was fully restored when they were genetically complemented (Li et al. 2014; Guo et al. 2009).

In the case of *P. fluorescens,* isolated from the bean rhizosphere, when mutated at random, it lost the ability to exert biocontrol on the fungus *Pythium ultimum*, which causes root rot of beans. The genes involved in this phenotype were identified as *gdh* and there was an open reading frame that appeared to belong to the *pqq* genes (Kremmydas et al. 2013).

### 9.2.4 PQQ and Systemic Resistance in Plants

The induction of systemic resistance in plants has been demonstrated to be a very efficient mechanism that promotes plant growth by confronting many different pathogens and herbivores, allowing systemic resistance to be classified as an environmentally friendly method to combat these agents (Mhlongo et al. 2018).

It is well known that *Enterobacter intermedium* induces systemic resistance in tobacco plants, but when the *pqq* gene is mutated, *Enterobacter intermedium* is unable to induce this resistance; moreover, under this condition, it does not produce gluconic acid. Of note, gluconic acid itself did not show any induction of systemic resistance to soft-rot disease (Han et al. 2008).

### 9.2.5 PQQ and Bacterial Mutualism

In a mutualistic relationship, organisms of different species benefit each other; previous studies have shown that, with PQQ, bacteria and plants can have such a

relationship (Goldstein et al. 1999). Also, PQQ can be involved in mutualistic interactions among bacteria.

In this regard, some bacteria cannot synthesize PQQ; it also appears that they produce apo-quinoproteins, which are not functional until PQQ is added exogenously. For example, when PQQ is added to *Acinetobacter lwoffii*, it seems that aldose sugars can be used as an auxiliary energy source, owing to the presence of apoglucose dehydrogenase (Van Schie et al. 1984). Another example involves *E. coli*, which synthesizes a quinoprotein glucose dehydrogenase apoenzyme and supplies an additional route for sugar metabolism, but this is functional only when PQQ is added exogenously or when PQQ biosynthesis genes are introduced into the bacterium (Hommes et al. 1984). *Pseudomonas testosteroni* synthesizes alcohol dehydrogenase (ADH) in its apo-form and metabolizes alcohol only when PQQ is added to the culture medium (Groen et al. 1986); another study showed that *Pseudomonas* metabolized polyvinyl alcohol only when PQQ was added (Shimao et al. 1984).

The question arises of why does a bacterium synthesize an inactive enzyme and depend on exogenously provided PQQ for its activity? A possible reason is that the bacteria live in communities where the presence of PQQ triggers the survival of other bacteria that are deficient in the synthesis of the cofactor. Therefore, a kind of mutualistic bacterial relationship is maintained, causing species to be preserved and bacterial diversity and ecological balance to be maintained.

### 9.2.6 PQQ and the Synthesis of Antimicrobials

One mechanism by which microorganisms regulate and maintain their populations is by the use of antimicrobials. In many cases, some antimicrobials, contrary to what might be supposed, help to ensure the preservation of bacterial diversity and maintain populations and ecological balance (Kerr et al. 2002; Kirkup and Riley 2004).

In the case of *P. fluorescens* CHA0, it is known that this bacterium produces several secondary metabolites, such as pyoluteorin and 2,4-diacetylphloroglucinol, which are critical antibiotics to control root diseases caused by soil-borne fungal pathogens. It has been determined that a site-directed mutation in the pqqFAB genes in *P. fluorescens* CHA0 to lack glucose dehydrogenase activity. Besides, this bacterium could not utilize ethanol as a carbon source and showed strongly enhanced production of pyoluteorin. Also, a pqqF mutant can grow in ethanol and produce pyoluteorin at levels shown by wild strain when PQQ is added to a final concentration of 16 nM, which indicates that PQQ negatively regulates antibiotic production and their biocontrol activity (Schnider et al. 1995).

*Pseudomonas kilonensis* JX22 is a bacterium that produces a wide range of antimicrobials and it is used as a biological control for several phytopathogenic fungi, e.g., *Fusarium oxysporum* f. sp. *lycopersici*. A mutation in the *pqqC* gene caused the loss of antifungal activity, which was recovered by complementation with the wild-type *pqqC* gene (Xu et al. 2014).

*Streptomyces rochei* strain 7434AN4 produces a secondary metabolite of a polycystic nature, called lankacidin, which exhibits significant antibacterial activities

against a wide variety of bacteria, and may have applications in agriculture. In this bacterium, a mutation in the *pqq* genes causes the non-synthesis of lankacidin, but when 2 μg/ml of PQQ is added to the mutant, the synthesis of the antibiotic lankacidin is provoked. Arakawa et al. (2005) have suggested that PQQ plays a crucial role in an oxidation process during lankacidin synthesis.

### 9.2.7 PQQ against Oxidative Stress

It is known that, under certain circumstances, plants release ROS, which have harmful effects on both the plant itself and microorganisms that coexist with the plant. Beneficial microorganisms stimulate the production of ROS in the plant, and they also stimulate the production of antioxidant agents (Rahman et al. 2018). Besides, the microorganisms possess mechanisms to eliminate ROS (Alquéres et al. 2013), in such a way that both the plant and the microorganisms can coexist.

Various phenotypes are associated with the production of PQQ by PGPB bacteria. These phenotypes can promote the growth of certain plants in different ways, among which are higher activities of catalase and superoxide dismutase (Khairnar et al. 2003). As a result, these phenotypes can protect against the attack of ROS derived from γ-irradiation and can preserve the DNA and proteins (Misra et al. 2004). Redox cycling systems result in repeated chemical reactions in which molecules acting as catalysts are repeatedly oxidized and/or reduced. It has been hypothesized that the PQQ molecule potentially has one of the largest numbers of catalytic cycles (number of repeated reactions), with about 20,000, mainly due to its chemical stability, compared with ascorbic acid, which has only four repeated reactions (Rucker et al. 2009; Paz et al. 1990). It has been suggested that PQQ exists as a reduced form, $PQQH_{(2)}$, throughout the cell and plays a role as an antioxidant, with an antioxidant power greater than those of vitamin C, cysteine, uric acid, and glutathione (Ouchi et al. 2009).

Treatment of cucumber leaf discs with PQQ or *P. fluorescens* B16, a producer of PQQ, resulted in the scavenging of ROS and hydrogen peroxide, suggesting that PQQ acts as an antioxidant in plants (Choi et al. 2008).

### 9.2.8 PQQ Involved in Swarming and Chemotaxis

Among the first events that occur during the microorganism-plant interaction is that the bacteria respond and move toward the plant. Chemotaxis and motility in the bacteria give them a competitive advantage for roots and rhizoplane colonization (Scharf et al. 2016); the swarming movement has been reported as important for the extension of colonization in plants (Sánchez-Contreras et al. 2002).

In this respect, in *P. aeruginosa* it has been determined that the *pqq* genes are down-regulated in tendril tip cells, and Tremblay and Déziel (2010) propose a model in which tendril tip cells function as “scouts”, whose main purpose is to

spread on uncolonized surfaces while the center population is in a biofilm-like state that allows permanent settlement of the colonized area.

Although *E. coli* is not considered to promote plant growth, several studies have been carried out with this bacterium using it as a genetic background for the expression of *pqq* genes from other bacteria. By using an *E. coli* strain, it was observed that, despite being unable to synthesize PQQ, the *E. coli* strain could activate, in the presence of exogenous PQQ, an apoglucose dehydrogenase, which seems to indicate that *E. coli* can take up PQQ present in the medium (Van Schie et al. 1985; Matsushita et al. 1997). In addition, PQQ in this bacterium can play the role of a chemoattractant, since, when present in concentrations of 10, 50, and 100 μM and with carbon sources such as glucose, fructose, mannose, and gluconate, a "swarming" movement of *E. coli* is caused (De Jonge et al. 1996).

### 9.2.9 PQQ Involved in Signal Transduction and UV-γ Radiation Stress Resistance

The resistance to UV-γ radiation that microorganisms can have is very important for them to be able to grow and to survive. It has been determined how radiation has a decisive impact both on plants and on the microorganisms that are associated with them (Paul et al. 2012); accordingly, mechanisms that may be involved in such resistance are important.

A quinoprotein called YfgL in *E. coli*, with protein kinase activity, has been reported to be involved in transduction and DNA strand break repair, and to enhance the UV resistance of *E. coli* (Khairnar et al. 2007). Likewise, PQQ activates a Ser/Thr protein kinase in *Deinococcus radiodurans* that improves the organism's resistance to γ radiation, possibly by regulating the differential expression of important genes for bacterial response to oxidative stress and DNA damage (Rajpurohit et al. 2013). PQQ has even been used to increase γ radiation resistance in animals (Xiong et al. 2011).

### 9.2.10 PQQ as a Growth Factor

Some bacteria, with their versatile metabolisms, can colonize different habitats, adapting their metabolism to replicate in specific host microenvironments. These adaptations are a consequence of the composition of their host niches, and this will cause that allows bacteria remain active and, in some cases, even modify the bacterial soil community structure (Kang et al. 2013).

PQQ has been shown to be an essential factor in stimulating the onset of bacterial growth (Ameyama et al. 1984), by decreasing the adaptive growth lag phase. In *Pseudomonas* sp. VMI5C, it has been shown that PQQ is essential for polyvinyl alcohol degradation (Shimao et al. 1984). The organism *Gluconacetobacter europaeus* can grow at a high concentration of acetic acid, owing to the stability of the PQQ-dependent ADH (Trček et al. 2006, 2007).

## 9.3 Discussion and Conclusion

PQQ promises to be a key molecule in many aspects of bacterial physiology and microorganism-plant interaction. As has been observed, PQQ affects the growth of several plants, which is sometimes associated with the solubilization of phosphates by the production of gluconic acid through glucose dehydrogenase that is dependent on PQQ as an antioxidant agent, but on other occasions the mechanism by which PQQ affects plant growth is unknown. Phosphate solubilization by bacteria requires PQQ; in other cases *pqq* genes and glucose dehydrogenase are required, enabling these bacteria to act as potential P-biofertilizers in plants. Of interest, it will be of value to investigate how the synthesis of PQQ and the expression of its genes regulate or influence swarming-like motility in bacteria.

PQQ has been shown to be crucial for its biocontrol activity in bacteria and fungi that has an impact on plants of agronomic interest, such as rice, grapes, sunflowers, and beans, but the mechanisms of this biocontrol activity are still to be clarified. Further, there is a report that PQQ induces a systemic response in tobacco, but its mechanism is unknown and needs to be explored in future research (Song et al. 2008).

Several antimicrobials have been reported to be influenced, either negatively or positively, by the presence of PQQ. These antimicrobials have effects on fungi and pathogenic bacteria, and the regulation of these antimicrobial mechanisms needs to be investigated and will be critical to driving the more rational use of these biocontrol agents in agriculture. Some studies have reported the synthesis of apo-quinoproteins in bacteria that cannot synthesize PQQ, and that depends on exogenous PQQ or even, the introduction of PQQ synthesis genes in order to enable it to effectively carry out the metabolism through these enzymatic quinoproteins, different substrates. Perhaps, in natural microenvironments of these bacteria, there are other PQQ-synthesizing bacteria that mitigate the deficiency of this cofactor, in a way that there is a type of bacterial mutualism that allows the non-PQQ synthesizing bacteria to maintain and preserve microbial diversity in these ecosystems in the presence of PQQ. On the other hand, regarding PQQ as a growth factor, it will undoubtedly be relevant to investigate, in different biological systems, the presence of PQQ-synthesizing bacteria and other organisms that cannot synthesize PQQ, but that can elaborate apo-quinoproteins; it will also be necessary to evaluate the ecological impact when PQQ-synthesizing bacteria change their populations.

PQQ has been shown to be a positive regulator that increases the activities of enzymes that combat ROS, such as catalase and superoxide dismutase. PQQ, compared with other antioxidant agents, tends to have the highest number of repeated redox reactions. Therefore, PQQ, by acting as a redox cyclic system, has an impact on enhancing plant growth and conferring protection to bacteria against ROS attack resulting from UV and $\gamma$ radiation of proteins and DNA. Also, it will be important to determine the signal transduction cascade and gene activation where PQQ acts to combat the effects of this type of radiation.

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# Bacterial Mechanisms Promoting the Tolerance to Drought Stress in Plants

10

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## 10.1 Introduction

All animals including human depend on plants as they produce oxygen and form the principal food for them. According to an estimation, 98% of the global food requirements are provided by 12 plant species and 14 animal species. Moreover, none of these 14 animals can supply the required substrates without the incorporation of the plants. By another estimation, more than 50% of the world energy intake is affiliated by crops consisting of wheat, rice, and maize. Therefore, reduction in plant productivity immediately affects the growth of a number of species that rely on plants as the nutrition basis (Orhan 2016).

The world food supply must increase considerably to certify food security for the growing population (Hanin et al. 2016). In fact, plant production is substantially affected by multiple environmental factors. Water is one of the most limiting factors for plant development, as well as for all life forms (Kavamura et al. 2013). Drought is a natural phenomenon that affects several parts of the world, causing social, economic, and environmental negative impacts (Kavamura et al. 2013). Water scarcity is among the main constraints on plant productivity worldwide (Delshadi et al. 2017) and is expected to expand with climatic changes (Rapparini and Penuelas 2014). Because drought is a multidimensional stress, plants respond to it at morphological, physiological, biochemical, and molecular levels (Kaur and Asthir 2017; Shrivastava and Kumar 2015; Wang et al. 2016). Thus worldwide, extensive efforts are on the development of the strategies to cope with abiotic stresses such as drought (Grover et al. 2011).

Plants undergo a variety of metabolic and physiological alterations in response to drought (water deficiency) (Kang et al. 2014a, b). Plant growth-promoting bacteria

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(PGPB) have been recognized to have an essential function in the growth and metabolism of plants to rescue plant growth in stressful conditions (Kang et al. 2014a, b; Bisen et al. 2015; Singh et al. 2016, 2017). Several strategies have been suggested for governing the detrimental effects of drought stress on plants. Among that the selection for tolerant varieties and genetic engineering are the most investigated approaches. Nevertheless, the development of new tolerant varieties is challenging due to the complexity of abiotic stress tolerance mechanisms and genetically modified plants cannot easily be approved based on the most national regulations (Kasim et al. 2012; Timmusk et al. 2014). The priming treatment can be considered as an alternative strategy to induce stress tolerance in the plant by using various chemical and biological agents as the stimulants (Kasim et al. 2012).

Consequently, the importance of exploitation of beneficial bacteria is emerging with the focus on issues such as sustainable agriculture, environmental preservation, and food security (Ilangumaran and Smith 2017). Selection, screening, and use of drought stress-tolerant PGPB to plants can help to overcome productivity restrictions in dry lands (Kaushal and Wani 2015).

The aim of this chapter is to explore the potential of plant-associated bacteria in drought stress protection and meanwhile to overview the possible mechanisms by which PGPB can improve the tolerance to drought stress in plants.

## 10.2 Worldwide Water Resource Limitation

By 2050, the world population is expected to reach to 9.2 billion (Rosegrant et al. 2009). As a result of population growth and enhanced demanded protein and energy per capita, global stress on water and land sources is manifold. Worldwide water consumption from irrigation, domestic, industrial, and livestock usages is expected to grow by 21% by 2050. The developing countries are expected to have a more dramatic increase in consumption (up to 25%), compared to the developed regions with an estimated 11% increase (Rosegrant et al. 2009).

Currently, agricultural production is accountable for the majority of global consumptive freshwater use (up to 85%) (Johnson et al. 2010). Although it creates a vast technological need to offer solutions for the effective use of the available water (Timmusk et al. 2013), any efforts to increase the adaptability to the low water activity in plants are a crucial parallel approach. Water scarcity affects all continent and around 2.8 billion people around the world at least 1 month annually (Yu 2016). In other words, almost 40% of the world population and huge area of ecosystems are travailing from water scarcity (Johnson et al. 2010), and more than 1.2 billion population even lack access to clean drinking water (Yu 2016). In a prediction by UN, one in four of the world's children will be in regions with extremely restricted water resources by 2040 as a result of climate change (Guardian 2017).

Water scarcity decreases crop yields and eventually may cause malnourishment even in the developing world (Johnson et al. 2010). Moreover, worldwide production of biologically derived energy and material sources (e.g., biofuels and biological textiles) is developing and can result in the expansion of the agricultural industry in the future. As a consequence of these pressures, water scarcity and land

degradation compete climate change as a main environmental concern in many areas of the world. Hence, there is a strong requirement for precise estimates of available water for future use and linked environmental impacts and for relating these to agricultural tools (Johnson et al. 2010).

Water resources inadequacy is a critical constraint to agriculture in many parts of the world. It often harms the soil through oversaturation and salt accumulation (Rosegrant et al. 2009; Fraiture et al. 2010). It is estimated that there are about 20–30 million hectares of irrigated lands severely affected by salinity on a global scale. An additional 60–80 million hectares are affected to some extent by water logging and salinity (Rosegrant et al. 2009). Hence, saline soils are estimated to extend at a rate of 7% in the world (Orhan 2016).

Despite the fact that drought is more prevalent and devastating than the salinity stress, plants' adaptation to both is substantially related (Kang et al. 2014b). The water scarcity presents the major challenge in securing enough water to meet human, environmental, social, and economic needs to support sustainable development. This is menacing human health and ecosystems' integrity; they represent a major concern for the water resource sustainability (International Hydrological Programme). Therefore, in an era of changing climates, there is a critical need for evolving tolerant plants to abiotic stresses specifically drought and salinity (Farrar et al. 2014).

## 10.3 Drought Stress in Plants

### 10.3.1 Effect of the Drought Stress on Plants

Drought is a multidimensional stress which triggers various plant reactions including morphological, physiological, biochemical to molecular levels (Kaur and Asthir 2017; Shrivastava and Kumar 2015; Wang et al. 2016). Drought stress harbors a decrease in water content, leaf pressure potential, closure of stomata, and a reduction in cell mitosis and in consequence cell elongation and growth. Plant growth is diminished because of the effect of the drought on numerous physiological and biochemical processes mainly photosynthesis, respiration, translocation, phytohormones production, adsorption of ions, sugar and nutrient metabolism, etc. (Farooq et al. 2009; Kaur and Asthir 2017; Reis et al. 2016).

Drought can lead to disturbed flowering process and grain filling that results in smaller and fewer grain production (Kaur and Asthir 2017). In the majority of the plant species, drought is associated with alterations in leaf anatomy and ultrastructure. However, harsh drought condition may cause the obstruction of photosynthesis and disruption of metabolism resulting in the death of plant (Kaur and Asthir 2017). The reactive oxygen species (ROS) such as superoxide radicals and $H_2O_2$ production (Kohler et al. 2008) is an initial step of plant defense flow to water stress and acts as a secondary messenger to prompt following defense reaction in plants (Kaur and Asthir 2017). The increased amounts of the ROS can cause extended damage by initiating lipid peroxidation, membrane deterioration, and degrading proteins, lipids, and nucleic acids in plants (Vurukonda et al. 2016). Drought stress can likewise

result in misfolding or unfolding of structural and functional proteins leading to denaturation and dysfunction (Kasim et al. 2012).

### 10.3.2 Drought Resistance Mechanisms in Plants

The sensitivity of plants to drought is determined by level and duration of stress, plant species, and their growing stages (Kaur and Asthir 2017; Cura et al. 2017). In theory, there are two types of drought avoider plants: (a) water savers which preserve water and (b) water spenders which compensate the transpirational losses with excess absorption. The plant anatomic and morphologic characteristics aid in increased water uptake and reduce water outgoings. Water uptake could be accelerated by a widespread root system with an extensive active surface area and optimum shoot/root ratio. However, water loss through transpiration can be much subjected to adjustment (Timmusk et al. 2013). Drought tolerance capacity of plants can be predicted by applying several drought-related characteristics, including root and leaf traits, osmotic balance capabilities, potential of water content, abscisic acid (ABA) content, and stability of the cell membranes as conventional indicators (Kaur and Asthir 2017).

The reaction of a plant to abiotic stress initiates by a sensation of the extracellular stress signal on receptors of the cell, consequenced by the regulatory networks, comprising signal transduction and expression regulation of stress-responsive genes that cause physiological response of tolerance of the plant to stress (Reis et al. 2016).

The secondary messengers including $Ca^{2+}$, ROS, ABA, phosphoglycerol, diacylglycerol, and transcriptional regulators are associated with signal-transmitting pathways to react to drought stress (Kaur and Asthir 2017). Furthermore, the plant hormonal apparatus is activated to transduce stress signals during altered osmotic potential (Khan et al. 2013).

At the mophological level, plants may adapt to drought stress by reducing the growth duration and elude the stress with the conservation of high tissue water content either by hindering water deprivation from plants or enhanced water absorption or both mechanisms. Some plants may lessen their surface area by shedding the leaf or generation of smaller leaves (Farooq et al. 2009).

At the molecular levels, numerous genes and transcription factors have been recognized that are involved in drought response, for instance, the dehydration-responsive element-binding gene, dehydrin's late embryogenesis abundant proteins, aquaporin, and heat shock proteins (Reis et al. 2016; Farooq et al. 2009). To ameliorate protein functionality, a widespread plant protective reaction is to express several heat shock proteins (HSPs) to restore the favorable folding of proteins required for proper structural and functional activity of proteins even during severe stress (Kasim et al. 2012). By moderation of the tissue metabolic activity, osmotic adjustment can act as one of the pivotal mechanisms in plant adaptation to drought as well. The osmotic compounds are also produced under drought condition which include compatible solutes such as glycine betaine, sugars (fructans and sucrose), amino acids (proline, aspartic acid, and glutamic acid), and cyclitols (mannitol and pinitol) (Kaur and Asthir 2017).

In principle, the antioxidant defense system of the plant cell comprises enzymatic and nonenzymatic mechanisms (Farooq et al. 2009). Enzymatic constituents consist of superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, and glutathione reductase (Farooq et al. 2009; Sandhya et al. 2010; Khan et al. 2013). The nonenzymatic components of antioxidant system include cysteine, reduced glutathione, and ascorbic acid (Farooq et al. 2009). The attenuation of ROS production during the drought state can provide plants to encounter water deficiency without extensive injury. The reduction of ROS synthesis highly depends on the effective energy dissipation mechanisms in the mitochondria (Kaur and Asthir 2017).

The plant growth-promoting bacteria (PGPB) or stress homeostasis-regulating bacteria (PSHB) (Sgroy et al. 2009) have the potential to produce the tolerance to drought in plants (Sandhya et al. 2010; Zelicourta et al. 2013). The mechanisms that PGPB provide to act in the mitigation of drought stress in plants are by production of polysaccharides, 1-aminocyclopropane-1-carboxylate deaminase, and phytohormones, inducing accumulation of osmolytes, volatile compounds, and antioxidants, upregulation or downregulation of stress-responsive genes, and modification in morphology of the root (Kaur and Asthir 2017). The early report on the enhancement of plant drought stress resistance by rhizosphere bacteria has been published in 1999, and some Gram-positive bacterial isolates including *Paenibacillus* sp. and *Bacillus* sp. were revealed to be effective in enhancing the plant tolerance to drought stress (Timmusk et al. 2013, 2014).

Despite the extensive studies on the plant drought response, there are still no economic practice or technologic tool to boost the crop production under drought (Wang et al. 2016). Therefore, finding efficient low-cost technologies to reduce effects of drought over crops is necessary to the maintenance of crop yields under water deficits, which is the major challenge, faced by agriculture (Furlan et al. 2017). Several strategies have been used in order to decrease the drought stress effects on plant growth, including traditional selection methods, plant genetic engineering, and recently application of plant growth-promoting bacteria (Tapias et al. 2012; Timmusk et al. 2014).

Drought stress tolerance in plant is a complex phenomenon containing by clusters of gene networks involved in drought stress responses which partially has been characterized (Saikia et al. 2018; Timmusk et al. 2014). The suitable phenotypes are also further challenging to be recognized due to plants are exposed to multiple environmental stressors in the field either simultaneously or sequentially (Timmusk et al. 2014).

Furthermore, it is currently not much promising that the gene engineering technology will progress fast enough to fulfill with multiplied food demands in the near future (Timmusk et al. 2013, 2014). Utilization of PGPB has become a promising alternative to withstand abiotic stresses (Tapias et al. 2012; Furlan et al. 2017; Egamberdieva et al. 2017a, b). An existing pattern in the nature, selection, screening, and development of stress-tolerant bacteria, thus, could be a worthwhile approach to neutralize the productivity restrictions of crop plants in stress-prone regions (Meena et al. 2017).

## 10.4 Plant Growth-Promoting Bacteria (PGPB)

### 10.4.1 Definition and Categorization

Plant growth-promoting bacteria (PGPB) are considered as free-living soil, rhizosphere (soil near the roots), rhizoplane (root surface), endophyte (reside inside the plant) (Bashan and Bashan 2005; Gopalakrishnan et al. 2015), and phyllosphere (the habitat provided by the aboveground parts of plants) (Whipps et al. 2008; Penuelas et al. 2011) bacteria which are beneficial to plants under some conditions (Bashan and Bashan 2005; Gopalakrishnan et al. 2015). The majority of PGPB activities have been investigated in the rhizosphere and to less extent on endophytic which reside in the leaf surface (Bashan and Bashan 2005).

PGPB can promote the plant growth in a direct or an indirect way (Saravanakumar et al. 2011; Sadeghi et al. 2011; Ahemad and Kibret 2014). They directly affect the metabolism of the plants or can be indirectly affected by PGPB by the production of components that are in deficit supply. These bacteria are capable of solubilizing phosphorus and iron, fixing atmospheric nitrogen, and producing plant hormones, such as auxins, gibberellins, cytokinins, ethylene, etc. Exceedingly, such supplementation can improve the plant's tolerance to other stresses than drought, including salinity, metal, and pesticides. The molecular mechanism that contributes to the plant growth can be a sole combination of mechanisms. The second group of PGPB, known as biocontrol PGPB, promotes indirectly the plant growth by inhibiting the damaging impact of the phytopathogenic microorganisms including the bacteria, fungi, and viruses (Bashan and Bashan 2005; Orhan 2016; Kang et al. 2014b; Ahemad and Kibret 2014). Indirect mechanisms consist of ACC deaminase, cell wall-degrading enzymes, antibiotic production, substrate competition, hydrogen cyanide, induced systemic resistance, siderophore production, and quorum quenching (Olanrewaju et al. 2017; Kang et al. 2014a, b).

The PGPB has been also categorized as extracellular PGPB (ePGPB) and intracellular PGPB (iPGPB). The known ePGPB belong to the genera *Bacillus*, *Pseudomonas*, *Arthrobacter*, *Erwinia*, *Caulobacter*, *Chromobacterium*, *Serratia*, *Micrococcus*, *Flavobacterium*, *Agrobacterium*, and *Hyphomicrobium*, and iPGPB consist of the genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium*, and *Allorhizobium* (Gopalakrishnan et al. 2015; Vandenberghe et al. 2017).

### 10.4.2 Site of Colonization in Plants

The plant-associated bacteria comprise endophytic, phyllospheric, and rhizospheric bacteria (Weyens et al. 2009; Glick 2014).

#### 10.4.2.1 Endophytic Bacteria

In addition to the rhizosphere populations, diverse communities of microorganisms live in plants with neutralism or commensalism interaction that are broadly referred to as endophytes. Bacterial endophytes have been isolated rather from all tissue

types of plant, and they can colonize in specific plant tissues, either inside the cells or in the intracellular fluids. These ancient interactions are not only evolutionary valuable evolved relations while are potential of precious value for sustainable plant production if these interrelationships subject to investigation.

The majority of endophytes exist in both states of free-living and endophytic. These endophytes are considered to represent a group of soil bacteria which colonize the plant without stimulating the host defense reaction. In order to transfer from the soil to the plant, the bacteria must in essence harbor competence in the rhizosphere area, ability to adhere to the root, followed by the establishment in the host plant. Following entering the plant, endophytes may be surrounded by a cell membrane and become either intracellular or remain extracellular. The motility and secretion of various extracellular enzymes mainly cellulases and pectinases are required attributes of bacteria which transform from free-living to endophytic lifestyles. However, endophytic bacteria do not induce detrimental reactions or cellular injury to the plant. Endophytic bacteria compared with pathogens usually have lower population size in the host plant tissues, and this may be a manner by which they skip the plant defenses. In fact, there are types of endophytic bacteria colonizing the host tissue internally, sometimes in high density which leads to the eliciting symptoms of plant damage. In addition to the scape from an immune reaction, useful endophytes partially act by activating the plant-induced systemic resistance (ISR) toward pathogenic bacteria in the site (Farrar et al. 2014).

The cultivation-independent analysis has revealed that a high number of unculturable species colonize plants endophytically and a variety of bacterial species has been isolated from plant tissues, such as seeds, roots, stems, and leaves so far (Sziderics et al. 2007). Major fraction of endophytic bacteria have been shown to have several beneficial effects on their host plant, and the mechanisms involved are probably similar to those have been described for rhizospheric bacteria (Sziderics et al. 2007). It is assumed that the endophyte infection can protect the host from abiotic stresses by improving tolerance to drought, the rate of photosynthesis, and growth (Collemare and Lebrun 2012). Interestingly, microbial functionality seems dependent on the plant colonization compartment (rhizosphere or endosphere), as the endosphere microbiome might harbor significantly more metabolic pathways and PGP phenotypes than those colonizing the rhizosphere (Wang et al. 2017).

In fact, bacterial endophytes have been isolated from virtually all studied plants. Endophytic *Bacillus subtilis* EPB5, EPB22, EPB 31 have been evaluated for their capacity to induce water stress-related proteins and enzymes in green gram (*Vigna radiata*) plants (Saravanakumar et al. 2011). However, a far deeper understanding of both the individual components and their interactions is required in order to exploit beneficial bacteria to optimize biomass production (Farrar et al. 2014).

#### 10.4.2.2 Phyllospheric Bacteria

The phyllosphere is the external parts of the plant that are above the ground, including leaves, stems, blossoms, and fruits (Weyens et al. 2009). The phyllosphere forms the largest biological interface on earth (Penuelas et al. 2011). Considering that the majority of the surface area available for colonization is located on the

leaves, this is the dominant tissue of the phyllosphere. The exposure to an extent and rapid fluctuations in temperature, irradiation, and water availability must be tolerated by the symbiont bacteria that reside the phyllosphere (Weyens et al. 2009). Bacteria and fungi in the foliar phyllosphere of *Quercus ilex* in Mediterranean forest in summer seasons and long-term drought were investigated (Penuelas et al. 2011).

#### 10.4.2.3 Rhizospheric Bacteria

The area of the contact between root and soil where soil is affected by roots was designated as "rhizosphere" (Tarkka et al. 2008; Kang et al. 2010). The name comes from the Greek *rhiza*, meaning root (Pujar et al. 2017). The rhizosphere concept was originally described as the narrow zone of soil surrounding the roots where bacterial populations are stimulated by root activities. The original concept has now been amended to include the soil surrounding a root in which physical, chemical, and biological properties have been changed by root growth and activity (Saharan and Nehra 2011). It was proved that the rhizosphere is much richer in bacteria than the surrounding bulk soil. This rhizosphere is supported by a substantial amount of the carbon fixed secreted by the plant, mainly as root exudates (Lugtenberg and Kamilova 2009; Kang et al. 2010).

## 10.5 Colonization of PGPB Under Drought Stress

The diversity and population size of the soil bacteria are influenced by the physicochemical conditions including temperature, water activity, and the existence and amount of salt and other chemicals along with the number and types of plants thriving in that soil (Glick 2012). Plant traits determine the conditions for microbial colonization mainly by the organic and inorganic compounds secreted from the roots. A precisely coordinated interaction between the variety of exudates excreted by the plant and individual characteristics of distinct microbial populations is a crucial aspect of driving selection (Moreira et al. 2016; Wang et al. 2017).

The small molecules such as sugars, amino acids, and organic acids that are exuded in large amounts from plant roots (i.e., 5–30% of all fixed carbon during photosynthesis) are usually consumed by bacteria (Olanrewaju et al. 2017).

The "initiation inoculum" of the soil microbiome will be influenced under drought stress by selective choice of desiccation-tolerant taxa, along with indirect transformed soil chemistry and diffusion rates. Like soil bacteria, plants also endure a set of physiological reactions to survive under the drought-induced damages. These responses consist of alterations in root morphology and root exudate profile in principle means by which plants attract bacteria. Therefore, the root microbiome diversity under drought is characterized by how drought shapes both the host plant and neighboring soils. These factors can affect reciprocally. The transformed soil nutrient cycles and subsequent modifications in the type of microbiome under drought can convey an indication for plant health, as plants rely on bacterial activity to make soil nutrients bioavailable. Correspondingly, drought-induced changes in plant exudate can influence the surrounding soil microbiome, by accelerating more

alterations to soil geochemistry that sequentially modify magnitude and directionality of soil community levels. As a result of this complication, a comprehensively integrated realization of the influence of drought on the root microbiome is yet not fully revealed (Naylor and Coleman-Derr 2017).

## 10.6 Mechanisms of Alleviation of Drought Stress by PGPB or Plant Stress Homeostasis-Regulating Bacteria (PSHB)

Plant growth-promoting bacteria (PGPB) could play a noteworthy role in the mitigation of drought stress in plants. The functionality of PGPB-mediated drought resistance may be related to the interaction between the used PGPB strain and soil type as well as the capability of the plants to accommodate the association of the PGPB populations naturally occurring in the soil. Coarse sandy or gravelly soils can allow the finer roots to grow, which increase soil penetration, and may finally confer the drought tolerance. In addition, the duration and intensity of the stress and stage of the plant's development at the point of drought exposure may also affect the efficiency of PGPB-mediated drought tolerance (Ngumbi and Kloepper 2016).

These beneficial bacteria colonize plants and confer drought tolerance by modification in root morphology in acquirement of drought tolerance (Vurukonda et al. 2016); production of exopolysaccharides (EPS) (Sandhya et al. 2009; Kavamura et al. 2013), phytohormones (Fahad et al. 2014), 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Saleem et al. 2007; Reed and Glick 2005; Glick et al. 2007), and volatile compounds (Vurukonda et al. 2016), inducing accumulation of osmolytes (Jha et al. 2011) and antioxidants (Gururani et al. 2013; Wang et al. 2012); and upregulation or downregulation of the genes involved in stress response (Vurukonda et al. 2016) (Fig. 10.1).

### 10.6.1 Alteration of Root Morphology in Acquisition of Drought Tolerance

The architecture of the root system is among the important mechanisms adopted by plants to endure the drought situation. Root system structure comprises root system topology, spatial dissemination of primary and lateral roots, and changes in the number and length of root diameters. Root morphological plasticity in response to soil physical conditions provides the plants an available tool to cope with the chemical and physical properties of the soil including the drought conditions. The modification in root features associated with preserving the plant productivity under drought conditions comprises proliferation in the ratio of roots with small diameters and a deeper root length. More numbers of thinner roots allow plants subjected to drought to excess the hydraulic conductance by enhancing the surface area in contact with soil water in parallel rising the extent of soil that can be used for water uptake (Ngumbi and Kloepper 2016).

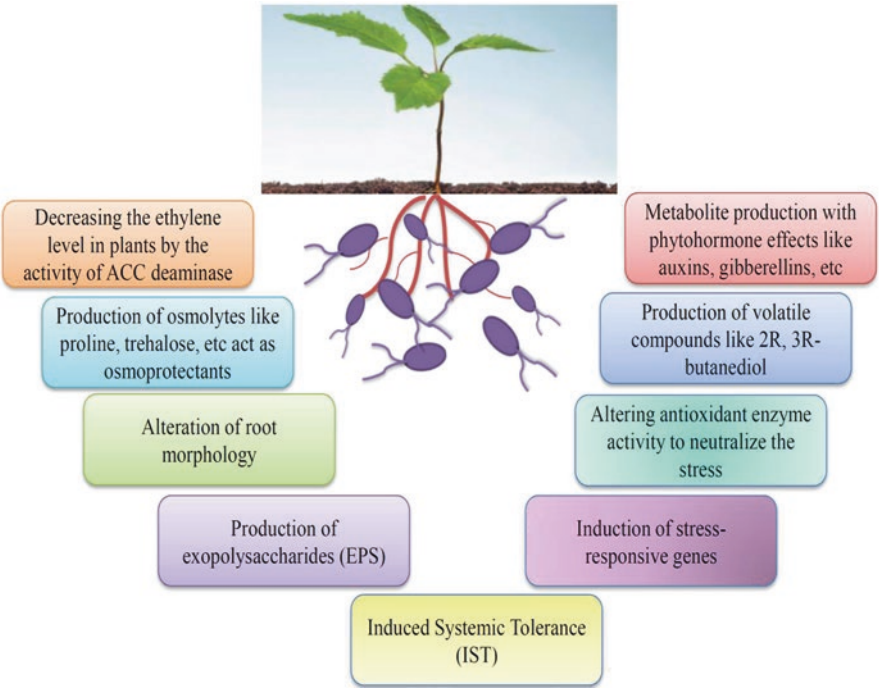


**Fig. 10.1** Mechanisms of plant growth-promoting rhizobacteria for imparting drought tolerance

The incorporation of the PGPB has been shown to accelerate the root growth and to modify its architecture. It has been supposed that the bacterial-induced alterations in root structure often lead to an extent in total root surface area, and subsequently to improved water and nutrient absorption, with an impact on the plant growth (Ngumbi and Kloepper 2016). In a study on the effect of *Alcaligenes faecalis* (AF3) on maize, 3 weeks after planting of the inoculated seeds, drought-stressed PGPB-treated plants had 10% enhanced root length compared to drought-stressed non-inoculated control group. This could show that alteration of the root net as a result of PGPB treatment results in an improved water uptake, and consequently treated plants show higher tolerance to drought stress (Ngumbi and Kloepper 2016).

It has also been shown that wheat plants treated with *Bacillus thuringiensis AZP2* could exhibit two to three times longer root hairs and longer and denser lateral roots following exposed drought stress (Ngumbi and Kloepper 2016).

In addition, plant's physiological state is controlled by the cell membranes, and rhizobacteria can affect the membrane transportation (Vurukonda et al. 2016; Sahin et al. 2015). Water scarcity changes the phospholipid pattern in the root, rises phosphatidylcholine, and diminishes phosphatidylethanolamine which results in unsaturation, but inoculation with *Azospirillum* prohibited these variations in wheat seedlings. As a whole, the bacterial elicited changes in the elasticity of the root cell

membranes are among the initial responses toward enhanced tolerance to water deficiency (Vurukonda et al. 2016).

### 10.6.2 Production of Exopolysaccharides (EPS)

Plants treated with exopolysaccharides (EPS) producing bacteria exhibit increased resistance to water and salinity stress due to improved soil texture (Ledger et al. 2016; Ilangumaran and Smith 2017). Microbial polysaccharides can attach the soil particles to construct microaggregates and macroaggregates. Plant roots and fungal hyphae fit in the pores between microaggregates and contribute to the stabilization of the macroaggregates (Shrivastava and Kumar 2015; Sandhya et al. 2009).

The EPS released by PGPB into the soil as slime materials which can be adsorbed by clay surfaces as a result of cation bridges, hydrogen bonding, Van der Waals forces, and anion adsorption mechanisms. This EPS film creates a protective capsule surrounding the soil aggregates. EPS supply a microenvironment that retains water and desiccate more slowly than the circumstances, thus avoiding the bacteria and plant roots from aridity (Vurukonda et al. 2016;Sandhya et al. 2009). EPS can also absorb the cations such as $Na^+$ therefore making it inaccessible to plants under saline conditions (Shrivastava and Kumar 2015; Upadhyay et al. 2011).

Particularly, the extracellular matrix made by PGPB can offer a range of beneficial macromolecules for plant growth and development. Biofilms have sugars and oligo- and polysaccharides that can also improve water availability in root medium. Additionally, some bacterial polysaccharides have a water retention capacity that can exceed severalfold of their mass (Timmusk et al. 2013, 2014). It has been demonstrated that even small polysaccharide alginate content in the biofilm facilitates the maintenance of hydrated microenvironment (Timmusk et al. 2014).

Accordingly, in a study an EPS-producing strain *Pseudomonas putida* strain GAP-P45 could form a biofilm on the root surface of sunflower seedlings and impart the plant tolerance to drought stress. The inoculated seedlings showed improved soil aggregation and root-adhering soil and eventually higher relative water content in the leaves (Sandhya et al. 2009; Vardharajula et al. 2011).

### 10.6.3 Metabolites with Phytohormone Effects

Phytohormones are synthesized in tissues of plants and are effective in quite a low amount after which are transported to their particular site of action. The hormone upon conveys to the targeted tissues prompts physiological alterations in plants such as lateral root development, flowering, fruit ripening, bud initiation, etc. The plant function is often the net consequence of the antagonistic or synergistic net of several hormones. Plant hormones are classified into five main groups: auxins, gibberellins, ethylene, cytokinins, and abscisic acid (Kang et al. 2014a, b). Phytohormones protect the plants against abiotic stress, and as a result, they can survive under stressful

**Table 10.1** PGPB phytohormonal activity in conferring drought tolerance of plants

| PGPB | Plant species | Effect |
|---|---|---|
| *A. brasilense* | Tomato | Nitric oxide acted as a signaling molecule in IAA |
| *Azospirillum lipoferum* | Maize | Gibberellins enhanced ABA amounts and relieved drought stress |
| *Azospirillum* sp. | Wheat | IAA improved root growth and lateral root development and enhanced uptake of water and nutrients under drought stress |
| *Phyllobacterium brassicacearum* | *Arabidopsis* | Increased ABA content leads to decreased leaf transpiration |
| *Bacillus subtilis* | *Platycladus orientalis* | Cytokinin production by PGPB raised ABA levels in shoots and enhanced the stomatal conductance |
| *P. putida* H-2–3 | Soybean | Secretion of gibberellins enhanced plant growth |
| *B. thuringiensis* | *Lavandula dentata* | IAA caused higher K-content and proline and reduced the glutathione reductase (GR) and ascorbate peroxidase (APX) |
| *Rhizobium leguminosarum* (LR-30), *Mesorhizobium ciceri* (CR-30 and CR-39), and *Rhizobium phaseoli* (MR-2) | Wheat | IAA produced by the consortia made better the growth, biomass, and drought resistance |

Adapted from Vurukonda et al. (2016)

conditions. Additionally, PGPB can synthesize phytohormones that motivate plant cell division and growth and make crops tolerant to the environmental stresses (Vurukonda et al. 2016) (Table 10.1).

#### 10.6.3.1 Abscisic Acid

Abscisic acid (ABA) is a naturally occurring sesquiterpenoid (Egamberdieva et al. 2017b). The abscisic acid is a stress hormone biosynthesized during water scarcity condition as cellular dehydration. ABA-induced regulates the expression of stress-responsive genes under abiotic stress and interposed signaling, resulting in stronger elicitation of resistance responses. Furthermore, ABA has been assumed to adjust the root development and water quantity under drought stress situations (Egamberdieva et al. 2017b).

In addition to ABA function in signaling, the most significant role of ABA is its action as an antitranspirant by the excitation of stomatal closure and lessening of canopy expansion (Vurukonda et al. 2016; Egamberdieva et al. 2017b; Vacheron et al. 2013). It was revealed that the elevation of ABA content in *Arabidopsis* inoculated by the PGPB *Phyllobacterium brassicacearum* strain STM196 could modulate the osmotic stress resistance in inoculated plants, causing reduced leaf transpiration (Vurukonda et al. 2016; Kaushal and Wani 2015).

Additionally, ABA can trigger developing a deeper root system and creating other root changes to intercede optimal water and nutrient attainment in plants exposed to stressful circumstances. Moreover, ABA retains the hydraulic conductivities of shoot and root to efficiently explore environmental water content, resulting in the retention of tissue turgor potential. Furthermore, ABA upregulates the antioxidant system and the accumulation of compatible osmolytes which conserve the relative water content (Egamberdieva et al. 2017b). It has been assumed that ABA conserves the balance of other hormones, including ethylene, causing the preservation of shoot and root growth in *Zea mays* (Egamberdieva et al. 2017b).

#### 10.6.3.2 Auxins

A number of identified auxins exist naturally as indole-3-acetic acid (IAA) is the most common (Olanrewaju et al. 2017) that is physiologically the most active auxin in plant growth and development (Vurukonda et al. 2016). In fact, throughout the literature, auxin is often interchanged with IAA. It is estimated that almost 80% of rhizosphere microorganisms have the ability to produce and release the auxin (Olanrewaju et al. 2017). It has been proposed that PGPB may support plants to modulate the abiotic stresses by supplying IAA for plants, which prompts plant growth in spite of the existence of inhibitory material (Glick 2012).

IAA increases the length of root, the root surface area, and the number of root tips, which result in an increased uptake of water and nutrients, therefore supporting plants to adapt with water scarcity (Shrivastava and Kumar 2015; Vurukonda et al. 2016; Kaushal and Wani 2015).

IAA and ACC deaminase enhance the plant growth synergistically. The excluded tryptophan from the roots can be absorbed by PGPB associated with the roots, where it is transformed into IAA. The diffused IAA from the bacterial source absorbed by plant cells and in combination with the plant inherent IAA induces the auxin signal transduction pathway which contains different auxin response factors. The plant cells growth and proliferation are prompted by that, while simultaneously some of the IAA molecules stimulate the expression of the gene encoding the ACC synthase enzyme. The activity of this enzyme leads to a raised level of ACC precursor and finally ethylene synthesized by the enzyme ACC oxidase (Glick 2012; Penrose and Glick 2011).

A number of biotic and abiotic stresses can promote the synthesis of IAA and induce the transcription of the gene for ACC synthase (Glick 2012). A fraction of this ACC may be scavenged by the PGPB which are associated with the plant that has the capability of producing the enzyme ACC deaminase and degraded to ammonia and ũFC;-ketobutyrate (Nadeem et al. 2014).

Therefore, PGPB that contain genetic information of ACC deaminase can act as a sink for the excess ACC. This root in the fact that, by the impose to an environmental stress, a decreased amount of ethylene is manufactured by the plant and the stress response of the plant is reduced (Glick 2015).

Following the increase in the quantity of ethylene in a plant, the transcription of auxin response factors is suppressed. The ethylene limits the transcription of auxin

response factors and as a result restricts both cell growth and proliferation in the absence of bacterial ACC deaminase, while in the presence of ACC deaminase, less ethylene is made. Therefore, when ACC deaminase exists, the transcription of auxin response factors is not repressed, and IAA can prompt cell growth and proliferation without parallel causing the accretion of ethylene. As a result, ACC deaminase reduces the inhibition of plant growth pursued by the ethylene and provides the state that IAA can increase the plant growth, both in the stressful and stressless conditions (Glick 2012).

The enhancement in leaf water content was induced by association of *Azospirillum* to wheat. This was related to the construction of plant hormones such as IAA by *Azospirillum* that elevated the root growth and formation of lateral roots, which consequently the water uptake and nutrient absorption of plants increase under the drought stress (Vurukonda et al. 2016).

#### 10.6.3.3 Cytokinin

Cytokinins such as zeatin (Z) (Sgroy et al. 2009) are compounds with a similar structure to adenine that is termed based on their influence on cytokinesis or cell division in plants. Other than plants, a number of yeast strains and a diversity of soil bacteria, including PGPB are able to synthesize the cytokinins. The overproduction of cytokinins in transgenic plants, particularly during periods of abiotic stress, is considerably protected from the harmful effects of abiotic stresses. The assessment of the protective activity of cytokinin-producing PGPB compared to cytokinin minus mutants will reveal their effect more comprehensively (Glick 2012).

#### 10.6.3.4 Gibberellins

Gibberellins (GAs) are omnipresent plant hormones that affect different stages of plant growth by regulating numerous physiological functions including seed germination, stem elongation, sex expression, flowering, fruiting, and senescence (Kang et al. 2014b). The exogenous applications of GA3 and GA4 have been shown to reclaim the plant growth and biomass production by countering the abiotic stresses in plants (Kang et al. 2014b). GAs cause improved root length, root surface area, and the number of root tips, causing an enhanced attraction of nutrients, thereby amending plant function under stress environments (Shrivastava and Kumar 2015;Vacheron et al. 2013).

The inoculation of rhizobacterium *P. putida* H-2–3 which can secrete gibberellins was shown to induce the physiological modifications in soybean plants leading to ameliorated growth under drought environments (Kaushal and Wani 2015). Production of ABA and gibberellins by *Azospirillum lipoferum* has also been reported to alleviate the drought stress in maize plants (Kaushal and Wani 2015).

#### 10.6.3.5 Salicylic Acid

Salicylic acid is the main phytohormone with a phenolic nature. It plays an important role in plant stress resistance by the activity regulation of the antioxidative enzyme (Egamberdieva et al. 2017b). SA moderates numerous physiological processes related to plant stress tolerance by stress-activated signal pathways and

response mechanisms (Egamberdieva et al. 2017b). It was reported the augmentation of plant by SA increased the plant growth of sesame in drought circumstance (Egamberdieva et al. 2017b).

### 10.6.4 Accumulation of Compatible Solutes

Plants adapt to drought stress by the metabolic adjustments that result in the aggregation of osmolytes (compatible solutes) such as proline, betaines, sugars, polyhydric alcohols, polyamines, quaternary ammonium compounds, and other amino acids and water stress proteins like dehydrins. PGPB are able to secrete osmolytes in reaction to drought stress, which works synergistically with inherent plant-produced osmolytes and prompts the plant growth (Vurukonda et al. 2016).

These small, uncharged, soluble molecules do not affect cellular function directly (Cura et al. 2017) while can reduce the hydric potential of cells by trapping water molecules or by retaining the water molecules they are already associated with (Furlan et al. 2017; Cura et al. 2017). In addition, compatible solutes can increase the stability and integrity of membranes and proteins, leading to lessening the cellular damage (Cura et al. 2017).

#### 10.6.4.1 Proline

Upregulation of proline biosynthesis pathway enhances proline amount which contributes in sustaining cell water station, conserving membranes and proteins from stress (Vurukonda et al. 2016), sweeping hydroxyl radicals, and moderating the NAD/NADH ratio (Marulanda et al. 2009). Higher proline accumulation in inoculated plants correlates with higher plant tolerance to water stress (Ngumbi and Kloepper 2016).

The inoculation of maize plants exposed to drought with PGPB *Pseudomonas putida* GAP-P45 amended the relative water content and leaf water potential by a concentration of proline (Vurukonda et al. 2016). *P. fluorescens* enhanced the amount of proline when maize plants were inoculated under drought stress (Vurukonda et al. 2016). Drought tolerance of *L. dentate* has been attributed to the PGPB *B. thuringiensis* (Bt) inoculation which was supposed to acquire through enhanced shoot proline accumulation (Vurukonda et al. 2016).

#### 10.6.4.2 Choline

Choline has a critical importance in plant stress tolerance, principally by increasing glycine betaine (GB) synthesis and aggregation. The investigation of the treatment of *B. subtilis* GB03 on *Arabidopsis* and *Klebsiella variicola* F2, *P. fluorescens* YX2, and *Raoultella planticola* YL2 on maize revealed improvements in biosynthesis and accumulation of choline. Choline as a precursor in GB anabolism promotes accumulation of GB; as a result, it elevates the leaf relative water content (RWC) and ultimately the plant biomass (Vurukonda et al. 2016).

A number of PGPB strains can induce accumulation of solutes such as GB under abiotic stress which controls plant stress responses by inhibiting water loss due to

osmotic stress. Correspondingly, inoculated plants with PGPB strains such as *B. subtilis* GB03 and *Pseudomonas* spp. considerably accumulated higher amounts of GB compared to uninoculated plants under osmotic stress. This might originate from upregulation of GB biosynthesis pathway by appending some key enzyme gene expression as PEAMT (phosphoethanolamine N-methyltransferase) (Vurukonda et al. 2016).

#### 10.6.4.3 Polyamines

Polyamines are aliphatic nitrogen mixtures which are ubiquitous in bacteria, plants, and animals. They control plant growth and development as well as plant reactions under drought stress by an active function in various metabolic and hormonal pathways (Kaushal and Wani 2015). Increased root growth due to cadaverine (polyamine) production by *A. brasilense* Az39 could induce the enhanced root growth of *Oryza* seedlings which caused the mitigation of the osmotic stress (Kaushal and Wani 2015; Vurukonda et al. 2016).

#### 10.6.4.4 Soluble Sugars

The accumulation of soluble sugars as osmolytes can also be adapted as a contributing mechanism to the osmotic amendment in the drought environment. It was assumed that starch hydrolysis increases the levels of monosaccharides. An enhancement in soluble sugar quantity in drought-stressed plants has been reported. Starch reduction and elevated sugar content were simultaneously detected in grapevine leaves under drought stress (Kaushal and Wani 2015).

Maize seedlings augmented with *Bacillus* strains inoculation showed elevated sugar content caused by starch degradation, thus made plants to tolerate the drought stress (Kaushal and Wani 2015). The enhanced soluble sugar quantity compared to uninoculated maize was observed in maize seedlings supplemented with *Pseudomonas* spp., representing that such inoculation causes the hydrolysis of starch, consequently providing sugar of osmotic regulation to alleviate the effect of drought stress (Kaushal and Wani 2015).

#### 10.6.4.5 Trehalose

Trehalose is a nonreducing disaccharide, consisting of two molecules of ūFC;-glucose that is widely distributed in bacteria, yeast, fungi, plants, insects, and invertebrates. Trehalose is recognized as a preserver against various abiotic stresses such as drought, high salt, and extreme temperature at high levels of concentration. Trehalose has a high structural stability and is tolerant to high temperature and acidity. Trehalose can form a gel phase as cells dry up, replacing water, consequently facilitate to expel the detriment from drought and salt. Furthermore, trehalose can protect proteins from degradation and aggregation caused by high- and low-temperature stresses (Glick 2012).

Treatments of plants with PGPB which overproduce trehalose have conferred drought (and other stress) tolerance. The inoculated beans with a genetically engineered overproduce of the trehalose (symbiotic *Rhizobium etli*) conferred the host more nodules, fixed more nitrogen, resulted in higher biomass, and recovered to a

greater amount from drought stress than inoculated plants with wild-type *R. etli* (Glick 2012).

Correspondingly, inoculated maize with the PGPB *Azospirillum brasilense* (modified to overproduce trehalose) was more drought tolerant and produced more biomass compared to plants treated with wild-type *A. brasilense*. The use of genetically manipulated PGPB to overproduce trehalose is simpler than engineering plants to achieve the same goal. Another advantage is that using a single engineered bacterial strain may effectively protect a large number of different crop plants (Glick 2012).

### 10.6.5 Production of Volatile Compounds

Production of volatiles is induced in plants exposed to a multitude of stresses. The stress-induced volatile compounds act as signals for beginning the systemic responses within the identical and in adjunct plants (Vurukonda et al. 2016).

The augmentation of wheat seedlings with *B. thuringiensis* AZP2 led to fivefold higher survival under intense drought. This tolerance was caused by a substantial decrease in emissions of volatiles and higher photosynthesis. This support that bacterial inoculation can improve plant drought tolerance by this mechanism. Volatiles are promising candidates of quick noninvasive indicator to evaluate crop drought stress and its alleviation during stress (Vurukonda et al. 2016).

*Pseudomonas chlororaphis* O6 which colonized root hinders water loss by the production of a volatile metabolite 2R,3R-butanediol. This volatile metabolite mediated stomatal closure. Bacterial volatile 2R,3R-butanediol stimulates the tolerance to drought stress in *Arabidopsis*. Additionally, *Arabidopsis* mutants illustrated that induced drought resistance required the signaling pathways of salicylic acid (SA), ethylene, and jasmonic acid. The induced drought resistance and stomatal closure pertained to Aba-1 and OST-1 kinase. Rise in free SA in plants colonized with *P. chlororaphis* O6 under drought stress and after 2R,3R-butanediol treatment proposes the initial function of SA signaling in induction of tolerance to drought stress. The volatile bacterial metabolite of 2R,3R-butanediol has shown as a major determining factor in promoting tolerance to drought through an SA-dependent mechanism in *Arabidopsis* (Vurukonda et al. 2016; Liu and Zhang 2015).

VOC treatment increased the level of PEAMT (phosphoethanolamine N-methyltransferase) transcripts. PEAMT is an essential enzyme in the biosynthesis pathway of choline and glycine betaine which mediate the VOC-induced plant tolerance to dehydration (Liu and Zhang 2015).

### 10.6.6 Antioxidants Effect to Neutralize the Stress

The systemic exposure of plants to drought stress can cause the generation of reactive oxygen species (ROS), including hydroxyl radicals (OH), superoxide anion radicals ($O^{2-}$), singlet oxygen ($O_2$) hydrogen peroxide ($H_2O_2$), and alkoxy radicals (RO). The reaction of the ROS with proteins, lipids, and deoxyribonucleic acid

leads to oxidative damage and impairing the proper functions of plant cells. In order to prevail these consequences, plants have antioxidant defense systems consisting of enzymatic and nonenzymatic components that render to prevent the concentration of ROS and diminish the oxidative damage occurring during drought stress. Enzymatic components consist of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). Nonenzymatic components include cysteine, glutathione, and ascorbic acid (Vurukonda et al. 2016; Kaushal and Wani 2015).

The consortia of PGPB comprising *P. jessenii* R62, *P. synxantha* R81, and *A. nitroguajacolicus* strainYB3 and strain YB5 enhanced plant growth and induced the stress-associated enzymes (SOD, CAT, peroxidase (POD), APX and lower level of $H_2O_2$, malondialdehyde (MDA)) under drought stress compared to control. These studies provide evidence on the influence of PGPB application in increasing the drought resistance of plants by modulating the antioxidants activity under water scarcity environment (Vurukonda et al. 2016).

PGPB species like *Azospirillum* sp. and *Pseudomonas* sp. increased the growth and biomass of canola plants by regulating the oxidative stress enzymes under salinity stress (Kang et al. 2014a). Inoculation of lettuce (*Lactuca sativa* L.) with PGPB *Pseudomonas mendocina* augmented an antioxidant CAT (catalase) under severe drought conditions, suggesting that they can be used in inoculants to alleviate the oxidative damage elicited by drought condition (Vardharajula et al. 2011).

### 10.6.7 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase

Ethylene is an ubiquitous hormone in all higher plants. This subject illustrates its importance in the regulation of normal cell progress and plant growth in addition to its vital role to counteract various levels of stress. Approximately all plant tissues and phases of growth are influenced by ethylene. Ethylene production in a specific plant is related to the existence and content of other plant hormones, temperature, light, gravity, nutrition, and the occurrence of different amounts of biotic/abiotic stress. The concentration of ethylene in plants is enhanced in reaction to the range of stresses including the existence of extreme temperatures, metals, chemicals (both organic and inorganic), extreme amounts of water, ultraviolet light, insect and nematode injury, and fungal and bacterial pathogens along with mechanical damages (Olanrewaju et al. 2017).

ACC oxidase enzyme produces ethylene more than its threshold level in the plant tissues, which can result in “stress ethylene” and influences the root and shoot growth in plants (Olanrewaju et al. 2017). ACC deaminase-producing PGPB favor to relieve “stress ethylene” situation and revive normal plant growth (Mayak et al. 2004b). Rhizospheric and phyllospheric organisms as well as endophytes, all of which can act as a sink for ACC produced as a consequence of plant stress by the synthesizing of ACC deaminase (Saleem et al. 2007). Plant ACC is sequestered and

catabolized by ACC deaminase-producing PGPB to nitrogen and energy substrate (Shrivastava and Kumar 2015; Cura et al. 2017).

The "stress ethylene" is being synthesized in two peaks. The first peak is a small portion of the quantity of the second peak. The first little peak which measures hard consumes much of the present 1-aminocyclopropane-1-carboxylate (ACC) in stressed plants and triggers the expression of genes that encode plant defensive/protective proteins. The second, much larger ethylene peak occurs when the level of ACC in response to stress increases. The second peak impairs consequent plant growth and initiates processes in the plant, for instance, senescence, chlorosis, and leaf abscission. The upregulated amount of plant ethylene considerably gets worse the effects of the causing stress that activates the ethylene response. So any treatment that reduces the quantity of the second peak of stress ethylene can also decrease/cease the deleterious effect of stress (Olanrewaju et al. 2017). In this regard, the ACC deaminase-producing bacteria can reduce the detrimental effect of the various stresses on plants by diminishing plant ACC amounts (and consequently plant ethylene levels). The ACC is being catabolized by ACC deaminase to α-ketobutyrate and ammonia in the PGPB (Olanrewaju et al. 2017; Tank and Saraf 2010; Saleem et al. 2007) (Fig. 10.2).

It was previously proposed that PGPB can absorb some of the tryptophan secreted by plants and transform the tryptophan to IAA, which is then exuded by the bacterium and soaked up by the plant. The enhanced amount of IAA can both assist plant growth and activate the expression of the plant enzyme ACC synthase simultaneously, leading to a raise at the level of ACC and therefore the concentration of ethylene within the plant. Consequently, PGPB that produce IAA from plant tryptophan can both stimulate and hinder plant growth (via the act of the ethylene that is ultimately synthesized). Fortunately, ACC deaminase-containing PGPB reduce the level of ACC in the plant by the act of ACC deaminase enzyme. As a consequence, IAA can improve plant growth without considerably inhibiting plant growth. Furthermore, by lessening the amount of ethylene in the plant, ethylene inhibition of auxin signaling pathway is pulled down, and the bacterial auxin enhances further growth of the plant. Therefore, ACC deaminase assists the action of bacterial IAA by the downregulation of plant ethylene amounts. The ACC is finally converted to ammonia and α-ketobutyrate (Olanrewaju et al. 2017). This model is depicted schematically in Fig. 10.2.

ACC deaminase production by endophytic PGPB can alleviate stress-related inhibition to a variety of environmental conditions (Ebels 2015). ACC deaminase-containing PGPB *Achromobacter piechaudii* ARV8 has revealed that considerably enhanced the fresh and dry weights of both tomato and pepper seedlings and decreased the ethylene construction under drought stress (Vurukonda et al. 2016; Saleem et al. 2007). ACC deaminase-producing *Pseudomonas fluorescens* prompted the length of roots of *Pisum sativum*, which resulted in higher absorption of water from soil in drought conditions. Enhanced growth, yield, and water-absorption competency of droughted peas was observed by the inoculation with *Variovorax paradoxus* (Vurukonda et al. 2016).

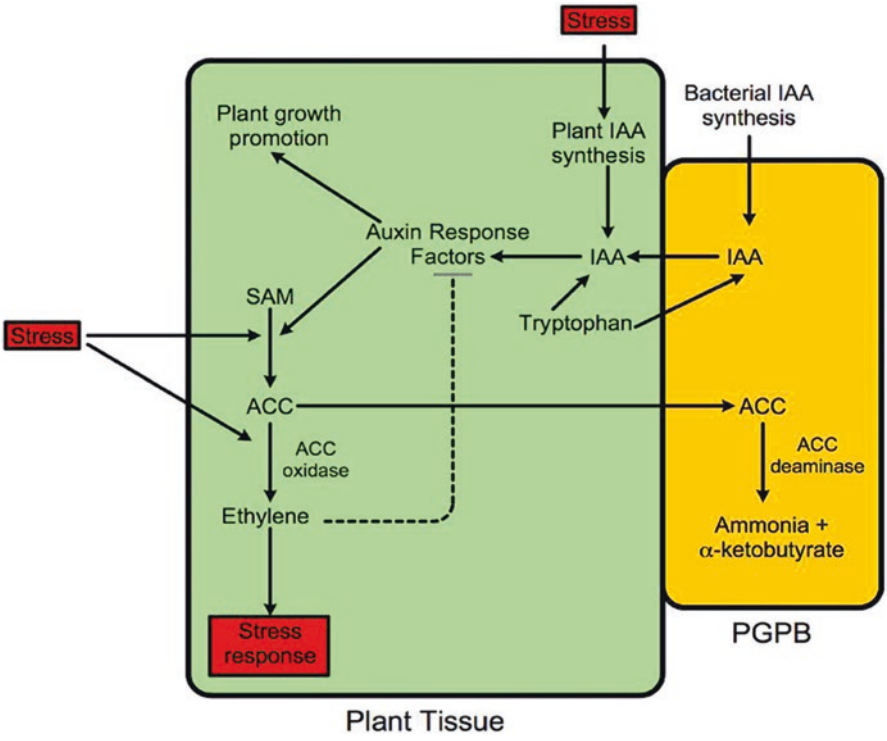


**Fig. 10.2** The induction of plant growth by an ACC deaminase-producing PGPB. Stress enhances both IAA and ethylene production within the plant which harbors a reduction in plant biomass production. The ACC deaminase-containing PGPB exhibit decreased levels of ethylene which allows the bacterial IAA to improve plant growth. Therefore, PGPB that synthesize both IAA and ACC deaminase counteract well the growth-limiting environmental stresses. The PGPB can care for plants against the suppressor effects of ethylene-producing stresses including drought, high salt, metal, flooding, temperature extremes and organic pollutants, insect and nematode predation, and both fungal and bacterial phytopathogens. *SAM* S-adenosylmethionine. (Olanrewaju et al. 2017)

### 10.6.8 Induction of Stress-Responsive Genes

Cell membrane proteins are at elevated hazard of denaturation because of their direct impose to the circumstance. Dehydration due to water scarcity induces protein aggregation, exposure of hydrophobic areas, modification in tertiary structure, and subsequent inactivation of enzymes or prohibition of their incorporation as structural proteins. HSPs are upregulated upon disposal to drought stress. HSPs, which are also termed chaperones, for instance, GroEL, DnaK, DnaJ, GroES, ClpB, ClpA, ClpX, sHSPs, and proteases, are implicated in responses to multiple of stress. The principal function of these proteins is to regulate the folding and refolding procedure of stress natured proteins. Clp family proteases are implicated in multiple stress reactions, suggesting they are important for ecological fitness of bacteria. Plant small heat shock proteins (sHSPs) act as molecular chaperones that assist

native folding of proteins and prevent irreversible aggregation of denatured proteins. Inoculated pepper plants with *Bacillus licheniformis* K11 exhibited enhanced transcription of genes Cadhn, VA, sHSP, and CaPR-10 during the drought stress (Lim and Kim 2013; Kaushal and Wani 2015).

Some PGPB alter plant gene expression, moderating drought tolerance-associated genes like ERD15 (Early Response to Dehydration15) (Mayak et al. 2004a, b; Bourque et al. 2016) or DREB (Dehydration Responsive Element Protein) (Bourque et al. 2016).

It was shown that elicitation of the drought-responsive gene ERD15 and ABA-responsive gene, RAB18, provides drought resistance in *A. thaliana* treated with *Paenibacillus polymyxa*. These genes, well-known as dehydrins (Group II late embryogenesis abundant proteins), are pertaining to drought and cold stresses and are mainly upregulated by the deficiency in cellular water content. Most of the dehydrins are supposed to act by the stabilization of hydrophobic interactions, for example, membrane structures or hydrophobic patches of proteins (Kaushal and Wani 2015).

The unusual expression of 93 genes in sugarcane, comprising drought-responsive genes such as MRB and WRKY transcription factors, is detected under drought stress. Nevertheless, co-treatment of the same plant with *Herbaspirillum* spp. and *Gluconacetobacter diazotrophicus* resulted in the induction of stress resistance and salicylic acid biosynthesis genes (Kaushal and Wani 2015).

It was shown that strain B26 of *B. subtilis* isolated from switch grass can contribute to the drought tolerance in *Brachypodium distachyon* by the upregulation of expression of drought-responsive genes, moderation of the DNA methylation procedure, and an enhancement in the soluble sugars and starch amount of the leaves. The strain B26 forming a close association with plants was also reported to thrive as a symbiosis strain and to synthesize several well-known lipopeptide toxins and phytohormones (Bourque et al. 2016).

### 10.6.9 Induced Systemic Tolerance

The term "induced systemic tolerance" (IST) has been suggested for PGPB-induced physical and chemical alterations that result in enhanced tolerance to abiotic stress (Shrivastava and Kumar 2015; Vardharajula et al. 2011).

The induced systemic resistance (ISR) is a common phenomenon against pathogens in plants that has been intensively investigated with considering the involved signaling pathways along with its potential application in plant protection. Provoked by a local infection, plants reacted with a salicylic-dependent signaling flow that results in the systemic expression of an extensive spectrum and long-standing disease tolerance that is practicable against fungi, bacteria, and viruses. Salicylic acid (SA) has a pivotal impact in the signaling pathway causing ISR. After infection, local and systemic endogenous concentration of SA enhances, and SA amounts rise in the phloem before the occurrence of ISR. The de novo production of SA in non-infected plant parts might contribute to the systemic expression of ISR (Saharan and Nehra 2011).

Compared to pathogens inducing SAR, even the nonpathogenic rhizobacteria inducing ISR can trigger another signal transduction pathway independent of the accumulation of the SA and activation of pathogenesis-related (PR) genes and following the precipitation of ethylene and jasmonic acid (Saharan and Nehra 2011). Interestingly, some of the volatile organic compounds (VOCs) that are emitted from *Bacillus subtilis* GB03 are recognized as the bacterial agent involved in IST (Vardharajula et al. 2011). In addition, some reports have suggested that some PGPB induce systemic tolerance (IST) in plants through enhanced antioxidant responses at the levels of enzyme activity and metabolite accumulation (Egamberdieva et al. 2017a).

## 10.7 Co-inoculation of PGPB for Mitigation of Drought Stress

As well as single strains of PGPB, its mixture with either mycorrhizal fungi or *Rhizobium* prompts the resistance of the plant to drought. Co-treatment of common bean (*P. vulgaris* L.) with a combination of *Rhizobium tropici* CIAT 899, *P. polymyxa* DSM36, and *P. polymyxa*-Loutit strains led to greater growth than lone inoculation of *Rhizobium*. Also, co-treatment showed higher nodulation and nitrogen content of plants inoculated with the sole *Rhizobium* under drought stress. Inoculated lettuce with a combination of PGPB strain *Pseudomonas mendocina* and an arbuscular mycorrhizal (AM) fungus (either *Glomus intraradices* or *Glomus mosseae*) showed considerably improved root phosphatase activity, proline content, and activities of NR, POD, and CAT in the leaves of lettuce under different levels of drought (Vurukonda et al. 2016).

## 10.8 Study of the Plant–Bacteria Interactions

The interactions of plant–bacteria consist of complex mechanisms within the plant cellular system. Currently, the investigation of plant–bacteria cooperation in terms of preservation against abiotic stresses is more critical consistently pressure of increasing climatic changes. Simultaneously, it is also crucial to make deeper understandings of the stress-alleviating mechanisms in crop plants toward the higher productivity (Meena et al. 2017).

It is obvious that any of the compounds manufactured can't be solely considered responsible for the detected drought stress resistance improvement. It is postulated that a variety of mechanisms are applied in the different growth levels and the drought resistance enhancement of the plants (Timmusk et al. 2013).

The feedback systems act on a diversity of levels: from DNA transcription to a signal transduction pathway within a cell to operate complicated interactions between systems of organisms. Taking advantage of the mathematical and computer modeling to quantify the interactions between constituents of a biological system is among system methods to make known the biological interactions of plant–bacteria. In order to recognize the complex behavior of the association and the processes of

PGPB and plant interaction, high-throughput, genome-wide research involving molecular networks along with high-resolution microscopy can also be performed (Timmusk et al. 2013).

Recent progress in "omics" technologies illustrates thoroughly the regulatory networks of stress reactions moderated by the PGPB (Ilangumaran and Smith 2017). Multi-omics technologies consisting of genomics, transcriptomics, proteomics, metabolomics, and phenomics incorporate assessments on the interaction of plants with bacteria and their interaction with peripheral environment and produce multi-dimensional information that can reflect what is occurring in real time within the cells (Meena et al. 2017).

Recently, meta-omics approaches comprising metagenomics, meta-transcriptomics, and meta-proteomics have been developed as capable techniques to investigate bacterial communities and function at a deeper level within the environment (Meena et al. 2017).

### 10.8.1 Genomics

Omics approaches have contributed to acquiring an improved understanding of the mechanisms of established plant–microbe interactions (Meena et al. 2017). The study of the association between the diazotroph *Gluconacetobacter diazotrophicus* PAL5 and sugarcane under drought stress by Illumina sequencing determined that bacterial treatment stimulated the ABA-dependent signaling genes providing drought tolerance in sugarcane (Vurukonda et al. 2016).

### 10.8.2 Metagenomics

The applying of the culture-independent method for the assay of microbial communities provides a comprehensive tool for the resolution of yet uncultured rhizospheric bacterial diversity. High-throughput metagenomic sequencing is demonstrating to be an exceptionally beneficial tool for a better understanding of PGPB populations.

Metagenomics also make known the hidden functional potential of microbial populations with regard to the affluence of the genes that are involved in specific metabolic processes related to stresses or stress mitigation mechanism. In an investigation on endophytes of the potato, two types of ACC deaminase genes (acdS) homologous to that of *P. fluorescens* for stress mitigation were discovered. Analysis of clones of metagenomic libraries contributed in recognition of whole acdS operon from uncultivated endophyte has shown a transcriptional regulator gene acdR at upstream of acdS. This operon was determined obviously in the genus *Burkholderia* (Meena et al. 2017).

The physiology of endophytic bacteria that exist inside roots is severely unknown as endophytes which are successfully cultured represent only a portion of the entire bacterial community that resides root interiors. With the aid of metagenomic

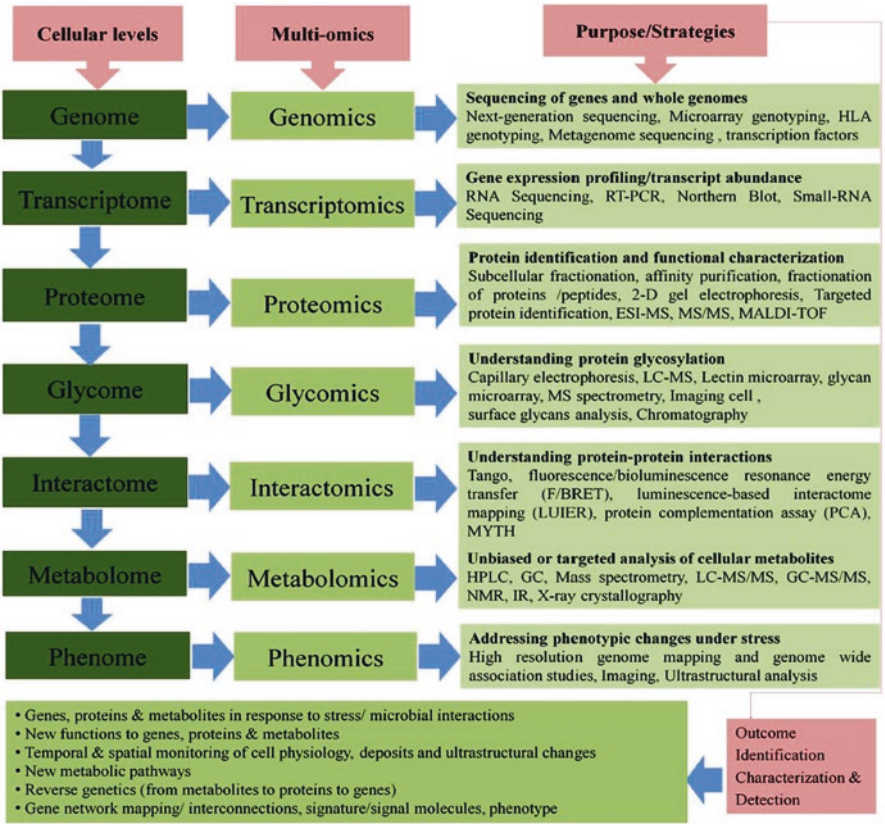


**Fig. 10.3** The multi-omics approaches in the investigation of the impact of abiotic stresses or effect of plant–bacteria interactions. (Adapted from Meena et al. 2017)

method, endophytic bacterial inhabitants of rice roots have been defined. Metagenomic sequences acquired from endophytic cell extracts proved that metabolic processes relating to the endophytic functional traits such as quorum sensing and detoxification of ROS are circumvented in enhancing the plant resistance to abiotic stress (Meena et al. 2017). To distinguish the microbiome composition and define its diversity and function, comprehensive methods, namely, metagenomics, meta-transcriptomics, and meta-proteomics, are needed to be implemented (Meena et al. 2017) (Fig. 10.3).

### 10.8.3 Transcriptomics

The transcriptome includes the whole set of transcripts that are expressed in a cell at a specific developing phase or under different environmental situations (Vurukonda et al. 2016). The comparison of transcriptome profiles can indicate the sets of

transcripts underlying the alterations between biologically diverse expressions in various conditions. Usage of mRNA sequence survey and microarray to make transcriptome level data is the main molecular approach used in the assessment of plant–microbe interactions (Meena et al. 2017).

Gene expression influenced by drought stress was newly characterized PGPB physiological roles with regard to resistance prompted by PGPB. At the transcriptional level, the positive effect of PGPB on improving plant resistance to drought was shown with the treatment of PGPB *Paenibacillus polymyxa* B2 on *Arabidopsis thaliana*. The expression study revealed that an mRNA transcription of a drought-response gene, Early Response to Dehydration15 (ERD15), was amplified in inoculated plants compared with uninoculated controls lacking the PGPB (Vurukonda et al. 2016; Yang et al. 2008).

The gene expression study on *Sinorhizobium meliloti* indicated the induction of genes for the stress reaction in IAA overproducing strains in comparison to wild-type strain-1021. This investigation compared the transcription profile of two *S. meliloti* strains. The coding genes of sigma factor RpoH1 and other stress responses were prompted in IAA overproducing strain of *S. meliloti* (Meena et al. 2017). Upregulation of stress-related genes APX1, SAMS1, and HSP17.8 in the leaves of wheat was recognized by real-time PCR (RT-PCR) analysis. The activity of enzymes of ascorbate–glutathione redox cycle enhanced in wheat when priming with *Bacillus amyloliquefaciens* 5113 and *A. brasilense* NO40 awarding drought tolerance to the plant (Vurukonda et al. 2016).

A number of drought signaling response genes were revealed by microarray analysis to downregulate in the *P. chlororaphis* O6-colonized *A. thaliana* in comparison to plants without bacterial priming under drought stress. The priming of plants resulted in upregulation of transcripts of the jasmonic acid-marker genes, VSP1 and pdf-1.2; salicylic acid-regulated gene, PR-1; and the ethylene-response gene, HEL (Vurukonda et al. 2016).

The effect of *Bacillus amyloliquefaciens* NBRISN13 inoculation on growth of the rice plant and expression analysis of related genes under salt stress was evaluated. Expression analysis by semiquantitative reverse transcriptase polymerase chain reaction (SQRT-PCR) revealed at least 14 genes implicating in SN13-mediated salt stress adaptation (Nautiyal et al. 2013). With RNA differential display on parallel RNA preparations from *P. polymyxa*-treated or untreated plants, changes in gene expression were investigated. From a small number of candidate sequences provided by this approach, one mRNA segment showed an intense inoculation-dependent increase. The corresponding gene was recognized as ERD15, previously identified to be drought stress-responsive (Timmusk and Wagner 1999).

### 10.8.4 Proteomics

Proteins play a vital function in expressing plant stress reactions since they directly harbor the phenotypic characteristics. Thus proteomics has become a potent technique for the investigation of physiological metabolism and protein–protein

interactions in microorganisms and plants. The implications of proteomics are significant for intra- and inter-microbial species and host–bacterium interplay. Such surveys result in obtaining a comprehensive insight of the regulation of the biological system by determination of several proteins as a signal that alerts the fluctuations in physiological station caused by stress or mitigating factors of stress. Thus, a comparative study of stressed versus non-stressed plants inoculated with bacteria can contribute to the recognization of protein targets and networks (Meena et al. 2017). Six differentially expressed stress proteins were known in pepper plants treated with *B. licheniformis* K11 in drought environment by 2-D polyacrylamide gel electrophoresis (2D-PAGE) and differential display polymerase chain reaction (DD-PCR). Particular genes of stress proteins (Cadhn, VA, sHSP, and CaPR-10) enhance more than a 1.5-fold in inoculated plants in comparison to the control plant (Vurukonda et al. 2016).

### 10.8.5 Metabolomics

Metabolomics implicates the describing of all the metabolites synthesized by an organism under the effect of adjusted environmental situations. Different metabolic pathways of the cell which reflects the presence of corresponding genetic information determine the metabolome of an organism. The metabolome alters largely with variations in the neighboring environment that stimulate direct physiological expression in a cell.

The analogous physiological state is expected in organisms which grow well under particular stress situations. Consequently, it is important to attain comprehensive perception of metabolome of an organism both in normal and under stress conditions for determining the presence/absence of signature metabolites. This will be useful in recognizing changes induced by the pathways and stimulation of typical stress-inducible genes. Metabolomics is progressively being applied for making comprehensive understanding into abiotic stress reactions. Currently, high-throughput advances of molecular recognition techniques have improved the metabolomics analysis that also shows the presence of diverse bioactive substances in plant metabolome (Meena et al. 2017). These facts relate to the findings regarding the identification of diverse signal molecules exuded by plants to attract and activate significant biochemical pathways in the microbial population that colonized plants.

*Trichoderma* spp. produce auxins which relieve stress and improve plant growth. *Trichoderma* synthesized two secondary metabolites (harzianolide and 6-pentyl-a-pyrone) which displayed auxin-like effects in pea stem and increase plant growth. Changing of environmental conditions induces variations in plant metabolism, also influences plants' secretion pattern and composition of secreted molecules, and, as a result, affects the variety and level of root colonization. Molecular signaling mechanisms of microorganisms in the rhizosphere are too influenced in the same way, but this is yet to be discovered (Meena et al. 2017).

Protective metabolites such as trehalose, glycine betaine, IAA, etc. accumulate in plants in reaction to abiotic stress conditions. The mechanisms acting in the

microbial cell relate to the conditions of the encompassing environment which affects the metabolome. It is obvious that the same must influence their general performance in the neighboring microenvironment and inside the ecosystem to a greater range in terms of the interactions within and between the residents in the ecosystem. Microbial metabolic products have enhanced plant growth both directly and indirectly. It is verified that various rhizospheric bacteria can synthesize plant growth-motivating biomolecules such as cytokinins, gibberellins, etc. (Meena et al. 2017). Newly the IAA manufactured by *Pseudomonas* sp., *Rhizobium* sp., *Enterobacter* sp., *Pantoea* sp., *Marinobacterium* sp., *Acinetobacter* sp., and *Sinorhizobium* sp. has been proved to affect the germination and seedling growth of wheat under saline stress (Meena et al. 2017).

The cellular metabolites of plant-colonized bacteria under the impact of stress analyzed by high-throughput mass spectrometry could show the amount of effect of stress source on the whole cellular homeostasis. The interaction between plants and soil microbial population signifies a bilateral process including root exudates and microbial-elaborated signal response molecules.

For rhizosphere supplementation with exogenous bacterial metabolites too, previous understanding on microbial metabolism is demanded. This consists of the balance of cellular richness, biomolecules synthesized in optimal conditions, quantifiable leak, contribution of plant signals in the cascade, and subsequent counter-reaction of microorganisms. The supplementation and enrichment of such biomolecules that are downregulated following the effect of the stressor could be attentive in the rhizosphere. A similar approach can be implemented to the eventual management of stressor-responsive biomolecules influencing the whole communication process between the host and bacteria (Meena et al. 2017). Novel analytical techniques like GC-MS and LC-MS have assisted in the analysis of low amounts of gibberellin in any cultures (Kang et al. 2014b).

## 10.9 Phylogenetic Distribution of Bacteria with Effect on Drought Tolerance

Bacterial phylogenetic diversity for soil communities may be dependent on the drought condition. With considering to drought context, a confusing factor that may participate in inconsistency is the absence of standardization of drought treatment which has been executed through a range of means (Naylor and Coleman-Derr 2017).

In contrast to microbial diversity, population composition is considerably influenced by drought. The imposed modifications in the soil microbial community under drought are likely to be a variation in relative amplitude, instead of complete elimination of drought-susceptible taxa and simultaneous emerging of tolerant ones. A commonly observed trend is a rise in the ratio of Gram-positive to Gram-negative bacteria in the drought environment. Definitely, in soils with limited moisture, prevalent relative richness alterations include declines in the most dominant Gram-negative phyla of *Proteobacteria*, *Bacteroidetes*, and *Verrucomicrobia* and

increases in the main Gram-positive phyla including *Firmicutes* and *Actinobacteria*. These alterations in relative abundance are provoked by one or a few members of a phylum; while relatively few groups change severely, most bacterial groups showed only minor alterations in reaction to drought. In a related study, an excess in *Actinobacteria* was detected (Naylor and Coleman-Derr 2017). Among the PGPB, the *P. fluorescens* and endophytic *Bacillus subtilis* have received special attention throughout because of their catabolic versatility, their excellent root-colonizing ability, and their capacity to produce a wide range of enzymes and secondary metabolites that favor the plant endure under varied biotic and abiotic stress conditions (Saravanakumar et al. 2011).

From the vast phylogenetically diverse microorganisms, Gram-positive bacteria are the most probable to be commercially applied in range of fields with limited water resources due to the endospore-forming ability that boosts the efficient colonization under drought stress conditions (Timmusk et al. 2014). The application of endospore-forming bacteria provides more reproducible results in various environments (Timmusk et al. 2013) (Table 10.2).

## 10.10 Biomarkers Used in Evaluation of Bacterial Colonization in Drought-Tolerant Plant

For screening the more stress-adapted resistant strains, assessing stress markers like proline or phytohormone production of bacterial cultures under the exposure to the elevated stress levels may assist (Marulanda et al. 2009). The analysis of the quantity of IAA is a proper indicator of bacterial efficiency principally under osmotic stress (Vardharajula et al. 2011). Significantly, the bacterial trait that is key in efficiency of PGPB in alleviating stress is the production of enzyme ACC deaminase (Glick 2014).

## 10.11 Cross-Resistance to the Other Abiotic Stresses

The different abiotic stresses exhibit some common signs, molecular damages, and alleviation strategies. For instance, drought and salinity stresses led to ionic and osmotic imbalance. The expression of drought and salinity tolerance genes both can restore the ionic and osmotic homeostasis via salt-regulated genes pathway or other associated pathways (Kang et al. 2014b). Drought and low temperature cause similar injuries such as the disintegration of the membrane, desiccation, and solute leakage. Crop plants in reaction to both stresses either activate detoxification signaling or induce stress genes which regulate molecular damage and mending of the cell membrane (Shinozak and Shinozaki 2000; Kang et al. 2014b).

**Table 10.2** Some of the effective PGPB in promoting plant growth under drought stress conditions

| Bacteria | Family/phyla (UniProt) | Plant | Mechanism | References |
|---|---|---|---|---|
| *Bacillus subtilis* | *Bacillaceae/Firmicutes* | *Platycladus orientalis* | Cytokinin production | Liu et al. (2013) |
| *Bacillus licheniformis* | *Bacillaceae/Firmicutes* | Pepper | Auxin and ACC deaminase production | Lim and Kim (2013) |
| *Pseudomonas fluorescens* | *Pseudomonadaceae/ Proteobacteria* | Foxtail millet (*Setaria italica* L.) | ACC deaminase and EPS production | Niu et al. (2018) |
| *Paenibacillus polymyxa* | *Paenibacillaceae/ Firmicutes* | *Arabidopsis thaliana* | Inducing expression of the drought stress gene Erd15 | Timmusk and Wagner (1999) |
| *Burkholderia* | *Burkholderiaceae/ Proteobacteria* | Wheat | Increase in activity of glutathione reductase (GR) | Naveed et al. (2014) |
| *Microbacterium* | *Microbacteriaceae/ Actinobacteria* | Pepper | Modulation of the plants glutamine and ketoglutarate | Vilchez et al. (2018) |
| *Azospirillum lipoferum* | *Rhodospirillaceae/ Proteobacteria* | Maize | Accumulating free amino acids and soluble sugars | Bano et al. (2013) |
| *Phyllobacterium brassicacearum* | *Phyllobacteriaceae/ Proteobacteria* | *Arabidopsis* | Elevating ABA content | Bresson et al. (2013) |
| *Ochrobactrum pseudogrignonense* | *Brucellaceae/ Proteobacteria* | Black gram (*Vigna mungo* L.) and the garden pea (*Pisum sativum* L.) | ACC deaminase production | Saikia et al. (2018) |

## 10.12 Successful Cases of the Field Studies

The effect of PGPB on crop productivity varies under laboratory, greenhouse, and field trials (Ahemad and Kibret 2014). The performance of efficient PGPB strains must be evaluated under field conditions where plants are more probable to tolerate cyclic drought rather than the continuous drought in the experiments (Ngumbi and Kloepper 2016).

Although, a small fraction of the studies have been conducted in the field, however, results are inconsistent with those of laboratory or greenhouse studies (Nadeem et al. 2014). With the aid of suitable monitoring systems, the restrictions of applying microbial inoculation in the fields can be significantly resolved. Therefore, the field trials along with the advanced molecular and biochemical monitoring systems suggested to be applied in parallel (Timmusk et al. 2013).

To investigate the impact of plant growth-promoting rhizobacteria (PGPR) on water stress, a field experiment was conducted in Iran during 2010 growing season. The effect of four types of bacterial strain consisting of *Pseudomonas* sp., *Bacillus lentus*, *Azospirillum brasilense*, and a combination of the three mentioned bacteria on proline, soluble carbohydrates, chlorophyll, and mineral content in basil was studied. Results showed water stress and different bacterial strain were substantially affected by proline and soluble carbohydrate accumulations in leaves of plants (Heidari et al. 2011). Also, the effects of PGPB inoculation under the water stress on antioxidant activity and photosynthetic pigments were investigated in basil plants by the field study. Application of rhizobacteria under water stress improved the activity of antioxidant enzymes and photosynthetic pigments in basil plants (Heidari and Golpayegani 2012).

A field experiment was conducted in Iran with soybean to evaluate the performance of different PGPB comprised of *Rhizobium japonicum*, *Azotobacter chroococcum*, *Azospirillum brasilense*, and a mixture of these inoculates on soybean antioxidant enzyme activity subjected to different irrigation regimes in 2012–2013 growing season (Zahedi and Abbasi 2015).

In another field experiment, the effects of selected PGPB including *Bacillus megaterium* TV 6D and *Bacillus subtilis* TV 12H on some physiological characteristics, plant growth, yield, and plant nutrient concentration of lettuce were monitored under different irrigation levels in Turkey. The results of the study demonstrated that PGPB inoculations could deduct the detrimental effects of lower irrigation conditions on the growth and yield of lettuce plants (Sahin et al. 2015).

The effect of selected PGPB on the growth, nutrient element content, chlorophyll content, and yield of strawberry plants under salinity condition stress was evaluated in the natural field. Field experiments were undertaken using a randomized complete block design with five PGPB strains consisting of *Bacillus subtilis* EY2, *Bacillus atrophaeus* EY6, *Bacillus sphaericus* GC, *Staphylococcus kloosii* EY37, and *Kocuria erythromyxa* EY43 and a control non-PGPB. PGPB inoculations could enhance the chlorophyll content, nutrient element content, and yield of strawberry plants. Priming with PGPB diminished the electrolyte leakage of plants under saline conditions. The leaf relative water content (LRWC) of plants was improved by the

inoculation of bacterial cells (Karlidag et al. 2013). Analogous mechanisms can lead to the stronger tolerance of the plant to the water deficiency in the environment. The effect of plant growth-promoting rhizobacteria (*Pseudomonas* spp.) on asparagus seedlings and germinating seeds imposed to water stress under greenhouse conditions has also been reported (Liddycoat et al. 2009).

A greenhouse study was conducted to assess the effect of biochar in combination with compost and *Pseudomonas fluorescens* under water deficit stress on the growth of cucumber. The results showed that water deficit stress significantly hindered the growth of cucumber, while the synergistic use of biochar, compost, and PGPB mitigated the negative impact of stress. The synergistic effect of biochar, compost, and PGPB caused remarkable increases in shoot length, shoot biomass, root length, and root biomass that were 88, 77, 89, and 74% more than uninoculated control, respectively (Nadeem et al. 2017).

## 10.13 Concluding Remarks

Sustainable food quality and reasonable cost might be a challenge for the increasing population in the next 50 years (Olanrewaju et al. 2017). Further, development of the arable land, insufficient managed water resources, and long-term effects of the climatic change could all contribute to possibly catastrophic consequences (Kasim et al. 2012). Numerous strategies have been introduced for modulating the effects of drought stress in plants and breeding for tolerant varieties, among which genetic engineering is the most focused approach (Ashraf 2010; Kasim et al. 2012). However, the complexity of tolerance mechanisms to abiotic stress makes the task of developing new tolerant varieties highly challenging and genetically modified plants are not adequately accepted in most countries. An alternative strategy is to induce stress tolerance by using various chemical and biological agents in a process known as priming (Kasim et al. 2012). One of the methods that might be subjected is the more extensive application of PGPB, initially in parallel, and possibly eventually in place of the present chemicals used in agriculture (Olanrewaju et al. 2017).

The application of PGPB is an integral component of modern agricultural practices. The agricultural chemicals are relatively inexpensive which has kept the use of PGPB at a limited scale however as a thriving approach in the development of organic agriculture (Glick 2012).

Although microbial inoculants are being extensively used to improve plant growth under controlled condition, the results inferred from these studies often do not attain a reasonable level of efficacy and consistency in natural field conditions that is required for their commercialization on a large scale. This might be due to the soil physicochemical parameters and microbial populations that establish a complex interaction (Keswani et al. 2014; Nadeem et al. 2014).

PGPB have the ability to colonize the roots and promote plant growth directly or through biological control of plant diseases and also involved in abiotic stress tolerance. The major challenges in this area of research lie in the identification of various strains of

PGPB and its properties. It is essential to dissect the actual mechanism of PGPB function in their efficacy toward exploitation in sustainable agriculture (Pujar et al. 2017).

Unfortunately, the interaction between associative PGPB and plants can be unstable and temporary. The achieved in vitro results cannot always be expected to be similar to the field conditions. The inconsistency in the performance of PGPB may be because of multiple environmental parameters that may influence their growth and execute their effects on the plant. The major environmental factors consist of soil characteristics, weather conditions, or the composition and activity of the indigenous microflora of the soil. To attain the optimum growth promoting interaction between PGPB and nursery seedlings, it is crucial to discover the mechanisms rhizobacteria putting their effects on the plant and whether the impacts are changed by various environmental factors, such as the activity of other microorganisms. Therefore, the principle challenge is the development of competent strains for the field conditions. Among promising approach is to explore soil microbial diversity for PGPB having a combination of PGP capability and enough adapted to certain drought soil environment (Saharan and Nehra 2011).

## 10.14 Future Prospects

Stressful circumstance can impose a negative impact on plant growth and development by causing nutritional and hormonal imbalances. However, the stress-induced detrimental impact on plant growth can be attenuated and/or minimized by the application of free-living microorganisms (Nadeem et al. 2014). In the last 30–40 years, a detailed perception of the way that PGPB increase plants growth is proposed, approaching the extensive application of these organisms more feasible in the near future (Olanrewaju et al. 2017).

However, the more common application of PGPB requires that a number of subjects to be addressed in advance. (A) New and optimized methods for the large-scale cultivation, storage, distribution, formulation, and utilization of stress-protectant bacteria demanding to be developed. (B) Sensible, safe, effective, and constant protocols for their application need to be agreed in all countries by keeping the regulatory obstacles of technology transfer at the minimum possible amount. (C) Broadly based movements of public training noticing the nature of stress-protecting agents (PGPB) must be initiated on safety and obligation of such natural treatments. (D) Following additional fundamental work to acquire a deeper understanding of the biochemistry, genetics, and physiology of these bacteria, it demands to be confirmed that these strains may necessitate some genetic manipulation and the application of such genetically manipulated strains will not make any new threats to humans or the environment. (E) It is expected that diverse crops and varying conditions require the application of bacteria which are either ectophytic or endophytic. It will be essential to define those conditions where either ectophytic or endophytic strains are most proper so that the most efficient mixture of plant and bacteria could be formulated. (F) Regarding that the growth of more than 90% of crop plants is improved by the interaction of plants with mycorrhizae, it is essential to understand the mechanism

by which bacteria and mycorrhizae interact in a manner that enhances the plant growth. (G) To the most possible extent, such technology should be developed in the public domain to limit the monopolization of the key know-hows by a few huge companies. Although there is still greatly more basic and applied work to be carried out, use of PGPB has already been effective on a rather small scale, in some countries. If the abovementioned issues are addressed, it is predicted that global agricultural practice can become sustainable and deeply efficient. This paradigm change in agriculture can be a reclaim that assists both the developing and the developed world (Glick 2012; Olanrewaju et al. 2017).

In addition to mentioned issues, for accessing fruitful PGPB in alleviating drought stress effects, the research should focus onto strains which are preferably indigenous from the stress-affected soils that could be applied as bio-inoculants for crops grown under stress conditions (Vurukonda et al. 2016). The future trend needs to be in introducing genetically altered PGPB rather than transgenic plants for propagating plant performance in drought environment, as it is more amenable to transform the bacterial cells instead of complicated higher macroorganisms. Moreover, rather than engineering individual crops, a single, engineered inoculant can be applied for a number of crops, particularly by the implementation of a non-specific genus such as *Azospirillum* (Kaushal and Wani 2015).

Genetic engineering can be applied to develop PGPB strains that are executable at low formulation doses and under a range of environmental states. It is urgent to develop more operative PGPB strains with longer shelf-lives to achieve sustainable crop production in dry lands (Kaushal and Wani 2015). Applications of bio-nanotechnology could also offer new insights into the development of carrier-based microbial inocula. Application of nano-material may improve the stability of PGPB formulations with regard to desiccation, heat, and UV inactivation (Kaushal and Wani 2015).

Commercial applications of PGPB are under evaluation and have been frequently productive; nevertheless, a more comprehensive perception of the microbial interactions leading to plant growth improvement will impact the success rate of their application in field conditions (Saharan and Nehra 2011). A majority of the mechanisms behind the plant–microbe interactions in the rhizosphere are not completely discovered. Challenges originate mainly in profiling the abundant range of processes existing in microbial communities. The discovery of this signal crosstalk is essential to improve the plant adaptation mechanisms and to enhance the ability of soil strains for stress mitigation in crops (Ngumbi and Kloepper 2016; Vurukonda et al. 2016).

In addition, the mechanism needs to be assessed in phytohormonal regulation (abscisic acid, salicylic acid, jasmonic acid, and gibberellins) during the PGPB interaction with crop host plants enduring abiotic stress, to further evolve strategies for sustainable crop production (Kang et al. 2014b). A very large number of molecular techniques are becoming accessible and being used to describe the molecular bases of the plant–microbiome interactions. In spite of the recent advancement and perspectives emphasized on microbial-facilitated drought resistance in plants, PGPB mechanisms offering drought tolerance to plants are not clearly revealed.

However, recent advancement implies that this approach has great potential to provide new awareness for sustainable food production compared to the alternative possible approach (Vurukonda et al. 2016).

With the aid of suitable monitoring systems, the limitations of applying microbial inoculation in fields can be substantially diminished. A spectrum of field trials has to be designed for this purpose, coupled with advanced molecular and biochemical monitoring systems (Timmusk et al. 2013).

Commercial applications need complex prerequisites in the field technology and in the commercial financing and intellectual property of the work. In order to motivate industrial investors, the application technologies of new bacteria are recommended to be patented. However, the academia and research sectors demand to publish the results as their main financial support originates from publications (Timmusk et al. 2013); this should not diminish the trend of the channel to the commercialization of stress-protectant inocula.

It is explicit that the underground resources of the plant rhizosphere could provide advantages associated with global water deficiency and climate change (Timmusk et al. 2013). Rhizospheric bacteria are potential resources for countering such abiotic stresses. Root bacteria perform important functions in retaining soil humidity and water management in arid soils (Daffonchio et al. 2015). Still, challenges must be resolved before the bacterial inoculants could be extensively applied in drought fighting practices. The implementation of the bacterial inoculation technology has multiple of constrictions in the formulation and delivery of the inoculants which should be resolved in case of each bacterial formulation product. As the inoculates are composed of living organisms, there is a specific host range where the growth promotion is more related on the explicit environmental factors such as optimal temperature, moisture, UV radiation, etc. Gram-positive bacteria, in general, are preferred as microbial inoculates as they are amenable to resist the low water activity, irradiation, and chemicals. Additionally, the endospores produced by some groups of them can that can resist under a spectrum of stress conditions in the field, offering higher durable or reproducible protection under the natural conditions. (Timmusk et al. 2013).

Ultimately, integrating assessment of PGPB strains into plant breeding strategies for drought tolerance purposes may aid the agricultural practices adapt to continued warming of the global climate (Ngumbi and Kloepper 2016).

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# *Bacillus* spp.: As Plant Growth-Promoting Bacteria

11

Estibaliz Sansinenea

## 11.1 Introduction

Agriculture is the most important resource to sustain global economy and environmental and social system. Because of the increase of the agricultural crops' global demand, the productivity of the crops should be improved. Unfortunately, plant pests and diseases, as well as weeds, are provoking heavy losses annually in agriculture. Therefore, chemical fertilizers and pesticides have been used over the years to increase nutrient needs of the crops and protect them against pests, causing a great environmental damage, creating pest resistance and having potential risks to human health. Due to this concern, it is a worldwide desire to reduce the use of chemical pesticides, and research is afforded to study alternative routes for management of plant pathogens. The use of biofertilizers in production plays an important role as a supplement to improve the growth and yield of several agricultural plants (Borriss 2011; Bisen et al. 2015; Mishra et al. 2015).

Plant growth results from interaction of roots with the environment. The environment for roots is the soil or planting medium, which provides structural support as well as water and nutrients to the plant. Soil microbes since their discovery in the late eighteenth century have been used extensively in crop production. Microbes actively involved in crop production are generally termed as plant growth-promoting bacteria (PGPB), whereas the bacteria isolated from the root zone are termed as plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova 2009). Plant growth-promoting rhizobacteria (PGPR) are able to facilitate plant nutrient acquisition and can also act as biocontrol agents by suppressing soilborne diseases (Bashan and de-Bashan 2010). The mechanisms by which these bacteria act are multiple and diverse (Martinez-Viveros et al. 2010). The beneficial interactions of the microbes with the plants, having implications in the agriculture (Almaghrabi

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et al. 2013; Dawwam et al. 2013), are supply of nutrients to crops; stimulation of plant growth, namely, producing phytohormones; biocontrol of phytopathogens; improving soil structure; bioaccumulation of inorganic compounds; and bioremediation of metal-contaminated soils (Verma et al. 2010; Singh et al. 2016, 2017). This chapter overviews *Bacillus* spp. which are among the most useful bacteria in agriculture, due to the different biotechnological uses of this interesting bacterium, focusing in its ability to promote plant growth.

## 11.2 Plant Growth-Promoting Rhizobacteria

The rhizosphere, volume of soil surrounding roots and influenced chemically, physically and biologically by the plant root, is a highly favourable habitat for the proliferation of microorganisms and exerts a potential impact on plant health and soil fertility (Castro-Sowinski et al. 2007). This zone is rich in nutrients when compared with the bulk soil due to the accumulation of a variety of plant exudates, such as amino acids and sugars, providing a rich source of energy and nutrients for bacteria (Shukla et al. 2011). The rhizosphere is the habitat for diverse range of microorganisms, and the bacteria colonizing this habitat are called rhizobacteria (Chaparro et al. 2013). They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turnover and sustainable for crop production (Beneduzi et al. 2012). Bacterial inoculants that help in plant growth are of two types: (a) symbiotic and (b) free-living (Ahmad et al. 2008). Plant growth-promoting rhizobacteria (PGPR) are free-living, soilborne bacteria, isolated from the rhizosphere, which enhance the growth of the plant and reduce the damage from soilborne plant pathogens when applied to seeds or crops.

PGPR can affect plant growth by various direct and indirect mechanisms (Vacheron et al. 2013), which can be acting simultaneously at different stages of the plants growth and are shown in Fig. 11.1. The direct mechanisms include their ability for nutrient supply (nitrogen, phosphorus, potassium and essential minerals) or modulating plant hormone levels (Figueiredo et al. 2016). The indirect mechanisms include the inhibitory effects of various pathogens on plant growth-producing antagonistic substances or by inducing resistance to pathogens and development in the forms of biocontrol agents, root colonizers and environmental protectors (Figueiredo et al. 2016). Therefore, utilizing PGPR is a new and promising approach for improving the success of phytoremediation of contaminated soils (Tak et al. 2013).

The direct mode of action of many PGPR is by increasing the availability of nutrients for the plants (Desai et al. 2011). This method involves solubilization of unavailable forms of nutrients, siderophore production and ammonia production.

Nitrogen is one of the most common nutrients required for the plant growth since it forms part of proteins, nucleic acids and other essential biomolecules. More than 80% of nitrogen is present in the atmosphere but is unavailable to plants. It needs to be converted into ammonia, and biological nitrogen fixation involves this conversion by microorganisms using a complex enzyme system. The most studied PGPR

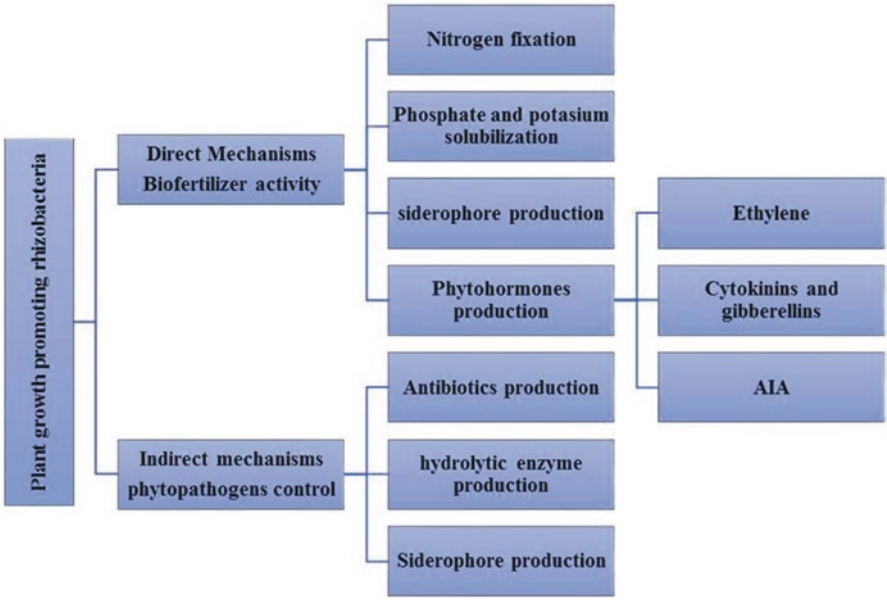


**Fig. 11.1** Direct and indirect mechanisms executed by PGPR that affect plant growth

are the rhizobia for their ability to fix nitrogen in their legume roots and *Azotobacter* and *Azospirillum* species that fix nitrogen in nonleguminous plants.

Phosphorus is the second mineral nutrient required for the growth of the plants, but it is used being in phosphate form, and there is small available amount of free phosphorus for the plant. Therefore, the use of phosphate-solubilizing microorganisms is very common. The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increase nutrient availability to host plants (Richardson 2001).

Iron is another essential nutrient of plants, but it is relatively insoluble in soil solutions. Iron exists in the oxidized ferric form ($Fe^{3+}$) at neutral pH (around 7) under aerobic conditions and forms various insoluble minerals; however, plant roots prefer to absorb iron as the more reduced ferrous form ($Fe^{2+}$). In response to this deficiency of iron, the microorganisms produce iron-binding siderophores that bind iron and can increase the availability of soluble iron in the soil surrounding the roots.

One of the direct mechanisms to promote plant growth is by production of phytohormones which include auxins, cytokinins, gibberellins, ethylene and abscisic acid (ABA). The indirect mechanisms include the production of inhibitory substances, which act against phytopathogens, increasing the natural resistance and releasing siderophores. Therefore, many PGPR increase the number and/or length of lateral roots (Combes-Meynet et al. 2011; Chamam et al. 2013) and stimulate root hair elongation. Consequently, the uptake of mineral sand water, and thus the growth of the whole plant, can be increased. Some of these effects, including

increased root and shoot biomass, are also documented for PGPR-inoculated plants growing in soil. Root is an organ with some distinct regions which have different roles. PGPR modify the root system architecture through their ability to interfere with the plant hormonal balance, as shown in Fig. 11.2. The hormonal balance includes cytokinin, ethylene, gibberellin, auxins and abscisic acid. The PGPR produce phytohormones and secondary metabolites which can affect these hormonal pathways (Vejan et al. 2016).

Auxins promote plant growth by different mechanisms such as cell enlargement, cell division, root initiation, root growth inhibition, increased growth rate, phototropism, geotropism and apical dominance. Indole-3-aceticacid (IAA) is the best-characterized auxin (Spaepen et al. 2007) produced by many plant-associated bacteria, including PGPR, which can stimulate primary root elongation or lateral roots or increase root hair formation depending of its concentration. The growth-promotion effect of auxin or auxin-like compounds by PGPR may require functional signalling pathways in the host plant.

Cytokinins are a class of phytohormones which are known to promote cell divisions, cell enlargement and tissue expansion in certain plant parts. Cytokinin production (especially zeatin) has been documented in various PGPR. Cytokinins can stimulate plant cell division, control root meristem differentiation and induce root hair proliferation (Riefler et al. 2006). Inoculation of plants with cytokinins has been shown to stimulate shoot growth.

Gibberellins (gibberellic acid) are a class of phytohormones most commonly associated with modifying plant morphology by the extension of plant tissue, particularly stem tissue. Gibberellins (GAs) constitute a large family of tetracyclic diterpenoid carboxylic acids, and some members operate as in higher plant growth hormones. They have been identified for the first time in 1926 in Japan as

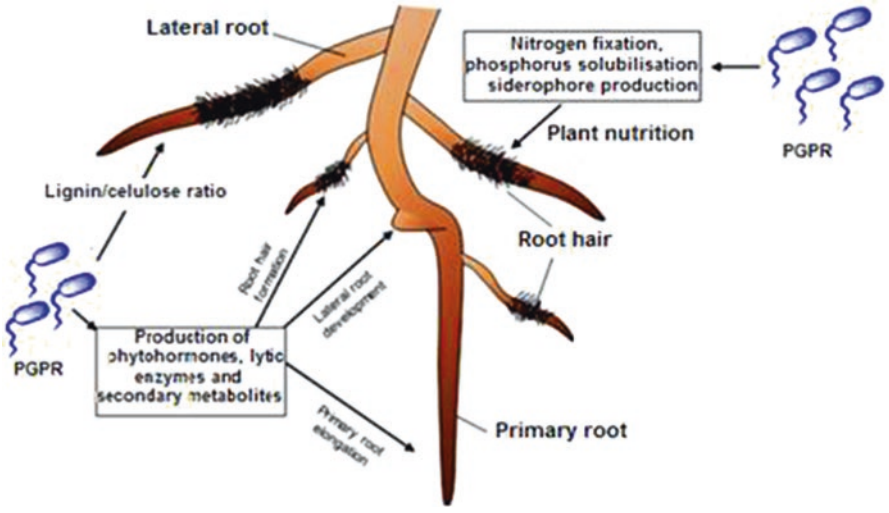


**Fig. 11.2** The influence of the PGPR on root development and growth

byproducts of the pathogenic fungus of rice *Fusarium fujikuroi* causing symptoms of overgrowth ("bakanae" disease) in rice seedlings. The compound GA3 was isolated for the first time by Japanese scientists, and this compound had the capacity to restore the normal growth of the dwarf mutant plants, leading to the suggestion that the GAs are natural plant hormones that regulate growth and development in higher plants (Tudzynski 2005). Gibberellins are associated with several processes of plant development such as germination, elongation of stems, flowering and fruit development (Gomi and Matsuoka 2003). They also promote the growth of roots, the abundance of root hairs and the delay on cellular ageing of plants. The effects of exogenous and endogenous gibberellins in the breaking of dormancy of seeds have been recognized in various species of plants; the application of gibberellins can replace the need for a specific temperature or light environmental stimulus. Two mechanisms of action of gibberellins in the germination process have been proposed: the first is its influence on the hydrolysis of food reserves, and the second mechanism of action consists of a direct effect on the growth potential of the embryo (Debeaujon and Koornneef 2000).There is little evidence of the gibberellin production by PGPR; however, there is a report providing evidence of the production of four gibberellins by *Bacillus pumilus* and *B. licheniformis* (Gutierrez-Manero et al. 2001).

Ethylene is the only gaseous phytohormone whose production in the plant can be induced by physical or chemical perturbation of plant tissues and is an important modulator of normal plant growth and development in plants as well as a key feature in the response of plants to a wide range of stresses. In relation with ethylene, the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase is responsible for the cleavage of the plant ethylene precursor, ACC, into ammonia and α-ketobutyrate to promote plant growth (Glick 2014).

PGPR inoculation increased the stress resistance and production of the crops, including tomato (Almaghrabi et al. 2013), lettuce (Kohler et al. 2009), wheat (Islam et al. 2014; Kumar et al. 2014), rice (Lavakush et al. 2014), soybean (Masciarelli et al. 2014), groundnut (Paulucci et al. 2015), broad bean (Younesi and Moradi 2014), maize (Rojas-Tapias et al. 2012) and chickpea (Patel et al. 2012). The increase in yields and other yield parameters can be different in different crops and environments and normally range from 25% to 65% (Mahmood et al. 2016).

## 11.3 *Bacillus* spp.: An Important Microorganism

*Bacillus* spp. have been widely used on biopesticide market around the world because of its capacity to produce many important products for food, pharmaceutical, environmental and agricultural industries with high impact in human activities. Recent studies have shown that these aerobic spore formers can produce fine chemicals with interesting biotechnological applications that open perspectives for new biotechnological applications of *Bacillus* and related species. The members of the genus *Bacillus* are often considered as microbial factories to produce a vast array of biologically active molecules, some of which are potentially inhibitory for fungal growth.

*Bacillus* species have a good secretion system and produce a variety of extracellular enzymes for the detergent, textile, food, feed and beverage industries. Among the enzymes of interest are amylases, pullulanases, β-glucanase employed in the brewing and bakery industries, β-galactosidase applied in beet sugar, pulp and paper industries, cellulases and xylanases in paper and pulp industry, chitinases used in food industry and esterases and lipases used in detergent industry.

*Bacillus* secondary metabolites include surfactants and bacteriocins among others. Lipopeptide biosurfactants are surface-active molecules that exhibit strong inhibition activity against several phytopathogens and have potential applications in agricultural, chemical, food and pharmaceutical industries. Some biosurfactants may be used as alternatives to synthetic medicines and antimicrobial agents (Shaligram and Singhal 2010). Their production is widely distributed among *B. subtilis*, *B. pumilus*, *B. licheniformis* (Tendulkar et al. 2007) and *B. amyloliquefaciens* strains (Wulff et al. 2002).

On the other hand, bacteriocins are proteins or ribosomal peptides with bactericidal activity towards species that are often closely related to the producer bacteria and display variable molecular weights, biochemical properties, inhibitory spectra and mechanisms of action. Although bacteriocin antimicrobial activity relies on pore formation, the spectrum of activity depends on the peptide; this observation implies that specific receptor molecules on the surface of target cells may generate differences in antimicrobial activity (Lee and Kim 2010). Bacteriocins are generally isolated from cultures under laboratory conditions. Several species of the *Bacillus* genus are bacteriocin producers, and there are some bacteriocins totally or partially characterized.

The polymers produced by microorganisms have been the subject of a growing interest because the problems originated by petroleum-based polymers on the environment. Biopolymers have broad areas of application ranging from food packaging to cosmetics and medicines as drug carriers. *Bacillus* spp. have been reported to produce polyglutamic acid (PGA), polylactic acid (PLA), polyhydroxyalkanoates (PHA) and exopolysaccharides (EPS).

Among *Bacillus* species, *B. thuringiensis* is the best known and best-studied entomopathogenic bacterium that produces parasporal protein crystals, which are selectively toxic to different species of several invertebrate phyla being safe to people, beneficial organisms and the environment. Microbial Bt biopesticides contain a mix of bacterial spores and δ-endotoxin crystals produced in fermentation tanks and formulated into solid powdery presentation or liquid sprays. The spore-crystal complex must be carried by suitable inert substance that can function to protect the spore-crystal complex or to increase availability to insects. Because of their high specificity and their safety for the environment, crystal proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture and forestry and in the home (Sansinenea 2012).

## 11.4 *Bacillus* spp. as Plant Growth-Promoting Bacteria

Multiple *Bacillus* spp. can be readily cultured from both bulk and rhizosphere soil on solid medium. Several *Bacillus* species have been identified as plant growth-promoting bacteria since they suppress pathogens or otherwise promote plant growth. Improvements in plant health and productivity are mediated by three different ecological mechanisms: production of antifungals that cause antagonism of pest and pathogens, secretion of compounds that promote the plant growth and stimulation of plant host defences inducing the plant systemic resistance (Lee et al. 2012).

The fungal antagonisms could be due by competition for niche and nutrients, stimulating the defensive capacities of the host plant and mainly by the production of antifungal compounds which seem to play an important role in the biological control of plant pathogens. Therefore, competition and antibiosis were the main mechanisms by which the *Bacillus* spp. inhibit the growth of phytopathogens (Živković et al. 2010). The mechanism of antibiosis between *Bacillus* spp. and other microorganisms has been shown to be a challenging topic. Microbial antagonism, commonly demonstrated by the development of a zone of inhibition between the two organisms when cultured together on a solid growth medium, is the basis for selecting microorganisms that produce antibiotics (Islam et al. 2012; Cawoy et al. 2015; Demain 2006). Some enzymes such as proteases, chitinases, glucanases, peptide antibiotics and small molecules can be secreted by various species, and many contribute to pathogen suppression. Peptide antibiotics and several other compounds toxic to plant pathogens have been recovered from several *Bacillus* strains (Yu et al. 2002). Many of antifungal compounds have been identified as mycobacillins, iturins, bacillomycins, surfactins, mycosubtilins, fungistatins and subsporins.

This has been exploited in the formulation of *Bacillus*-based products active against fungi. On the market, several *Bacillus*-based biofungicidal commercial products are available, based on *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus* and *B. subtilis*. They are employed to control fungal diseases, like root diseases (such as tomato damping-off, avocado root rot and wheat take-all), foliar diseases (cucurbit and strawberry powdery mildews) and postharvest diseases. In the next years, a great increase is expected in this field of application for biofungicidal bacilli. Few examples are AvoGreen and Biobest based on *B. subtilis* or Ballad Plus and Sonata based on *B. pumilus*.

*Bacillus amyloliquefaciens* is closely related to *B. subtilis* but is distinguished by its ability to form biofilms and to support plant growth and to suppress plant pathogens living in plant rhizosphere. It is successfully commercialized as biofertilizer by ABiTEP GmbH (Borriss 2011). *B. amyloliquefaciens* strains are distinguished by their potential to synthesize nonribosomally a huge spectrum of different secondary metabolites, many of them with antibacterial and/or antifungal action (Schneider et al. 2007).

*Bacillus* is also a producer of zwittermicin A, a potent antibiotic and antifungal compound. (+)-Zwittermicin A is a highly polar, water-soluble aminopolyol

antibiotic isolated from the soilborne bacterium *B. cereus* with significant activity against phytopathogenic fungi. The rising interest in zwittermicin A as a "green" biopesticide has stimulated studies of its unique biosynthesis, mechanism of action and organic synthesis (Sansinenea and Ortiz 2012). Zwittermicin A has been proven to be difficult to isolate in substantial quantities due to its highly polar, charged nature at physiological pH and sensitivity to alkaline conditions. Zwittermicin A was found to have a high activity against the oomycetes and their relatives, the algal protists, and a moderate activity against some Gram-negative bacteria and many plant pathogenic fungi such as *Alternaria*, *Fusarium*, *Helminthosporium* and *Ustilago* (Silo-Suh et al. 1998). Shang et al. reported that zwittermicin A provided the strongest inhibition of germination of the cysts and elongation of germ tubes in *Pythium torulosum*. Entomologists have identified and utilized diverse strains of entomopathogenic species that have potential for biological control.

Iron is an essential element for nearly all living systems. However, iron is not a freely available nutrient but exists in the oxidized ferric form ($Fe^{3+}$) at neutral pH (around 7) under aerobic conditions and forms various insoluble minerals. In response to this deficiency of iron, one of the most widely utilized mechanisms of microbial iron acquisition is the production and secretion of siderophores, low molecular weight iron chelators that bind ferric iron in the environment with extremely high affinity and shuttle it into the cells. Thus, siderophores act as extracellular solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation. Thus, the presence of siderophore-producing microorganisms in the rhizosphere contributes to plant health by complexing iron and making it less available to phytopathogens that are generally not able to produce comparable Fe-transport systems (Arguelles-Arias et al. 2009; Chen et al. 2009).

Plants and microorganisms abound with natural chemicals; many of them are volatiles. Ethylene, a molecule from other chemical development, was the first gaseous hormone discovered. Selected PGP *Bacillus* strains release a blend of volatile components (VOCs). The volatiles 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, released by PGPR *B. subtilis* GB03 and *B. amyloliquefaciens* IN937A, trigger enhanced plant growth by regulating auxin homeostasis (Ryu et al. 2003).

The well-documented plant growth-promoting effect by root-colonizing *Bacillus* (Kloepper et al. 2004) is at least partially due to the bacterial production of plant hormones such as IAA, cytokinins and gibberellins (Bottini et al. 2004). GC-MS verifies gibberellin production by *B. pumilus* and *B. licheniformis* (Gutierrez-Manero et al. 2001). Such compounds (eg. auxins, gibberellins, and cytokinins) play different roles in processes including plant cell enlargement, division, and enlargement in symbiotic roots and non-symbiotic roots as well.

Phytases are enzymes that sequentially remove phosphate groups from myoinositol1,2,3,4,5,6-hexakisphosphate(phytate), the main storage form of phosphate in plants. Besides their ability to make phytate phosphorus available, elimination of chelate-forming phytate, which is known to bind nutritionally important

minerals ($Zn^{2+}$, $Fe^{2+}$ and $Ca^{2+}$), is another beneficial effect of extracellular phytase activities of *Bacillus* spp. (Makarewicz et al. 2006).

The study of the processes that regulate the interaction between bacteria and plants is a recent and fundamental topic of biology since the bacteria can communicate by molecular signals through a process called quorum sensing (QS), and at the same time the plants have developed some mechanisms to receive these chemical signals. QS is a form of cell-cell communication between bacteria and plants mediated by small diffusible signalling molecules (autoinducers); these include acylated homo-serine lactones (AHLs) for Gram-negative bacteria and peptide-signalling molecules for Gram-positive bacteria. Plants respond to these signals in different ways. Plant-bacteria communication can take place through different compounds, some of which mimic the activity of endogenous phytohormones. Cyclic dipeptides and their derivatives, diketopiperazines, constitute a novel class of small molecules synthesized by microorganisms that have different biological functions such as antifungal, antibacterial or plant growth promoters.

Although the diketopiperazines are notable bioactive molecules, there is little information concerning its biosynthesis in bacteria and their role in communication with plants. There is a study which reports that diketopiperazines have an important role in the communication between cells called quorum sensing (Ortiz-Castro et al. 2011), modulating the auxin signalling to promote plant growth. The diketopiperazines consist of a ring containing two peptide bonds, and this cyclic structure has a great stability and resistance to human digestion. This last property allows that these dipeptides are used as scaffolding for drugs, besides having a series of interesting biological properties, including antiviral, antibiotic properties and antitumor activity. Some of these compounds are extracted from many both marine and terrestrial organisms and have proved to be promising for several routes in the pharmaceutical industry and with multiple functions (Arachchilage et al. 2012).

The induced resistance constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens exist (van Loon and Glick 2004). Plants can acquire enhanced level of resistance to pathogens after exposure to biotic stimuli provided by many different PGPRs. This is often referred to as rhizobacteria-mediated ISR. Induced resistance is a physiological "state of enhanced defensive capacity" caused by specific environmental stimuli, whereby the plant's innate defences are potentiated against subsequent biotic challenges. Besides ISR, there is another defined form of induced resistance so-called systemic acquired resistance (SAR), which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved. SAR can be triggered by exposing the plant to virulent, avirulent and non-pathogenic microbes (Choudhary and Johri 2009). Fewer published accounts of ISR by *Bacillus* spp. are available which showed that specific strains of the species *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides* and *B. sphaericus* can reduce the incidence or severity of various diseases on a diversity of hosts.

## 11.5 Conclusion

During the past century, industrialization of agriculture has provoked a significant and essential productivity increase, which has led to a greater amount of food available to the general population. Chemical fertilizers, herbicides and pesticides have resulted in negative environmental impacts, and one way is the use of plant growth-promoting rhizobacteria (PGPR). PGPR can alter root architecture and promote plant development. These organisms perform their positive effects in plants producing phytohormones like auxins, gibberellins, cytokinins and certain volatiles, siderophores, antifungals, fixing nitrogen and solubilizing phosphorous and other nutrients among others. The important role that PGPR play in agriculture can be clearly deduced from the extensive research published until now.

The knowledge of the mode of action of siderophore, lytic enzymes, antibiotic production and mechanism of quorum sensing and its activity of antagonistic bacteria could help in using successfully *Bacillus* spp. as a growth-promoting bacteria, since *Bacillus* spp. are spore-forming bacteria, and this causes a long-term viability that facilitates the development of commercial products. *Bacillus* spp. have been a very used bacterium as a biopesticide. It has been employed from different points of view. One of them is as a pesticide against insects that are pest of crops; another as antagonist of other microorganisms by antimicrobial (antifungals) secretion, thus avoiding damage to the plant; and finally as a promoter of plant growth and can even present the three possibilities together. The present review indicates the role of *Bacillus* spp. as PGPR with biological promotion of different characteristics of plant growth. Most of the PGPR isolates significantly increased plant height, root length and dry matter production in various agricultural crops like potato, tomato, maize, wheat, etc.

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# 12 Secondary Metabolites from Cyanobacteria: A Potential Source for Plant Growth Promotion and Disease Management

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## 12.1 Introduction

Cyanobacteria belong to the most diverse group of Gram-negative photosynthetic prokaryotes in terms of their morphology, physiology, and metabolism (Codd 1995). Due to its aerobic as well as anaerobic nature, cyanobacteria show rapid growths in different habitats. In eutrophic surface water, cyanobacteria are able to form intense blooms. This bloom-forming process can be caused by increased levels of nutrients, like phosphorus and nitrogen due to anthropogenic influence. Cyanobacteria have a number of special properties, like their ability to fix nitrogen using the enzyme nitrogenase (Ressom et al. 1994), and many of them also have the ability to form several toxic metabolites. Cyanobacteria contain five functional groups of toxins named cytotoxins, neurotoxins, hepatotoxins, dermatotoxins, and irritant toxins (lipopolysaccharides). In the aquatic ecosystem, with exception of the cytotoxic cylindrospermopsin, these toxins are mainly present within cyanobacterial cells but can be released in high concentrations during cell lysis (Saker and Grifiths 2000).

Cyanobacteria belong to the Gram-negative group of bacteria having properties of photolysis mediated evolving of oxygen. These are cosmopolitan prokaryotes that have been survived and boomed on the earth for over two billion years with the formation of oxygenic environment (Sergeeva et al. 2002). The most common cyanobacterial structures in the fossil record include stromatolites and oncolites (Herrero and Flores 2008). The fossil of oxygen producing stromatolites has been reported around 2.8 billion years ago (Olson 2006). Cyanobacteria can survive in almost every habitat such as from oceans to freshwater, soil to bare rocks, deserts to ice shelves, and hot springs to Arctic and Antarctic lakes as well as in the form of endosymbionts in plants, lichens, and several protists (Baracaldo et al. 2005). In some of these habitats, they form dominant microflora in terms of total biomass and

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productivity. Because of persistent survival in varied habitats, cyanobacteria display a range of secondary metabolites, each with specific purpose to compete successfully for their sustenance on the planet. Several species of cyanobacteria produce photoprotective metabolites such as scytonemin and mycosporine-like amino acids (MAAs) that play significant role in screening of ultraviolet radiation (Sinha and Häder 2008). It produces various enzymes such as superoxide dismutase, catalase, and peroxidases. The production of scavengers such as vitamins B, C, and E as well as cysteine and glutathione is also observed which quench or scavenge UV-induced excited states and reactive oxygen species (ROS) (Vincent and Quesada 1994). Biochemically active (bioactive) metabolites have been also studied in marine and freshwater as well as in extensive and intensive aquaculture systems. In diverse array of cyanobacterial secondary metabolites, there are certain groups which cause undesirable tastes and odors (Smith et al. 2008). The odorous metabolites are produced by certain cyanobacteria from marine and freshwater habitats. These are harmful for several organisms including humans by means of alliterating the quality of drinking water and recreational activities (Dittmann and Wiegand 2006). The examples of toxic metabolites include compounds such as microcystin, anatoxin, and saxitoxin, which exhibit hepatotoxicity and neurotoxicity (Karl and Cyril 2008). Cyanobacterial toxins also show allelochemical properties, and their applications such as algaecides, herbicides, and insecticides have been also investigated. These allelochemicals (e.g., microcystin, lyngbyatoxin A, cyanobacterin, etc.) could also involve in defense against potential predators and grazers (Berry et al. 2008). The ability of cyanobacteria to synthesize numerous complex secondary metabolites such as peptides, depsipeptides, polyketides, alkaloids, etc. has fascinated the researchers for their pharmaceutical and biotechnological exploitations (Thajuddin and Subramanian 2005; Sielaff et al. 2006; Spolaore et al. 2006). These compounds may be exploited as drug leads, mainly formed through large multimodular nonribosomal peptide synthetase (NRPS), polyketide synthase (PKS), and mixed NRPS-PKS enzymatic systems (Wase and Wright 2008). Several indole alkaloids have been reported, from simple carbolines such asbauerines and nostocarboline as well as from complex polycyclic structures such as hapalindole, welwitindolinone, and ambiguine in cyanobacteria (Van Wagoner et al. 2007). Few cyanobacteria also lead to the production of iron chelators (siderophores) such as schizokinen, synechobactin, and anachelin. The protease inhibitors such as cyanopeptolins, micropeptin, and oscillapeptin from certain cyanobacteria and their selectivity for trypsin/chymotrypsin have also been described. In the present scenario, cyanobacteria are recognized as a potential source of toxins as well as novel bioactive compounds with pharmaceutical applications (Raja et al. 2008; Abed et al. 2009) as several compounds are demonstrated to have antibacterial, antiviral, antifungal, algicide, and cytotoxic activities (Rao 1994; Issa 1999; Schlegel et al. 1999; Schaeffer and Krylov 2000).

Research activities involving investigations on plant metabolites and metabolites from other groups of organisms were undertaken not only for a better understanding of their nature but also to discover new metabolites for possible use in humans for different fields of interest. And the common way to discover biologically active

metabolites is to screen the extracts or isolate compounds from different natural sources. In the context of these research activities, microalgae, for example, cyanobacteria, were regarded to be a rich source for various metabolites of pharmaceutical or toxicological interests like primary metabolites such as proteins, fatty acids, vitamins, or pigments (Borowitzka 1995) and secondary metabolites with different bioactivities (antifungal, antiviral, antibiotic, and others) or cyanotoxins like the hepatotoxic nodularins and microcystins or the neurotoxic like saxitoxins and anatoxins (Carmichael 1992; Rinehart et al. 1994). Most of the cyanobacterial metabolites are accumulated in the cyanobacterial biomass. Moreover, cyanobacteria too excrete various organic compounds into their environment.

## 12.2 Cyanobacterial Secondary Metabolites

Cyanobacteria secondary metabolites are low molecular weight organic molecules which are not essential for normal growth, development, and reproduction of organism. They facilitate to face stress environment and reproductive process. Tremendous increase in the discovery of secondary metabolites is due to the use of analytical techniques like advanced ultra-performance liquid chromatography, which can be a better option than high-performance liquid chromatography. These secondary metabolites are associated with toxic, hormonal, and antimicrobial effects (Patterson et al. 1994). Some of these too take part in the treatment or prevention of multitude biological disorders. Many of the deadly diseases did not have any cure until these products were discovered. Secondary metabolites are commonly divided into structural classes related to their biosynthesis. This classification has its limitations because a number of compounds have building blocks from more than one biosynthetic pathway and some compounds that appear closely related can have completely different biosynthetic origins. The important classes of cyanobacterial secondary metabolites are the polyketides and non-ribosomal peptides. The other structural classes are alkaloids, terpenoids, shikimate-derived molecules, and amino glycosides (Davies and Ryan 2011). Secondary metabolites in cyanobacteria confer an evolutionary benefit to the producing organism. In the simplified environment of the laboratory, cyanobacteria often do not depend on the entire capabilities of their secondary metabolome, and thus the products of most of the biosynthetic gene clusters could not be observed. Improvements in de novo genome sequence technologies have resulted in a dramatic increase in the number of complete genomes available for well-known producers of natural products. These data have revealed that many members of these groups produce only a small fraction of the natural products encoded by their genomes under the standards of laboratory conditions. The biosynthetic pathways of natural product that are not often expressed are referred to as the "silent metabolome," therefore, potentially representing a vast reservoir of undiscovered small molecules. Epigenetic enzymes like histone deacetylases (HDACs) and DNA methyl transferases (DNMTs) play a crucial role in gene regulation of biosynthesis clusters (Schmitt et al. 2011). A recently studied approach is genome mining which is used to discover natural product, while it is also possible

to recognize the biosynthetic gene cluster from genome sequence data for a known compound produced by a microorganism. But, the converse approach of predicting the exact structure of a natural product from sequence data is often not possible. The factors which lead to this problem are complexity in forecast of post-assembly, modification, ambiguous cyclization patterns, biosynthetic domain skipping, and non-colinearity of few biosynthetic enzymes. Although bioinformatics tools are available to analyze genome data, identify biosynthetic clusters of natural product with a low level of accuracy, to predict the structure of the encoded compound which concludes that there is room for significant advancement in this field. There are possibilities to identify silent gene clusters in natural product produced by microorganisms through subtractive analysis by comparing the observed compounds to predicted biosynthetic pathways using existing bioinformatics tools (Schmitt et al. 2011). In recent time, mass spectrometry based on metabolomics has come forward as an efficient tool for the recognition of metabolites in complex biological systems as well as identification of novel metabolites.

## 12.3 Role of Cyanobacterial Secondary Metabolites in Plant Diseases Management

Ethanol extracts of the blue green alga *Anabaena circinalis* exhibit antimicrobial activity against the fungus *Aspergillus flavus*. The other blue green algae *Nostoc muscorum* has wide range of activities on both Gram-positive and Gram-negative bacteria in addition to the fungus *A. flavus* (Shaieb et al. 2014). Aqueous, methanol, n-propanol, and petroleum ether extracts of 40 cyanobacterial isolates belonging to 9 genera had been earlier examined showing inhibitory activities against five fungal plant pathogens, *Aspergillus flavus*, *Aspergillus niger*, *Colletotrichum musae*, *Fusarium oxysporum*, and *Paecilomyces lilacinus* (Pawar and Puranik 2008). In an experiment, it has been reported that the aqueous extract of one of the dominant species of cyanobacteria *Spirulina platensis* demonstrates antifungal activity against the fungus *A. flavus* (Shaieb et al. 2014). In vitro and in vivo fungal growth, spore sporulation, and fungal infection of the wilt pathogen in tomato seeds were significantly inhibited by cyanobacterial extracts. *Nostoc commune* FA-103 extracts showed the potential to suppress *Fusarium oxysporum* f. sp. *lycopersici* (Kim 2006). Algae are one of the chief biological agents that have been studied for the control of plant pathogenic fungi, particularly soilborne pathogens (Hewedy et al. 2000). *Anabaena* spp. (Moore et al. 1986; Frankmolle et al. 1992), *Scytonema* spp. (Chetsumon et al. 1993), and *Nostoc* spp. (Bloor and England 1989) were shown to be efficient in the control of damping-off as well as the growth of the soil fungus *Cunninghamella blakesleeana*. The aqueous extract from cyanobacteria and algae cells when applied to seeds showed protection from damping-off fungi such as *Fusarium* sp., *Pythium* sp., and *Rhizoctonia solani* (Kulik 1995). In a previous study, Kim (2006) reported antifungal activities in 29 strains of the 298 microalgal strains tested. *Nostoc commune* FA-103 was selected as the subject of this study because of its broad-spectrum antifungal activity on plant pathogenic fungi,

especially *F. oxysporum* f. sp. *lycopersici* (Borowitzka 1995). They reported that the extracts of *Nostoc muscorum* significantly inhibit the growth of *Candida albicans* and *Sclerotinia sclerotiorum*. Nonetheless, Kulik (1995) reported that the growth of *R. solani* on PDA was significantly inhibited by using *N. muscorum* extract. The maximum inhibition of *Fusarium* growth in soil was 81% with *Anabaena flosaquae*. In addition, the growth activities of *F. oxysporum* f. sp. *betae*, *F. oxysporum* f. sp. *lycopersici*, and *F. oxysporum* f. sp. *vasinfectum* were strongly inhibited with increasing concentration of cyanobacterial extracts (Moussa and Shanab 2001). In vitro and in vivo growth, sporulation, and sclerotial production were significantly inhibited with *Nostoc muscorum*. In vivo studies showed that *F. oxysporum* was very sensitive to cyanobacteria species *Nostoc muscorum* filtrates. They have potential for the suppression of phytopathogenic fungi such as the sugar beet pathogens *Fusarium verticillioides*, *Rhizoctonia solani*, and *Sclerotium rolfsii* (Rizk 2006). Abo-Shady et al. (2007) also reported that cyanobacteria filtrates strongly inhibit the phytopathogenic fungi isolated from leaves, stems, and roots of Faba bean. Mycelial growth of several plant pathogenic fungi such as *Fusarium oxysporum*, *Penicillium expansum*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Verticillium albo-atrum* was inhibited by the methanol extracts of the cyanobacterium *Nostoc* strain ATCC 53789 (Biondi et al. 2004). The reduced disease severity coupled with improved plant growth elicited by cyanobacterium *Anabaena* spp. treatments illustrated the utility of such novel formulations in integrated pest and nutrient management strategies for *Fusarium* wilt challenged tomato crop (Prasanna et al. 2013). Biological control of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) causing wilt disease in tomato was studied in vitro as well as under pot conditions. Methanol extract of *Nostoc linckia* and *Phormidium autumnale* showed moderate and minor zone of inhibition (33.3% growth inhibition). In spite of all these investigations and researches, more efforts are required in search of more strains of cyanobacteria including genetically modified strains to ensure maximum production of the desired products (Table 12.1).

## 12.4 Role of Cyanobacterial Secondary Metabolites in Plant Growth Promotion

Cyanobacteria generally known as BGA (blue-green algae) is not a true eukaryotic algae; it is a Gram-negative prokaryotes that are able to perform nitrogen fixation and oxygenic photosynthesis. It can easily grow in ponds, lakes, rivers, and any other wetlands. This BGA has the quality to improve soil fertility and enhance plant growth. Cyanobacteria are a rich source of enzymes, fibers, carbohydrates, proteins, vitamins, etc. Among all vitamins, the most abundant are vitamins A, C, B1, B2, and B6 and niacin, and the minerals like iron, magnesium, iodine, potassium, and calcium are commonly found in BGA. The proper use of particular cyanobacterial strain in agriculture purpose shows beneficial effect on crop production (Higa and Wididana 1991). Cyanobacteria enhance plant growth by some different mechanisms such as fixing atmospheric nitrogen and producing plant beneficial hormones,

**Table 12.1** Biocidal activity of cyanobacteria against plant pathogens

| Sl. No. | Cyanobacteria | Extract | Plant pathogens | References |
|---|---|---|---|---|
| 1. | *Fischerella muscicola* | Fischerellin | *Uromyces appendiculatus* (brown rust), *Erysiphe graminis* (powdery mildew), *Phytophthora infestans*, and *Pyricularia oryzae* (rice blast) | Hagmann and Juttner (1996) |
| 2. | *Nostoc muscorum* | aBis (2, 3-dibromo-4, 5-dihydroxybenzyl) – BDDE | *Sclerotinia sclerotiorum* (cottony rot of vegetables and flowers) and *Rhizoctonia solani* and *Candida albicans* | Borowitzka (1995) |
| 3. | *Tolypothrix byssoidea* | Antifungal peptides dehydrohomoalanine (Dhha) | Antifungal activity against the yeast *Candida albicans* | Jaki et al. (2001) |
| 4. | *Oscillatoria redekei* syn. *Limnothrix redekei* HUB 051 | Antibacterial fatty acids a-dimorphecolic acid, a 9-hydroxy-10E, 12Z-octadecadienoic acid (9-HODE), and coriolic acid | Inhibited the growth of the Gram-positive bacteria *Bacillus subtilis* SBUG 14, *Micrococcus flavus* SBUG 16, and *Staphylococcus aureus* SBUG 11 and ATCC 25923 | Mundt et al. (2003) |
| 5. | *Nostoc* sp. | Cryptophycin | Natural pesticides against the fungi, insects, and nematodes | Biondi et al. (2004) |
| 6. | *Anabaena subcylindrica*, *Nostoc muscorum*, and *Oscillatoria angusta* | Efficient algal filtrate concentration (EAFC) | *Alternaria alternata*, *M. phaseolina*, and *F. solani* | Abo-Shady et al. (2007) |
| 7. | *Spirulina platensis*, *Oscillatoria* sp., and *Nostoc muscorum* | | *Cercospora beticola* causing leaf spot of sugar beet | Mostafa et al. (2009) |
| 8. | *Calothrix elenkenii* | Ethyl acetate extract | *Pythium aphanidermatum* | Manjunath et al. (2010) |

(continued)

**Table 12.1** (continued)

| Sl. No. | Cyanobacteria | Extract | Plant pathogens | References |
|---|---|---|---|---|
| 9. | *Lessonia trabeculata* | Ethanolic extracts | Reduced number and size of the necrotic lesion in tomato leaves following infection with *Botrytis cinerea* | Jimenez et al. (2011) |
| 10. | *Gracilaria chilensis* (red algae) | Aqueous or ethanolic extracts | *Phytophthora cinnamomi* | |
| 11. | *Durvillaea antarctica* | Crude extracts | Tobacco mosaic virus (TMV) in tobacco leaves | |
| 12. | *Anabaena variabilis* RPAN59 and *A. oscillarioides* RPAN69 | Antifungal | *Pythium debaryanum*, *Fusarium oxysporum lycopersici*, *F. moniliforme*, and *Rhizoctonia solani* | Chaudhary et al. (2012) |
| 13. | *Anabaena variabilis*, *S. platensis*, and *Synechococcus elongatus* | Butanol extract | *Aspergillus niger* and *Alternaria solani* | Tiwari and Kaur (2014) |
| 14. | *Nostoc muscorum* and *Oscillatoria* sp. | Norharmane and α-iso-methyl ionone | *Alternaria porri* (purple blotch of onion) | Abdel-Hafez et al. (2015) |

vitamins, and enzymes (Higa 1991). The fixed nitrogen may release in the form of polypeptides, auxin-like substances, ammonia, free amino acids, or vitamins (Subramanian and Sundaram 1986). More particularly, the hormones that are released by cyanobacteria are abscisic acid (Marsalek et al. 1992), auxin (Ahmad and Winter 1968), cytokinin (Rodgers et al. 1979), gibberellins (Singh and Trehan 1973), and vitamin B in particular (Grieco and Desrochers 1978). Most studied cyanobacterial effect on plant growth was paddy and wheat, where the use of BGA helped in increasing the germination rate, root and shoot growth, and chlorophyll content in both crops' growth (Misra and Kaushik 1989a, b; Obreht et al. 1993). Plant growth promotion activities of cyanobacteria were first observed in rice and wheat crops. In 1995, Likhitkar and Tarar observed that the total length of plants, seedlings, radicals, and dry weight was significantly increased in *N. muscorum*-treated cotton seed. Similar results were observed by Adam in 1999 when lentil, maize, sorghum, and wheat seeds were soaked in live inoculums, and boiled algal extract or filtrate extract of *N. muscorum*, *N. calcicola*, and *Anabaena vaginicola* from Iranian terrestrial helped in promoting growth in several herbaceous plants, vegetables including *Satureia hortensis*, *Cucumis sativus*, *Mentha spicata*, *Cucurbita maxima*, and *Solanum lycopersicum* (Shariatmadari et al. 2013; Hashtroudi et al. 2013). Another morphological and biochemical parameters were tested by Haroun and Hussein (2003) in *Lupinus termis* when treated with *A. oryzae*

and *Cylindrospermum muscicola* extracts. Culture filtrates of *Cylindrospermum* increase nitrogenous compound contents, photosynthetic activity, and carbohydrate in plants. Some cyanobacteria secrete some components that were attributed to gibberellic acid. It was also known to inhibit chlorophyllase activity, and for this reason, both chlorophyll a and b and total chlorophyll and total pigments increased (Martinez et al. 1996). Osman et al. (2010) analyzed some protein bands through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and it was observed that there was a change in the gene expression pattern after cyanobacterial treatment in plants. A huge number of cyanobacterial species like *Nostoc muscorum*, *Anabaena variabilis*, *Tolypothrix tenuis*, and *Aulosira fertilissima* are considered as an efficient biofertilizers. Some cyanobacterial strains like *Nostoc* and *Anabaena* are able to colonize in rocks and soil and have nitrogen fixation capability of up to 20–25 kg/ha, and *Aulosira*, *Anabaena*, *Tolypothrix*, etc. are also used as inoculums for rice crop. The second most important macronutrient for plant growth is phosphorus. Cyanobacteria can produce phosphatise enzymes that help in solubilization of the organic phosphorus and help in plant growth. After death of cyanobacteria, the phosphate present in cell wall is released in soil and easily serves as a nutrient for the plants. Fuller and Roger (1952) observed that phosphorus uptake was significantly increased in the algal-treated plant compared to untreated plants. A study of Rogers and Burns (1994) showed that the cyanobacteria inoculums improved the water holding capacity and aeration of soil that helped in improving soil fertility and increasing plant growth (Table 12.2).

## 12.5 Cyanobacterial Extract in Defense Activation Against Biotic and Abiotic Stresses

Cyanobacteria are ubiquitous in nature as they are found in saline water, marine water, and freshwater and terrestrial environments and having symbiotic association with plants, animals, protista, etc. (Gupta et al. 2013). They are known to produce various bioactive compounds, and their utilization as biological agents showed them as best antiviral, antifungal, antibacterial, and anti-inflammatory properties which have promising application in agriculture, food, and various industries. The role of cyanobacterial extract of *Calothrix elenkenii* was tested against *Pythium aphanidermatum* and found potential inhibitor of pathogenic fungi by treating seeds of some vegetable crops with ethyl acetate extract (Manjunath et al. 2010). Some other researchers also shed their ideas in improving nutrient uptake that leads to enhancing defense enzyme activities in plants. Various cyanobacterial strains such as *Anabaena variabilis* RPAN59 and *A. laxa* RPAN8 are good in defense enzyme expression and fungicidal and hydrolytic enzymatic activities (Prasanna et al. 2013). Defense enzymes, viz., polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL), and pathogenesis-related enzymes like β-1,3 glucanase and chitosanase were observed to be highest in the roots of a 14-day-old tomato seedlings under the action of cyanobacterial strains. And they further stated that the highest correlation of defense enzymes and hydrolytic enzymes is associated with

**Table 12.2** Cyanobacterial metabolites and their mechanism in plant growth promotion

| Secondary metabolites | Cyanobacteria | Mechanism | Reference |
|---|---|---|---|
| Cytokinins | *Chroococcidiopsis*, *Anabaena*, *Anabaenopsis*, *Cylindromum*, etc. | Nitrogen fixation | Hussain and Hasnain (2011) |
| 6-benzyl adenine (6-BA) Thidiazuron (TDZ) Kinetin (KIN) | *Anabaena* | Increase the organic matter of the soil and nitrogen fixation | El-Bahbohy et al. (2014) |
| Phytohormones | *Acutodesmus* | Enhanced plant growth, biostimulant, nitrogen fixation | Garcia-Gonzalez and Sommerfeld (2016) |
| IAA | *Anabaena*, *Oscillatoria*, *Synechocystis* | Nitrogen fixation | Bergman et al. (1997) |
| Auxin | *Anabaena*, *Plactonema*, *Chlorogloeopsis*, *Cylindrospermum*, *Glactothece*, *Synechocystis*, *Anabaenopsis*, *Calothrix*, *Nostoc*, etc. | Plant growth promotion | Ahmad and Winter (1968), Mohan and Mukherji (1978), Selykh and Semenova (2000), Sergeeva et al. (2002) |
| Gibberellins | *Anabaenopsis*, *Cylindromum* | Plant growth promotion | Mohan and Mukherji (1978) |
| Vitamin B12 | *Cylindrospermum*, *Tolypothrix*, *Nostoc*, *Hapalosiphon*, etc. | Plant growth promotion | Venkataraman and Neelakantan (1967), Okuda and Yamaguchi (1960), Misra and Kaushik (1989a) |

phosphorus uptake, whereas the nitrogen uptake was highly correlated with hydrolytic enzyme production (Prasanna et al. 2013). Priya et al. (2015) showed utilization of cyanobacterial strain *Calothrix elenkenii* in flooded rice field which resulted increase in plant growth as well as enhancement in some plant defense enzyme expression levels. The tropical spray and root application of such strains also resulted in increased accumulation of phytochemicals such as glucosinolates, alkaloids, terpens, polyphenols, etc. Cyanobacteria not only act against biotic stress, but their association in salt- affected soils is also well studied (Apte and Bhagwat 1989; Singh and Dhar 2010). Multiple approaches are made by cyanobacteria in the regulation of immune responses against salt stress (Pandhal et al. 2009; Nikkinen et al. 2012). Their colonization in association with plant helps them to act against stressed soil condition by producing diverse biologically active metabolites in soil and thereby inducing systemic acquired responses by combating abiotic stresses. In

order to maintain physiological properties of plants in salt stress condition, the application of cyanobacteria upregulates the phytohormone producing genes associated with cytokinin, indole-3-acetic acid (IAA), and gibberellic acid (GA) production which plays a major role in stabilizing the growth (Singh 2014). The cytokinin and IAA production were observed in rice roots under the influence of the endophytic *Nostoc* (Hussain et al. 2013). The *Oscillatoria angustissima*, *Cylindrospermum* sp., and *Anabaenopsis* sp. produce gibberellin-like substances and provide the phytohormonal signaling under stress conditions (Tsavkelova et al. 2006). The production of salicylic acid, jasmonic acid, and its various metabolites plays effective role in regulation of immune responses to abiotic and biotic stresses (Khan et al. 2012). The antioxidant production, viz., superoxide dismutase and peroxidase, by the application of cyanobacterial extract in rapeseed and rice was demonstrated by Chen et al. (2004). Because of its ability to promote growth and production of defense response (Grzesik et al. 2017) assessed the application of *Microcystis aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120 (cyanobacteria), and *Chlorella* sp. (green algae) singly for their utilization as foliar biofertilizers in order to improve plant growth and yield through enhanced physiological performance of the plants.

## 12.6 Conclusion

The use of cyanobacterial products can provide us a better future by limiting the use of inorganic chemical products for the management of plant pathogens which are causing prominent diseases in agriculture crops. These cyanobacterial extracts are having more potential in battling against the biotic and abiotic stress responses by activating the defense enzymes to provide resistance response in plants to withstand various stresses.

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# 13 Biological Control of Nematodes by Plant Growth Promoting Rhizobacteria: Secondary Metabolites Involved and Potential Applications

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## 13.1 Introduction

Plant-parasitic nematodes are one of the most destructive agronomic pests. Because of their nature, nematodes are difficult to manage and detect, as the appearance of affected crops can resemble other pathogenic diseases or nutrient deficiency. Current estimates put crop losses to nematodes worldwide, of at around USD 157 billion per year (Singh et al. 2015). For several decades, the control of plant-parasitic nematodes on agricultural crops has depended on chemical pesticides. These chemicals are in general very toxic with high potential to pollute the environment. Specifically, methyl bromide, which was widely used as soil fumigant from the 1960s, was shown to contribute to the depletion of the ozone layer. As a result, its use was banned under the Montreal Protocol in 2005 (Meadows 2013). Since then, research into alternate products has become a priority. In this context, the biological control agents have arisen as an environmentally friendly alternative (Beneduzi et al. 2012).

Rhizobacteria and nematode populations cohabit in plant root systems. These organisms affect each other's functioning along with the health of the plants whose rhizosphere they colonize (Singh et al. 2016, 2017). Several rhizobacterial strains are able to control nematode populations using different mechanisms of action, improving plant health and yield. For example, *Pasteuria penetrans* is a nematode parasite that can control *Meloidogyne incognita* on tomato and cucumber and *M. arenaria* on Snapdragon (Kokalis-Burelle 2015); *Bacillus nematocida* can control nematodes by producing extracellular proteases that can destroy their cuticles (Niu et al. 2006), and *B. thuringiensis* produce

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Cry proteins that are toxic to these phytopathogens (Bravo et al. 2007). In all cases, the production of virulence factors is vital for the performance of bacterial biological control activity. These factors are secondary metabolites.

Bacteria produce a wide range of secondary metabolites that have several important ecological functions, for example, stimulating competition against other bacteria or eukaryotic organisms, operating as metal transporting agents or as facilitators of symbiotic relations with other organisms (Demain and Fang 2000). The aim of this chapter is to review the secondary metabolites produced by rhizospheric bacteria that have been identified as being involved in the control plant-parasitic nematodes. In general, secondary metabolites can act directly or indirectly on nematode populations. Direct mechanisms affect nematode integrity through the production of lytic enzymes, toxins, gases, volatile organic compounds, and other metabolites or through indirect mechanisms inducing other rhizospheric factors that can reduce the nematode population helping the plant overcome the infestation.

## 13.2 Secondary Metabolites with Direct Nematocidal Activity

### 13.2.1 Lytic Enzymes: Chitinases, Proteases, and Glucanases

Among the secondary metabolites produced by rhizobacteria, lytic enzymes have attracted the attention of scientists since the initiation of research into the biological control of nematodes (Miller and Sands 1977; Galper et al. 1990). Lytic enzymes are an attractive proposition because nematodes have a very simple structure with an outer cuticle of keratin and collagen-like proteins that function not only as a skin but also as an exoskeleton maintaining and defining the shape of the organism (Johnstone 1994). In the same way, nematode egg shells are composed mainly of chitin fibrils inserted in a protein matrix, with the chitin complex as the major barrier against fungal infections (Wharton 1980). Extracellular enzymes that digest the main chemical components of the nematode cuticle and eggshell have been studied in potential nematode control bacteria (Tian et al. 2007; Yoon et al. 2012; Yang et al. 2013). Table 13.1 summarizes some examples of the reported rhizobacterial lytic enzymes with nematicidal activity.

Chitinases produced by *Lysobacter capsici* have been found to degrade the eggshell of *Meloidogyne* spp. causing a decrease in hatching (Jung et al. 2014). In a pot trial, *Streptomyces cacaoi* GY525 producing chitinase and β-1,3-glucanase inhibited hatching and caused mortality of *Meloidogyne incognita* J2 stages, reducing the population of J2 in soil and the number of nematode egg masses in tomato plant roots (Yoon et al. 2012). Similarly, El-Hadad et al. (2010) reported *Bacillus megaterium* strain PSB2-inhibited root colonization by *M. incognita* and caused 100% mortality of J2 stages. The authors detected high production of lytic enzymes like proteases, chitinases, and gelatinases by the isolate that could be considered virulence attributes.

**Table 13.1** Rhizobacterial lytic enzymes with nematicidal activity

| Rhizobacteria | Enzymes | Nematode | Reference |
|---|---|---|---|
| *Serratia marcescens Streptomyces griseus* | Chitinases | *Meloidogyne hapla* | Mercer et al. (1992) |
| *Paenibacillus illinoisensis* KJA-424 | Chitinases | *Meloidogyne incognita* | Woo Jin et al. (2002) |
| *Pseudomonas fluorescens* CHA0 | AprA extracellular protease | *M. incognita* | Siddiqui et al. (2005) |
| *Brevibacillus laterosporus* | Alkaline serine protease BGL4 | *Panagrellus redivivus* | Huang et al. (2005) |
| *Bacillus* sp. RH219 | Alkaline serine protease Apr219, neutral protease Npr219 | *Panagrellus redivivus* | Lian et al. (2007) |
| *Bacillus nematocida* B16 | Alkaline serine protease Bace16, neutral protease Bae16 | *Caenorhabditis elegans* | Niu et al. (2010) |
| *Streptomyces cacaoi* GY525 | Chitinases, glucanases | *M. incognita* | Yoon et al. (2012) |
| *Bacillus thuringiensis* | Metalloproteinase Bmp1 | *C. elegans* | Luo et al. (2013a, b) |
| *Lysobacter capsici* YS1215 | Chitinases, proteases | *M. incognita* | Lee et al. (2014) |
| *Bacillus firmus* DS-1 | Sep 1 serine protease | *M. incognita*, *C. elegans*, soybean cyst nematode | Geng et al. (2016) |
| *Alcaligenes faecalis* ZD02 | Extracellular serine protease | *M. incognita*, *C. elegans* | Ju et al. (2016) |
| *P. fluorescens* FP805PU | Collagenase, chitinases, lipases | *Xiphinema index* and *M. ethiopica* | Aballay et al. (2017) |
| *Brevibacterium frigoritolerans* FB37BR | Collagenase, proteases, chitinases, lipases | | |

Proteases have also been widely studied in nematode antagonistic bacteria, especially serine and cysteine proteases. The extracellular alkaline serine protease BLG4 from *Brevibacillus laterosporus* has been very well characterized as virulence factor; BGL4-deficient mutants were 57% less effective than the wild strain at controlling nematodes (Tian et al. 2006). Serine proteases with the ability to degrade nematode cuticles from other rhizobacteria and nematophagous fungi have shown a high percent similarity (97–99% sequence match) to those of *Brevibacillus* (Tian et al. 2006). This fact suggests that these proteases are highly conserved across microbial species. The role of proteases in the biological control of nematodes was also demonstrated by Siddiqui et al. (2005) using mutants of *Pseudomonas fluorescens* CHA0 for the gene *apr A* which encodes an extracellular protease with nematicidal activity. Mutants had no nematocidal activity.

Other virulent proteases like extracellular alkaline serine protease Bace16 and neutral protease Bae16 have been described in different *Bacillus* species with a Trojan horse-like mechanism. Through this mechanism, once the rhizobacteria

reach the intestine of the worm, they secrete Bace16 and Bae16, both of which target vital intestinal proteins, killing the nematode (Lian et al. 2007; Niu et al. 2010). Combinations of diverse kinds of proteases may occur in the nematicidal spore-forming Bacilli group (Zheng et al. 2016) and in other rhizobacterial groups.

Even when phytoparasitic nematodes have a higher lipid content, few studies have focused on lipases as potential virulence factors. Castañeda-Alvarez et al. (2016) performed an in vitro study and reported strains belonging to *B. thuringiensis*, *B. megaterium*, and *B. amyloliquefaciens* with strong lipase activity that caused mortality of the nematode *Xiphinema index*; they also found that *B. megaterium* FB133M with no lipase activity displayed the lowest nematicidal effect. Other lytic enzymes like glucanases, cellulases, and pectinases from *Pseudomonas* spp. have been reported to be involved in the control of *M. incognita* (Krechel et al. 2002). However, their specific role has not been addressed as they are secreted together with proteases and other secondary compounds that can overlap in activity. In general, lytic enzymes play a crucial role in the rhizobacterial activity against nematodes due to their different mechanisms of action and the relatively simple physiology and structure of nematodes.

### 13.2.2 Cry Toxins from *Bacillus thuringiensis*

During sporulation, *Bacillus thuringiensis* strains produce endotoxins called Cry proteins which are toxic to a large number of insect species (Maagd et al. 2001). It has been found that some Cry proteins are toxic to plant-parasitic nematodes (Bravo et al. 2007; Guo et al. 2008). Fifty-four families of Cry toxins have been identified; among them Cry5, Cry6, Cry12, Cry13, Cry14, Cry21, and Cry55 have been described with nematicidal activity (Bravo et al. 1998; Marroquin et al. 2000; Frankenhuyzen 2009).

The mechanism of action reported for Cry proteins affecting nematodes is similar to the one described in insects. The toxin attaches to the epithelial cells of the nematode intestine inducing the formation of pores and vacuoles and ending with the degradation of the intestine (Marroquin et al. 2000). Iatsenko et al. (2014) reported two novel plasmid-encoded protoxins (Cry21Fa1 and Cry21Ha1) from *B. thuringiensis* DB27 that also display nematicidal activity.

### 13.2.3 Other Secondary Metabolites Produced by *Bacillus*

Among the wide range of bacteria described as active against nematodes, members of the *Bacillus* genus are the most thoroughly studied. Other secondary compounds from *Bacillus* strains (different than lytic enzymes and Cry proteins) have been reported with nematode control activity. Mendoza et al. (2008) reported that *B. firmus* produced unidentified metabolites during culture that significantly reduced egg hatch of *M. incognita* and controlled *Radopholus similis*. Similarly, dichloromethane-soluble metabolites produced by *B. cereus* and *B. subtilis* showed in vitro activity

against *M. exigua* J2; these compounds were identified by HPLC and mass spectrometry as uracil, 9H-purine, and dihydrouracil, the latter being the most effective (Oliveira et al. 2014).

The peptide plantazolicin, product of the gene RBAM_007470, was identified as the nematicidal factor from *B. amyloliquefaciens* strain FZB42 (Liu et al. 2013). Other nematode control-related metabolites produced by *B. cereus* strain S2 were identified by LC-MS as C16 sphingosine and phytosphingosine. Sphingosine induced reactive oxygen species in the intestinal tract of *C. elegans* and destroyed the genital area of the nematode with the consequent inhibition of reproduction (Gao et al. 2016).

In *B. thuringiensis*, Liu et al. (2010) reported a mechanism of action different from that of Cry toxins. The bacterium produces an adenine nucleoside derivative called thuringiensin (β-exotoxin) with insecticidal and nematicidal abilities that inhibits RNA polymerases by competing with the ATP molecule for binding sites. *B. thuringiensis* strains expressing thuringiensin can kill nematodes with a higher mortality rate than those not expressing the molecule (Zheng et al. 2016).

#### 13.2.3.1 2,4-Diacetylphloroglucinol (DAPG)

The polyketide antibiotic 2,4-diacetylphloroglucinol (DAPG) is produced by some strains of the plant growth-promoting rhizobacteria *P. fluorescens*. A DAPG-overproducing strain inhibited *M. incognita* gall formation on the root systems of mungbean, soybean, and tomato plants, whereas a mutant strain, DAPG-deficient, did not show such activity (Siddiqui and Shaukat 2003). It has been shown that DAPG does not affect all nematodes in the same way. A study by Meyer et al. (2009) found that DAPG exposure decreased the hatch of *M. incognita* eggs but had no effect on its J2 stage; it stimulated hatching of *C. elegans* eggs and was toxic to adults of *Xiphinema americanum*. However, other nematodes tested (*Heterodera glycines* eggs and J2, *Pristionchus scribneri* juveniles and adults, *Pratylenchus pacificus* eggs and adults, and *Rhabditis rainai* eggs and adults) were not affected by the metabolite.

Different authors have suggested that the biocontrol activity of phytoparasitic nematodes exerted by DAPG is due to synergistic action with other metabolites produced by the rhizobacteria, like HCN and pyoluteorin or inducing agents of systemic resistance in plant roots (Siddiqui and Shaukat 2003). DAPG alters the plasma membrane and vacuolization and causes cell content disintegration in fungi (de Souza et al. 2003), but its activity on nematodes is unknown.

#### 13.2.3.2 Gaseous Compounds: $H_2S$, $NH_3$, and HCN

Some gaseous compounds released by rhizobacteria mainly as a result of amino acid metabolism have been reported to be effective in the control of nematodes (McSorley 2011).

$H_2S$ can be produced in large amounts by some bacteria as the result of the metabolism of peptides rich in cysteine or other sulphurated amino acids or by the activity of sulfate-reducing bacteria (Carbonero et al. 2012). Early work of Rodriguez-Kabana et al. (1965) described a decrease in nematode populations due

to $H_2S$ formation in flooded soils resulting from the growth of sulfate-reducing bacteria on organic substrates. More recently, Marin et al. (2010) reported the PGPR strain *Tsukamurella paurometabola* C-924 had the potential to control plant parasitic nematodes through the release of $H_2S$ and chitinases.

Ammonia released by ammonifying bacteria during the breakdown of soil organic matter can result in reduced phytoparasitic nematode populations (Rodriguez-Kabana 1986). In this sense, the practice of amending soil with organic matter high in ammonia content like urea could increase the release of ammonia by rhizospheric bacteria, with consequent decrease in nematodes (McSorley 2011). The production of ammonia by rhizobacteria has been included among the strategies to select strains with biological control abilities as this compound can not only control nematodes, it could also serve as a nitrogen source for plants improving plant nutrition, enhancing yields, and triggering crop tolerance to phytoparasites (Mota et al. 2017).

Another gas that has been described by Siddiqui et al. (2006) as "an antagonistic factor that contributes to biocontrol of *Meloidogyne javanica*" is cyanide. Siddiqui et al. (2006) demonstrated a mutant of *P. fluorescens* CHA77, unable to produce cyanide, did not exert the nematicide activity that the wild strain exhibited. In the same way, *P. aeruginosa* PA01 caused irreversible paralysis of nematodes by releasing hydrogen cyanide (Gallagher and Manoil 2001). More recently, Nandi et al. (2015) performed a binary choice assay, where *C. elegans* were allowed to choose for grazing among colonies of *Pseudomonas chlororaphis* PA23 wild strain producing cyanide or the HCN nonproducer mutant. It was found that hydrogen cyanide, produced by *Pseudomonas chlororaphis* PA23, repelled *C. elegans* as the *hcn* mutant was preferred over the wild type.

#### 13.2.3.3 Volatile Organic Compounds (VOCs)

Volatile organic metabolites are usually lipophilic liquids with high vapor pressures. Due to their nature, they freely cross membranes and are released into the soil environment with little restrictions. Similarly, they can move with relative facility through the soil pores extending their area of action and reaching potential targets (Pichersky et al. 2006). Rhizobacteria can also produce volatile compounds that potentially are able to control nematodes; however, their nematicidal activity has only been studied in vitro or in pots probably due to the difficulties of managing these substances in the open field.

An assay performed using compartmented Petri dishes and a pot experiment showed that the VOCs producer *Bacillus megaterium* strain YMF3.25 significantly decreased egg hatching and reduce infection by *M. incognita*. Gas chromatograph/mass spectrometer analysis revealed at least six compounds that could be involved in the nematode control activity (Huang et al. 2010). In the same way *Lysinibacillus mangiferahumi*, isolated from mango rhizosphere soil, also exhibited nematicidal activity versus *M. incognita* through the production of VOCs (Yang et al. 2012).

More recently, Xu et al. (2015) reported five different bacterial strains that, when incubated independently in sealed Petri dishes with *C. elegans* or *M. incognita*, progressively reduced nematode movement until they stopped completely and

irreversibly at 24 h. The active compounds were identified as acetophenone, S-methyl thiobutyrate, dimethyl disulfide, ethyl 3,3-dimethylacrylate, nonan-2-one, 1-methoxy-4-methylbenzene, and butyl isovalerate.

#### 13.2.3.4 Lactic Acid and Amino Acids

In the search for active compounds for biological control, some other rhizobacterial secondary metabolites have been found with a direct nematicidal effect. *L. capsici* YS1215, isolated from soil by Lee et al. (2014), produced lactic acid (2-hydroxypropanoic acid) in culture medium that inhibited the egg hatching of *M. incognita*. In the same way, amino acids present in the culture media of *P. macerans* induced mortality of J2 stages of *Meloidogyne exigua* and reduced the nematode population in coffee plants to levels comparable to the chemical pesticide aldicarb (Oliveira et al. 2009).

## 13.3 Secondary Metabolites with Indirect Nematocidal Activity

### 13.3.1 Metabolites Inducing Nematode-Trapping Fungi

There are around 200 species of nematode-trapping fungi. They can develop specific structures like adhesive nets, branches, and mechanical trap rings like to capture, kill, and digest soil nematodes (Liu et al. 2009). Rucker and Zachariah (1986) found that different bacterial species can influence trap production by the fungi *Dactylaria brochopaga* and *Arthrobotrys conoides*.

Li et al. (2011) performed a bioassay to screen soil samples for trap-inducing bacteria using the fungus *Arthrobotrys oligospora*. They found 18 isolates able to induce fungal traps and identified induction activity was due to bacterial cells and their metabolites. Recently, Su et al. (2016) found that volatile organic compounds and ammonia released by bacteria can induce trapping structures on fungi. Similarly, Wang et al. (2014) reported urea as the metabolite produced by bacteria that can trigger the shift in *A. oligospora* from saprophytic to nematode-trapping form and ammonia as the signal molecule that initiates the lifestyle modification in the fungus.

### 13.3.2 Secondary Metabolites Involved in the Development of Induced Systemic Resistance (ISR) in Plants Against Nematodes

Rhizobacteria can suppress a disease, like a nematode infestation, by inducing a resistance mechanism in plants (van Loon et al. 1998). Induced systemic resistance (ISR) involves expression of defense-related genes and other compounds that help plants overcome pathogen attack. To trigger ISR, one or more bacterial metabolites need to be recognized by root cell receptors (Beneduzi et al. 2012). Several

ISR-inducing compounds have been identified: lipopolysaccharides, siderophores (Van Loon et al. 1998), flagella, N-acyl-homoserine lactones, antibiotics, and exopolysaccharides (Vleesschauwer and Höfte 2009).

The induction of systemic resistance against nematodes is one of the approaches that has been studied in the search for environmentally friendly alternatives for their control. *Bacillus subtilis* triggered ISR in eggplants inhibiting *M. javanica* infection while increasing ascorbate peroxidase, superoxide dismutase, and phenylalanine ammonia lyase activities (Abbasi et al. 2014). *P. fluorescens* CHA0 and salicylic acid induced resistance on tomato plants against the root-knot nematode *M. javanica* (Nikoo et al. 2014). Similarly, Kempster et al. (2001) reported induction of resistance to the clover cyst nematode, *Heterodera trifolii*, on white clover (*Trifolium repens*), when inoculated the pectinolytic *P. fluorescens* P29 or *B. cereus* B1.

Finally, the bacterial enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase, involved in the ethylene pathway, is known to help crops stand abiotic and biotic stresses like pathogenic nematodes. This enzyme degrades ACC, the precursor of ethylene, lowering the hormone levels in plant tissues (Glick 2014). Nascimento et al. (2013) reported bacterial ACC deaminase as a crucial attribute in decreasing populations of *Bursaphelenchus xylophilus*, which is the causal agent of pine wilt disease. Therefore, plant inoculation with rhizobacteria producing ACC deaminase may enhance plant resistance to nematode infestation (Gamalero and Glick 2015).

## 13.4 Biocontrol Potential of Secondary Metabolites

A review published by Siddiqui and Mahmood almost 20 years ago (1999) stated that the lack of commercial interest in bacterial inoculants to use as biocontrol agents for plant-parasitic nematodes was a major problem to research advancement in this area. Nowadays, with the ban on use of many nematicide fumigants, like methyl bromide, due to human and environmental toxicity, the need to find alternatives for controlling nematodes has become a priority (Zasada et al. 2010). Biological control agents and their related metabolites have been the focus of the search for environmentally friendly alternatives. Among the biological control agents on the market or in an advanced research/development stage, rhizospheric bacteria have gained a preeminent place. In this sense, the secondary metabolites produced by rhizobacteria are a potential source of a new generation of pesticides.

To achieve the introduction into the market of secondary metabolites as pesticides for the control of nematodes, some challenges need to be overcome. The most important are costs. It is more expensive and time-consuming to produce and commercialize a molecule (USD 256 million) than to develop and market a biological agent (USD 20–50 million) (Olson 2015). Other difficulties needing to be addressed are the infield stability of the metabolites, the spectrum of target pathogens, the interaction with plants and other organisms, and its effect on the environment. However, the use of these compounds could help to overcome problems related to survival of biocontrol agents, as they frequently fail when introduced into a new ecosystem because of the competition with autochthonous populations. In the same

way, the study of the metabolic pathways that lead to the production of these compounds can help to discern the conditions needed to naturally trigger their production and consequent activity in the rhizosphere.

Finally, some of these metabolites have been the starting point in developing transgenic plants with inbuilt resistance to nematodes. For example, *B. thuringiensis* Cry5B toxins expressed on tomato roots can make the plant resistant to attack by *M. incognita* (Li et al. 2008). The application of molecular and metabolomic techniques, as well as bioinformatics, is improving our understanding of rhizosphere processes, but the function of many metabolites with biocontrol potential still remains unknown.

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# 14 A Deeper Insight into the Symbiotic Mechanism of *Rhizobium* spp. from the Perspective of Secondary Metabolism

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## 14.1 Introduction

Growth of an organism is determined by mineral nutrient availability, and among all the mineral nutrients, nitrogen is the most crucial for plant growth as it is a component of proteins, nucleic acids and other cellular constituents. Atmosphere comprises about $10^{15}$ tonnes of gaseous nitrogen out of which about $1.4 \times 10^8$ metric tonnes of nitrogen is fixed biologically all over the globe every year. This accounts for about 90% of the total nitrogen being fixed in terrestrial environment, and the rest 10% is fixed by lightning (Postgate 1982; Zahran 1999). An additional $1.4 \times 10^8$ metric tonnes of nitrogen being fixed each year by utilization of nitrogenous fertilizers, fossil fuels and planting of legumes (Vitousek et al. 1997; Gage 2004). The prokaryotes are the so far only known source of biological nitrogen fixation being carried out by 87 species in 38 genera of bacteria, 2 genera of archaea and 20 genera of cyanobacteria (Dixon and wheeler 1986). Nitrogen fixation can be accomplished by both free living (*Clostridium, Azotobacter*, *Beijerinckia*, *Rhodospirillum* and *Chromatium*) and symbiotic nitrogen-fixing bacteria (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium* and *Frankia*). Symbiotic nitrogen fixation in Leguminosae family is associated with class alphaproteobacteria, family *Rhizobiaceae*, whereas filamentous, gram-positive actinomycete, *Frankia*, induces nodules on a variety of woody plants from the family Betulaceae, Casuarinaceae, Rosaceae, Myricaceae, Rhamnaceae, Elaeagnaceae, Coriariaceae and Datiscaceae (Benson and Clawson 2000).

*Rhizobium* is a genus of gram-negative motile bacteria which has the ability to fix atmospheric nitrogen. *Rhizobium* species forms a symbiotic nitrogen-fixing association with roots of leguminous plants such as soybean, pea and alfalfa. An equivalent term used by other researchers is 'root nodule bacteria' (RNB) (Zakhia

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et al. 2004; Howieson and Brockwell 2005). Soil-inhabiting bacteria, *Rhizobium*, form specific root structure, nodules generally of two types, determinate and indeterminate. Differ mainly in that indeterminate nodules are elongated in shape and have persistent meristem that continuously form new nodule (Handberg and Stougaard 1992).

## 14.2 Historical Perspective of *Rhizobium*

Beijerinck (1888) was the first to isolate and culture microorganism from root nodules of different legume species and named it as *Bacillus radicicola*. Later on, the name *Rhizobium* was proposed by Frank (1889) for nitrogen-fixing bacteria of legumes. The word *Rhizobia* is derived from the Greek word *rhíza*, meaning "root", and *bios*, meaning "life". The term *Rhizobium* is usually used as a singular form of rhizobia. Genera other than *Rhizobium* were identified later; this includes *Sinorhizobium* (Chen et al. 1988), *Bradyrhizobium* (Jordan 1982) and *Mesorhizobium* (Jarvis et al. 1997). Nobbe and Hiltner (1896) developed the technology for inoculation of legume with *Rhizobium* spp. and granted US patent for it (Das et al. 2017). Mass production of *Rhizobium* inoculants began in 1895 in the USA, mostly by employing peat-based inoculants (Roughley and Vincent 1967). Besides peat-based formulation used worldwide, vermiculite, mineral soil, bentonite, perlite and coal are used as rhizobial inoculants (Stephans and Rask 2000; Temprano et al. 2002; Das et al. 2017).

## 14.3 Rhizobial Genome

Rhizobium has a large and complex multipartite genome with genome size varying from 5.4 to 9.2 Mb and plasmid number ranges from 0 to 7 (MacLean et al. 2007). The genome organization reflects the adaptive potential and the lifestyle of species (MacLean et al. 2007; González et al. 2006). Comparative genomic studies reveal the evolutionary pattern of rhizobia-legume symbiosis. Outcomes of genome comparisons were quite interesting as it revealed that no gene is common and specific to all rhizobia (Amadou et al. 2008; Laranjo et al. 2014).

## 14.4 Rhizobium: Plant Symbiosis

The bacteria colonize plant cells within root nodules and convert atmospheric nitrogen into ammonia, a process known as nitrogen fixation (O'Gara and Shanmugam 1976). The ammonia is used by the plants as a nitrogen source. In turn the rhizobia are supplied with nutrients (Lodwig and Poole 2003) and are protected inside the nodule structure (van Rhijn and Vanderleyden 1995). However, in ineffective nodules no nitrogen is fixed, yet rhizobia are still supplied with nutrients, and in this case, the rhizobia could be considered parasitic (Denison and Kiers 2004). Other

genera of rhizobium such as *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium* have also got the ability to fix nitrogen. The rhizobium-legume association is unique and specific in that each rhizobial strain has definite host range varying from narrow to exceptionally wide (Perret et al. 2000).

### 14.4.1 Mechanism of Root Nodule Formation

The process of nodule formation involves a complex series of steps (Vincent 1974; Newcomb 1981a, b). Plants of Leguminosae family usually secrete a variety of organic compounds such as amino acids and flavonoids which are recognized by bacterial NodD protein. Rhizobium is generally chemotactic towards the plant roots due to the secretion of such compounds (Bergman et al. 1988; Caetano-Anolles et al. 1988; Kurrey et al. 2016). Nodulation takes place due to specific and complex interaction between the plant and the *Rhizobium*. The initial attachment usually involves a protein called "rhicadhesin" which is found on the surface of all leguminous plants. Upon binding of these compounds with NodD protein, nodulation genes get activated. *Rhizobium* secretes Nod factors, lipochito-oligosaccharides which get recognized by the leguminous plant, and triggers early step of nodulation (Pawlowski and Bisseling 1997; Spaink 1992). Host specificity of rhizobia is determined by terminal sugar residues of lipochito-oligosaccharides secreted by rhizobia (Denarie and Cullimore 1993; Fisher and Long 1992; Stokkermans and Peters 1994). When the root hair of the plant comes in contact with bacterium, the growing root hairs get curled and form a pocket for the particular rhizobia (Mylona et al. 1995). The bacteria invade the plant by forming a new infection thread. The infection threads progress towards the primordium, and the bacteria are released into the cytoplasm of the host cells, surrounded by a plant-derived peribacteroid membrane (PBM) (Verma and Hong 1996). This separation usually occurs to suppress plant defence responses which are likely to harm the bacteria. The bacteria produce cytokinin which facilitates division of plant cells to form nodules and the nodule formation initiates on the root hairs. Afterwards, the nodule primordium develops into a mature nodule. The bacteria differentiate into their endosymbiotic form, which is usually known as bacteroid. Bacteroids, altogether with the surrounding PBMs, are called symbiosomes (Roth and Stacey 1989; Guan et al. 1995).

Rapid cell division starts in the infected tissue. The area of $N_2$ fixation is usually pink or red in colour due to the presence of "leghaemoglobin" required for active oxygen transport (Appleby 1984; Kannenberg and Brewin 1989). The formed nodule establishes a direct vascular connection with the host for nutrient uptake. In the process of nodule formation, certain genes called *nod* genes are involved and are known as nodulin genes (van Kammen 1984). The "early nodulin genes" encode products which get expressed before the commence of $N_2$ fixation and are involved in infection and nodule development. However, the "late nodulin genes" interact with the bacterium and aid in metabolic specialization of the nodule (Nap and Bisseling 1990).

### 14.4.2 The Infection Thread

The invasion of root tissues is initiated by intracellular 'tunnels' known as infection threads, which initially arise in root hair cells (Callaham and Torrey 1981). In uninfected root hairs, the nucleus is paired to the tip by microtubules which facilitate new wall material to the growing apex (Lloyd et al. 1987; Ridge 1988). The bacterial infection usually removes the nucleus from the tip and facilitates the pathway for incorporation of wall precursors. Initially, the infection thread develops as an invagination of root hair wall, and the nucleus migrates towards the base of the root hair. The new wall material synthesized is thereafter directed to the tip of the invagination to produce an interior growing cylinder of wall material bounded by a membrane, and the bacteria embedded in a matrix (Gage 2004). Infection thread structures develop subsequently in the underlying cortical cell layers and facilitate the bacteria in the infection thread to spread from one cell to adjacent cell (Libbenga and Harkes 1973). During this process of tissue invasion, the wall of the infection thread limits the rhizobia to the extracellular space, thus preventing its contact with the plant plasma membrane (VandenBosch et al. 1989). Cell invasion can only arise by endocytosis from unwalled infection droplets that evolve from infection threads at a particular stage of development.

As cell divisions in the plant root facilitate the formation of body of the nodule, the infection threads start penetrating individual target cells within the nodule. The bacteriods are released into the plant cytoplasm itself, enveloped in plasma membrane of the plant (Robertson et al. 1978). Thereafter, the bacteria and plant cells differentiate and initiate symbiotic nitrogen fixation and metabolite exchange (Sutton et al. 1981; Verma and Long 1983) (Fig. 14.1).

## 14.5 Rhizobia as Biocontrol Agent and Biofertilizer

*Rhizobium* spp. has boosted legume production worldwide by enhanced nitrogen fixation, plant growth promotion and suppression of soilborne pathogens such as *Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp., and *Macrophomina phaseolina* in both legumes and nonlegumes (Table) (Antoun et al. 1978; Malajczuk et al. 1984; Chakraborty and Purkayastha 1984; Ehteshamul-Haque and Ghaffar 1993; Nadia et al. 2007; Das et al. 2017). Ehteshamul-Haque and Ghaffar (1993) deployed biocontrol potential of *Rhizobium leguminosarum*, *Sinorhizobium meliloti* and *Bradyrhizobium japonicum* by soil drenching and seed coating of sunflower, okra, mung bean and soybean. Antimicrobial activity of *Rhizobium* spp. strains ORN 24 and ORN 83 has been exploited against *Pseudomonas savastanoi*, olive knot disease (Maurad et al. 2009). Buonassisi et al. (1986), inoculated seeds of snap bean with *Rhizobium leguminosarum* bv. *phaseoli* (isolated from nodules of commercial snap bean) to control fusarium foot rot of beans caused by *Fusarium solani* f. sp. *phaseoli*. Inoculation of pea and sugar beet seeds with *R. leguminosarum* bv. *vicieae* strain R12 significantly reduced the occurrence of pythium damping-off (Bardin

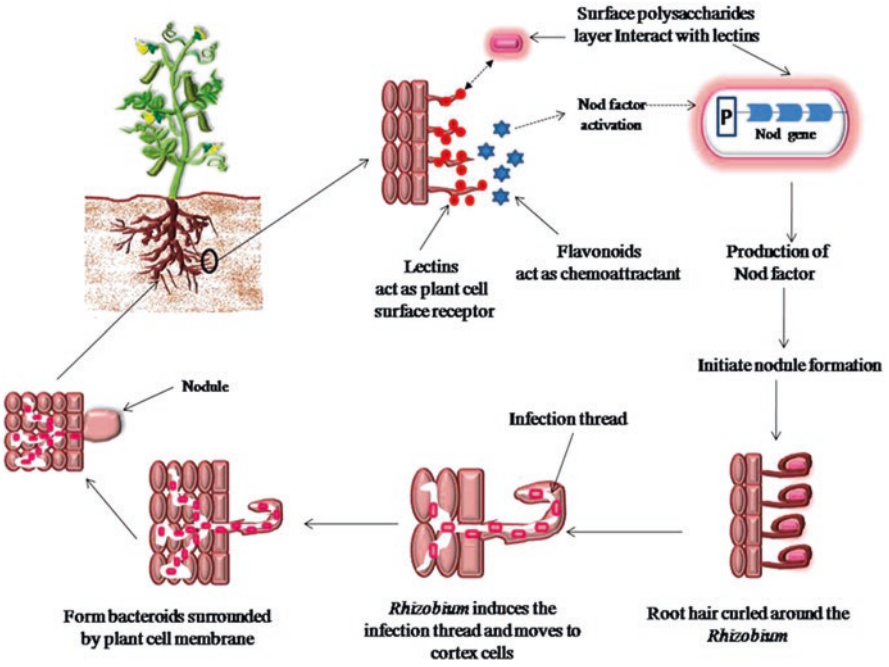



**Fig. 14.1** Mechanism of nodule formation in rhizobium-legume symbiosis

et al. 2004). Different strains of *Rhizobium* were reported to reduce incidence of root rot of chickpea, *Rhizoctonia solani*, and increased the nitrogen fixation, phosphorus uptake and plant growth (Hemissi et al. 2011). Seed treatment of chickpea with PGPR + *Mesorhizobium ciceri* provided enhanced plant growth (seedling emergence and shoot length) and reduced fusarium wilt of chickpea significantly over their single treatment (Kumari and Khanna 2014). Co-inoculation of common bean with *Rhizobium* and *Pseudomonas* strains was reported to have increased number of nodules and produce higher yield (Sancheza et al. 2014) (Table 14.1).

## 14.6 Mechanism of Biological Control by Rhizobia

The mechanism associated with biological control of phytopathogens by rhizobia consists of antibiotic production, siderophore production, HCN production, production of lytic enzymes, phosphate solubilization, competition and induction of plant defence (Arora et al. 2001; Huang and Erickson 2007). Antagonistic activity against a wide range of pathogens is due to its ability to produce wide range of secondary metabolites such as HCN, siderophore, rhizobitoxin, lytic enzymes, IAA production and phosphate solubilization (Antoun et al. 1978; Presmark et al. 1993; Nautiyal, 1997; Biswas et al. 2000; Deshwal et al. 2003; Pandey and Maheshwari 2007).

**Table 14.1** Biological control potential of *Rhizobium* spp.

| S. N. | Producer | Host | Target plant pathogen | Disease manage | References |
|---|---|---|---|---|---|
| 1 | *Rhizobium japonicum* | *Glycine max* | *Fusarium solani* | Root rot | Al-Ani et al. (2012) |
| | | | *Macrophomina phaseolina* | Charcoal rot | |
| 2 | *Rhizobium* sp. | *Cicer arietinum* | *Fusarium oxysporum* f. sp. *ciceris* | Wilt | Arfaoui et al. (2005) |
| 3 | *Rhizobium meliloti* | *Arachis hypogaea* | *Macrophomina phaseolina* | Root rot | Arora et al. (2001) |
| | | | *Pythium* sp. | Brown rot of groundnut | Bardin et al. (2004) |
| | | | *Fusarium solani* f. sp. *phaseoli* | Wilt | Buonassisi et al. (1986) |
| 4 | *Mesorhizobium loti* MP6 | *Brassica juncea* | *Sclerotinia sclerotiorum* | Sclerotinia rot | Chandra et al. (2007) |
| | | | *Rhizoctonia solani* | Root rot | Dubey and Maheshwari (2011) |
| | | | *Fusarium oxysporum F. solani* | Wilt | |
| | | | *Fusarium oxysporum* f. sp. *lentis* | Wilt | Essalmani and Lahlou (2002) |
| 5 | *Rhizobium* sp. | *Phaseolus vulgaris* | *Fusarium solani* f. sp. *phaseoli* | Wilt | Estevez de Jensen et al. (2002) |
| 6 | *Rhizobium* sp. | *Arachis hypogaea* | *Sclerotium rolfsii* | Stem rot | Ganesan et al. (2007) |
| 7 | *Rhizobium* sp. | *Glycine max* | *Cylindrocladium parasiticum* | Red crown rot | Gao et al. (2012) |
| 8 | *Rhizobium leguminosarum* bv. *viciae* | *Pisum sativum Lens culinaris* | *Pythium spp.* | Root rot | Huang and Erickson (2007) |
| 9 | *Rhizobium* sp. | *Olea europaea* | *Pseudomonas savastanoi* | Olive knot | Kacem et al. (2009) |
| 10 | *Sinorhizobium fredii* KCC5 | *Cajanus cajan* | *Fusarium udum* | Wilt | Kumar et al. (2010) |
| 11 | *Ensifer meliloti, Rhizobium leguminosarum* | *Trigonella foenum-graecum* | *Fusarium oxysporum* | Wilt | Kumar et al. (2011) |
| | | | *Phytophthora cinnamomi* | Root rot | Malajczuk et al. (1984) |
| 12 | *Rhizobium* sp. NBRI9513 | *Cicer arietinum* | *Fusarium spp.* | Wilt | Nautiyal (1997) |
| | | | *Rhizoctonia bataticola* | Dry root rot | |
| | | | *Pythium* sp. | Damping-off | |

(continued)

**Table 14.1** (continued)

| S. N. | Producer | Host | Target plant pathogen | Disease manage | References |
|---|---|---|---|---|---|
| 13 | *Rhizobium* sp. | *Glycine max* | *Macrophomina phaseolina* | Charcoal rot | Omar and Abd-Alla (1998) |
| 14 | *Bradyrhizobium* sp. | *Helianthus annuus* | *Rhizoctonia solani* | Collar rot | Siddiqui et al. (2000) |
| 15 | *Rhizobium* sp. | | *Macrophomina phaseolina* | *Charcoal rot* | Romesh Sagolshemcha et al. (2017) |
| 16 | *Rhizobium* sp. | *Vicia faba*, *Cicer arietinum*, *Lupinus albus* | *Fusarium oxysporum Fusarium solani,* | Wilt | Shaban and El-Bramawy (2011) |
| | | | *Macrophomina phaseolina* | *Charcoal rot* | |
| | | | *Rhizoctonia solani* | *Rot* | |
| | | | *Sclerotium rolfsii* | Collar rot | |
| 17 | *Bradyrhizobium japonicum* | *Solanum lycopersicum* | *Macrophomina phaseolina* | Charcoal rot | Siddiqui and Shaukat (2002) |
| | | | *Fusarium solani* | Wilt | |
| | | | *Rhizoctonia solani* | Damping-off, root rot, stem rot and stem canker | |
| 18 | *Rhizobium leguminosorum* | *Cicer arietinum* | *Fusarium oxysporum* f. sp. *ciceris* | Wilt | Singh et al. (2010) |
| 19 | *Rhizobium sp.* RS12 | *Cicer arietinum* | *Macrophomina phaseolina* | Dry root rot | Smitha and Singh (2014) |

### 14.6.1 Antibiotic Production

Antibiotic production is one of the major mechanisms of biological control of phytopathogens. Several workers have reported different rhizobial strains to produce variety of antibiotics (Ligon et al. 2000; Raaijmakers et al. 2002; Deshwal et al. 2003; Bardin et al. 2004; Chandra et al. 2007; Das et al. 2017). Hirsch (1979) reported that 97 strains of *R. leguminosarum* produces bacteriocins, characterized as small and medium based on their size. *R. leguminosarum* plasmid pRL1J1 carries genes for nodulation and bacteriocin production, encodes for medium bacteriocin (Hirsch et al. 1980). *R. leguminosarum* bv. *trifolii* T24 produces a potent antibiotic, trifolitoxin that promote clover nodulation have been reported by Triplett and Barta (1987). Different strains of *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *R. meliloti*, *B. japonicum* and *S. meliloti* have been reported to secrete diverse group of antibiotics having potential for inhibition of phytopathogens (Chakraborty

and Purkayastha 1984; Bardin et al. 2004; Deshwal et al. 2003; Hafeez et al. 2005; Chandra et al. 2007; Gopalakrishnan et al. 2015) (Table 14.2).

### 14.6.2 Production of Antimicrobial Secondary Metabolites

#### 14.6.2.1 HCN Production

HCN are volatile, secondary metabolite produced during the early stationary phase of rhizobacteria (Rezzonico et al. 2007; Knowles and Bunch 1986). HCN is inhibitor of various metalloenzymes such as cytochrome C oxidases of respiratory electron transport. It disrupts the energy supply to the cell and is highly toxic; even at low concentration, it has deleterious effect on growth and development of aerobic plant pathogens (Corbett 1974; Gehring et al. 1993; Deshwal et al. 2003; Siddiqui et al. 2006; Martínez-Viveros et al. 2010). Beauchamp et al. (1991) and Antoun et al. (1998) have reported that 12.5 and 3% of the total strains of rhizobia screened were HCN producers, respectively. HCN production has also been reported in *Mesorhizobium loti* MP6, retarding the growth and development of *S. sclerotiorum* causing white rot in *Brassica campestris* (Chandra et al. 2007). Six *Rhizobium* spp. strains (an isolate from root nodules of chickpea) has been reported to produce HCN, reducing the incidence of chickpea wilt by *Fusarium oxysporum* f. sp. *ciceris* (Arfaoui et al. 2006).

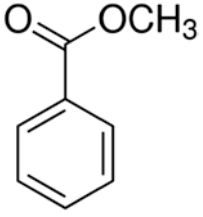
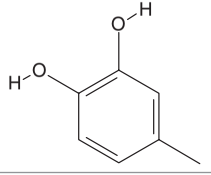
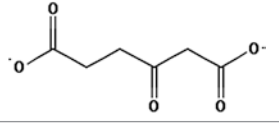
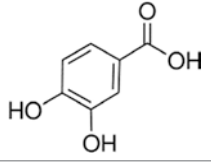
#### 14.6.2.2 Siderophore Production

Iron is one of the key components of metabolic molecules such as ribonucleotide reductase, cytochromes, etc. (Guerinot 1994). Some microbes are equipped with the ability to produce siderophores, an iron-binding compound of low molecular weight (Matzanke 1991; Andrews et al. 2003). Siderophores scavenges iron ($Fe^{3+}$) from environment under iron stress condition which in turn determines the colonization of bacteria on plant roots leaving pathogens (Crowley and Gries 1994; Siddiqui 2006; Martínez-Viveros et al. 2010). Rhizobia has been endowed with the ability to produce a range of siderophores varying from catechol and hydroxamate type (Modi et al. 1985; Roy et al. 1994; Persmark et al. 1993), rhizobactin type (Smith et al. 1985), citrate type (Guerinot et al. 1990), phenolate type (Patel et al. 1988), vicibactin type (Carson et al. 1992), anthranilic acid (Rioux et al. 1986) to dihydroxamate type (Carson et al. 2000). Arora et al. (2001) reported that *M. phaseolina* causing charcoal rot of groundnut was inhibited by siderophore-producing strains of *Rhizobium meliloti* under in vitro condition. Seed treatment with hydroxamate siderophore producer, *Mesorhizobium loti* MP6, reduced the occurrence of white rot of *Brassica campestris* (Chandra et al. 2007).

### 14.6.3 Lytic Enzyme Production

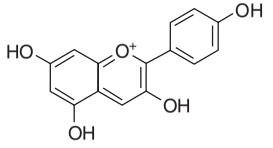
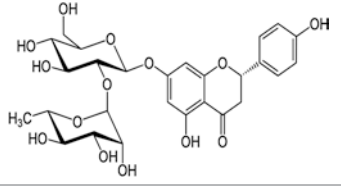
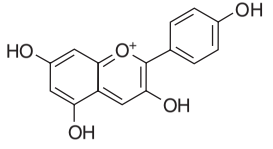
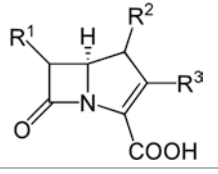
Chitinases, cellulases, β-1,3-glucanase β-1,4-glucanase, β-1,6-glucanase, proteases, pectinase and amylases are some of the lytic enzymes produced by microorganisms

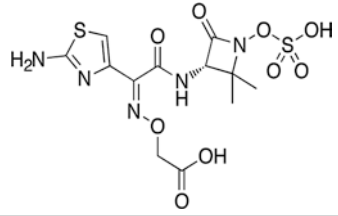
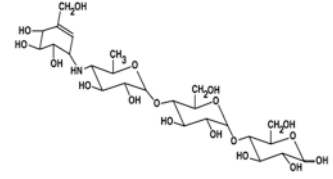
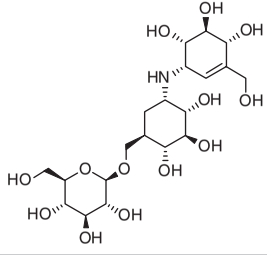
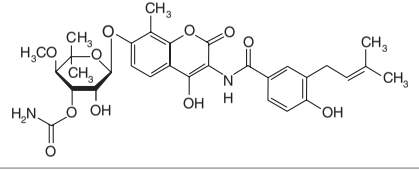
**Table 14.2** Representative list of secondary metabolites of important *Rhizobium* species (KEGG database accessed on April 25, 2018)

| *Rhizobium* species | Type of secondary metabolite | Biosynthesis pathway | Structural formula |
|---|---|---|---|
| 1. *Rhizobium leguminosarum* bv. *viciae* 3841 | a) Methylbenzoate | Xylene degradation |  |
| | b) Methylcatechol | Benzoate degradation |  |
| | c) 3-Oxoadipate | Catechol ortho cleavage |  |
| | e) 3,4-Dihydroxybenzoate | Terephthalate degradation |  |

(continued)

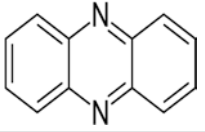
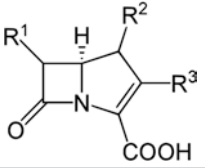
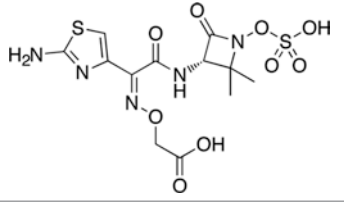
**Table 14.2** (continued)

| *Rhizobium* species | Type of secondary metabolite | Biosynthesis pathway | Structural formula |
|---|---|---|---|
| | f) Pelargonidin | Flavonoid biosynthesis |  |
| | g) Naringenin | Flavanone biosynthesis |  |
| | h) Paspaline | Paspaline biosynthesis |  |
| 2. *Mesorhizobium opportunistum* | a) Carbapenem | Carbapenem biosynthesis |  |

| | | | |
|---|---|---|---|
| | b) Monobactam | Monobactam biosynthesis |  |
| | c) Acarbose | Acarbose biosynthesis |  |
| | d) Validamycin | Validamycin biosynthesis |  |
| | e) Novobiocin | Novobiocin biosynthesis |  |

(continued)

**Table 14.2** (continued)

| *Rhizobium* species | Type of secondary metabolite | Biosynthesis pathway | Structural formula |
|---|---|---|---|
| | f) Phenazine | Phenazine biosynthesis |  |
| 3. *Azorhizobium caulinodans* | a) Carbapenem | Carbapenem biosynthesis |  |
| | b) Monobactam | Monobactam biosynthesis |  |

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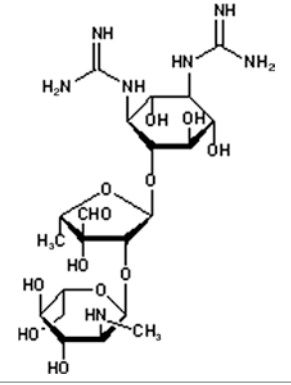
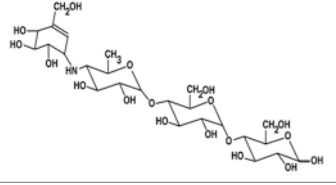
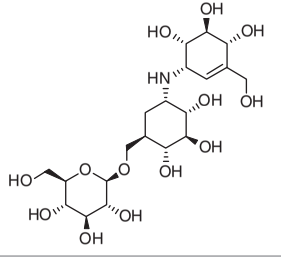
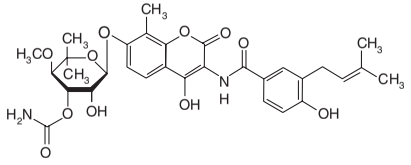
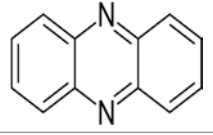
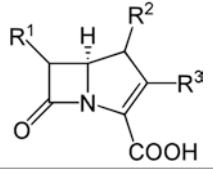
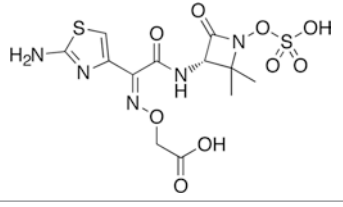
|---|---|---|---|
| | c) Streptomycin | Streptomycin biosynthesis |  |
| | d) Acarbose | Acarbose biosynthesis |  |
| | d) Validamycin | Validamycin biosynthesis |  |

(continued)

**Table 14.2** (continued)

| *Rhizobium* species | Type of secondary metabolite | Biosynthesis pathway | Structural formula |
|---|---|---|---|
| | e) Novobiocin | Novobiocin biosynthesis |  |
| | f) Phenazine | Phenazine biosynthesis |  |
| 4. *Sinorhizobium meliloti* 1021 | a) Carbapenem | Carbapenem biosynthesis |  |
| | b) Monobactam | Monobactam biosynthesis |  |

| | | |
|---|---|---|
| c) Streptomycin | Streptomycin biosynthesis |  |
| d) Acarbose | Acarbose biosynthesis |  |
| e) Novobiocin | Novobiocin biosynthesis |  |

for disease reduction (Chatterjee et al. 1995; Diby et al. 2005; Gupta et al. 2006; Ruiz Duenas and Martinez 1996; Szekeres et al. 2004). There are reports of rhizobial isolates producing chitinase to inhibit pathogenic microbes (Chernin et al. 1955; Mazen et al. 2008). Mazen et al. (2008) reported that seed treatment with chitinase-producing *Rhizobium* spp. alone or co-inoculated with mycorrhizal fungi leads to reduction of damping-off of fababean. *Rhizobium* strains isolated from *Sesbania sesban* has been reported to be produce chitinase (Sridevi and Mallaiah 2008). *R. leguminosarum* isolate TR2 and *Ensifer meliloti* isolate TR1 and TR4 showed β-1,3-glucanase and chitinase activity, respectively, and inhibited fusarium wilt of fenugreek (Kumar et al. 2011). *Rhizobium* sp. Strain RS12, with chitinase-producing ability, suppresses diseases of chickpea caused by *F. oxysporum*, *S. sclerotiorum* and *M. phaseolina* by preventing mycelia growth and development (Smitha and Singh 2014).

### 14.6.4 Phosphate Solubilization

Phosphorus is present in soil in immobile for and thus become unavailable to microbe and plant (Gyaneshwar et al. 2002). Group of rhizobia have been reported to be potent phosphate solubilizers, some of them as *R. leguminosarum* mobilizes phosphorus making it available to plant (Rodriguez and Fraga 1999; Mehta and Nautiyal 2001). *Rhizobium* inoculated *P. vulgaris* showed significant difference in acid phosphatase activity in its rhizospheric zone (Makoi et al. 2010). Bradyrhizobium strains that have been reported by Deshwal et al. (2003) for their ability to produce siderophores, phosphate solubilization and IAA, conferring it strong root colonizing, growth promotion and vigorous antagonistic activity against *M. phaseolina* (charcoal rot of peanut). Co-inoculation of *Rhizobium* and phosphate solubilizing bacteria have been reported to have synergistic effect increasing nodulation, shoot and root nitrogen and phosphorus content (Rugheim and Abdelgani 2009).

## 14.7 Induction of Plant Defence Mechanisms

Systemic resistance in host is induced by up regulating the expression of defence-related genes encoding for antioxidant enzymes, hydrolytic enzymes and pathogenesis-related proteins. Defence-related enzymes such as polyphenol oxidase, L-phenylalanine ammonia lyase, peroxidase, chalcone synthase and isoflavone reductase play crucial role in induction of plant defence to pathogenic attack (Arfaoui et al. 2005; Dutta et al. 2008). Rhizobia have ability to induce defence arsenal by triggering production of plant defensive enzymes, phytoalexins, phenolics and flavonoids (Mavrodi et al. 2001; Yu et al. 2002). Phenolics plays a crucial role in plant defence by activating plant defence genes, acting directly as structural barriers and modulating the pathogenicity, preventing growth and spread of pathogens (Ramos et al. 1997 and Dihazi et al. 2003). Mishra et al. 2006 reported that inoculation of rice with strains of *Rhizobium leguminosarum* bv. *phaseoli* and *R.*

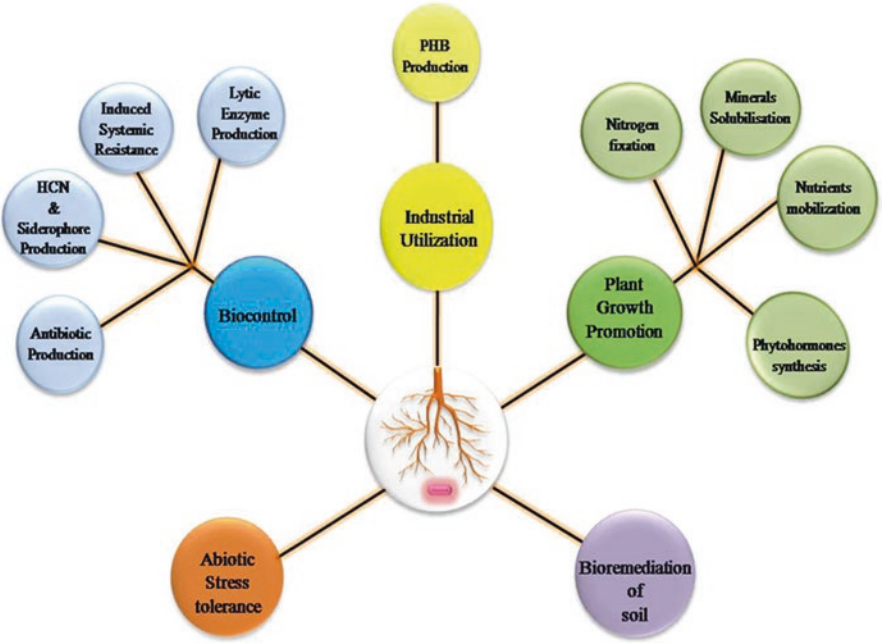



**Fig. 14.2** Multifaceted role of *Rhizobium* sp.

*leguminosarum* bv. *trifolii* induces production of phenolics such as ferulic acid, gallic acid and tannic and cinnamic acids, reducing infection by *Rhizoctonia solani*. Induction and accumulation of phytoalexins such as medicarpin and maackiain in response to *Rhizobium* species in planta, protect it from phytopathogens (Weigand et al. 1986; Weidemann et al. 1991). A phytoalexin, glyceollin have been reported to be produced by *Rhizobium* and *Bradyrhizobium* sp. in soybean, which has antimicrobial activity against plant pathogens (Phillips and Kapulnik 1995) (Fig. 14.2).

## 14.8 Microbial Secondary Metabolites and Its Importance

Microbial secondary metabolites are low molecular weight compounds, indispensable for growth of producing microbes but play an important role in nutrition, health and economy of the society (Berdy 2005; Ruiz et al. 2010). Microbial secondary metabolites varied widely in its chemical nature from peptides, polyketides, lipids, steroids, terpenoids and carbohydrate to alkaloids (O'Brien and Wright 2011). They include pigments, toxins, antibiotics, pheromones, antitumor agents, enzyme inhibitors, effectors of ecological competition and symbiosis, receptor antagonist and agonists, immunomodulating agents, pesticides, cholesterol-reducing drugs and growth promoters of plants and animals (Demain 1998). These metabolites are not synthesized during logarithmic growth phase but are synthesized during subsequent

production stages; stationary phase (idiophase) and metabolites known as idiolites (Demain and Fang 2000; Gonzalez et al. 2003; O'Brien and Wright 2011). Production of secondary metabolites are brought about by addition and biosynthesis of an inducer or exhaustion of nutrients, generate signal which regulate metabolic pathways leading to chemical differentiation (Bibbs 2005; Ruiz et al. 2010). Microbial secondary metabolites are major source of essential agricultural products and contributes to about half of the pharmaceutical market (Demain and Sanchez 2009). In addition to its use as anti-infective drugs, they are used as immunosuppressants to facilitate organ transplantation (Verdine 1996; Barber et al. 2004; Demain and Sachez 2009). Autoinducers of secondary metabolites includes oligopeptides of gram-positive bacteria, N-acylhomoserine lactone of gram-negative bacteria and butanolides of the actinomycetes (Kawaguchi et al. 1988; Demain 1998).

## 14.9 Rhizobial Formulations

Field applicability of rhizobium for its better exploitation at large scale is determined by a formulation with appropriate inoculum load. Survivability in higher number and for longer period in commercial formulation is major objective of developing an inoculants formulation. Mainly two types of commercial formulation of *Rhizobium* are available in market, they are solid and liquid. Solid inoculants are prepared by blending broth culture with an appropriate carrier material. Selection of carrier material is determined by a number of factors such as survivability of rhizobial cells on carrier material, cost-effectiveness and accessibility, pH buffering moisture absorbing capacity, etc. (Date and Roughley 1977; Brockwell and Bottomley 1995). Peat-based application of rhizobial inoculants is the most widely used method for application of rhizobia worldwide since 1895. A diverse range of carriers such as soil material (peat, clay, charcoal) (Chao and Alexander 1984; Beck 1991; Temprano et al. 2002), perlite (Ronchi et al. 1997; Khavazi et al. 2007), vermiculite (Graham-weis et al. 1987), plant by-products (sawdust, peanut shell, corn cobs) (Sparrow and Ham 1983) and composts (Kostov and Lynch 1998) are used all over the world (Singh et al. 2016; Singh et al. 2017).

Other formulations such as liquid, granular and biofilm-based formulation have been studied, but of all formulations only solid- and liquid-based formulations have been exploited commercially. Liquid formulations are based on broth culture with oil in water suspensions or mineral and organic oil as carriers (Albareda et al. 2008; Bashan 1998). Granular formulations such as peat prills (Fouilleux et al. 1996), peat inoculants coated on sand (Chamber 1983), perlite/alginate beads (Bashan 1986; Hedge and Brahmaprakash 1992) and polymer-coated beads (Brockwell et al. 1980) have been studied. Biofilm-based formulation is latest and efficient one having greater stability under abiotic and biotic stresses. Bacteria may be grown on carrier material to form biofilm or trapped by a fungal matrix (Seneviratne 2003; Seneviratne et al. 2008; Triveni et al. 2013; Prasanna et al. 2013; Jayasinghearachchi and Seneviratne 2004).

## 14.10 Conclusion and Future Prospects

Currently, there is an increasing threat to agricultural sustainability, soil and groundwater contamination. Biofertilizer and biocontrol agents are used as a highly efficient alternative to chemical fertilizers and chemical pesticides, respectively. *Rhizobium* with promising biofertilization and biocontrol ability can be exploited for increasing legume and nonlegume production. Studies regarding secondary metabolites of *Rhizobium* need to be explored for its greater benefit for agriculture. Genetic engineering approaches can also be used to incorporate genes for secondary metabolites in rhizobial strains lacking it but have potential for biocontrol. Although a number of rhizobial biofertilizer such as solid and liquid formulations are available, better commercial formulations such as polymer and biofilm based need to be urgently introduced in the market.

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# Metabolites of Plant Growth-Promoting Rhizobacteria for the Management of Soilborne Pathogenic Fungi in Crops

15

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## 15.1 Introduction

Soilborne pathogenic organisms are those pathogenic organisms which inhabit and partly or fully complete their life cycle in the soil environment by causing various diseases in plants and cause extensive damage. These diseases caused by soilborne pathogens are collectively known as soilborne diseases. Soilborne diseases occur in a wide variety of plants such as fruits and vegetables, ornamental plants, trees, and shrubs. Fungi, oomycetes, nematodes, viruses, and few parasitic plants have been considered as causative agents for the soilborne diseases. Diseases caused by soilborne pathogens are one among the most significant biological stress to the plants. Soilborne fungi such as *Rhizoctonia*, *Fusarium*, *Macrophomina*, *Sclerotiana*, *Sclerotium*, *Gaeumannomyces graminis* including oomycetes *Pythium* and Phytophthora are the major causal agents of significant soilborne plant diseases (Mathivanan et al. 2006; Jayaprakashvel and Mathivanan 2011). Hence, soilborne pathogenic fungi (SBPF) are considered as one of the major limiting factors for the growth and yield of crop plants world over. These SBPF may cause severe damage to crop plants and could incite rot diseases in seedlings and vascular systems and roots of crop plants (Mishra et al. 2015).

Rhizosphere of plants is one of the most dynamic and competitive ecosystems. Both beneficial and harmful microorganisms constantly compete with each other in the rhizosphere region because of the root exudates and other

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growth-promoting substances found in the vicinity of rhizosphere, produced largely by the plants. Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR) (Kloppper and Schroth 1978; Singh et al. 2016a, b, 2017). These PGPR could enhance plant growth either by direct mechanisms such as production of plant growth-promoting hormones and indirect mechanisms such as suppressing the growth of pathogenic microorganisms *in planta.* Antagonistic PGPR have received tremendous attention because of the immense potential in reducing plant diseases, especially strains of the PGPR genera such as *Bacillus*, *Pseudomonas*, and *Burkholderia*. Further, many of the PGPR are now commercialized as bioinoculants for the improvement of plant growth and disease control in agriculturally important crop plants (Kloppper and Schroth 1978; Watt et al. 2006; Bouizgarne 2013; Berendsen et al. 2012; Beneduzi et al. 2012; Gouda et al. 2018; Roeland et al. 2012; Singh 2013).

The antimicrobial metabolites of the PGPR or other bioinoculants have received much attention in the past few decades. While it is always a challenge to maintain a desirable population of PGPRs or other bioinoculants in the bioformulations, it is envisaged that the microbial metabolites could be the potential choice for the control of plant diseases. *Pseudomonas* is one of the most important soil microbial communities which are present abundantly in almost all agroecosystems. Pseudomonads are having high rhizosphere competence and greater metabolic diversity which makes them much suitable organisms for biological control of plant diseases. Several efficient strains of *Pseudomonas*, especially fluorescent pseudomonads, have been isolated and characterized for their potential antimicrobial metabolites such as phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, etc. *Bacillus* is another most interesting soil microorganism with reference to plant disease control. *Bacillus* spp. are gaining much attention in recent years due to their longer shelf life because of their ability to produce the endospores that are tolerant to heat and desiccation. This sporulating feature of *Bacillus* spp. complemented with their metabolic diversity to produce wide variety of metabolites makes them efficient biocontrol agents of plant diseases especially on the soilborne plant pathogenic fungi. Besides antifungal metabolites, PGPR are also reported to produce siderophores, iron-chelating substances that compete with soilborne pathogenic fungi for the iron in the rhizosphere. Hydrogen cyanide is the volatile antifungal antibiotic produced by some of the PGPR. PGPR are also found to produce biosurfactants which could act against the fungal pathogens especially soilborne pathogens belonging to oomycetes group.

Since the microbial metabolites of the PGPR have prominent role in the plant disease management, studies have been done to characterize the metabolites, and efforts are also made to optimize the production. Genetic engineering approaches have also been made to enhance the production of bioactive secondary metabolites of the PGPR. Hence, this chapter provides an overview of PGPRs used in the management of plant diseases caused by SBPF (with reference to agriculturally important crop plants), metabolites of the PGPR, and recent studies on enhancing the production of metabolites by the PGPR.

## 15.2 Soilborne Plant Pathogenic Fungi (SBPF)

Soilborne diseases of agriculturally important crop plants especially on the roots are one of the most pressing problems for a long time. The plant diseases caused by the soilborne pathogenic fungi (SBPF) are very difficult to predict, detect, and diagnose because of the high complexity in disease onset, pathogen perpetuation, and diverse ways for pathogen dispersal. Soil itself is very complex in nature, and the interactions between crop plants and pathogenic fungi are too complicated.

Soilborne diseases of crop plants include the following major type of infections:

1. *Pre-emergence damping-off*: SBPF such as *Fusarium*, *Phytophthora*, *Pythium* and *Rhizoctonia*, and *Sclerotium* could cause decay of plant seedlings in the soil before they emerge above soil surface, a condition known as preemergence damping-off. These pathogens would survive both in wet and dry conditions in soil for longer duration due to their special resting structures such as sclerotia and spore.
2. *Post-emergence damping-off*: SBPF could infect crop plants after the seedling emerges on soil surface where cotyledons, stems, and roots of tender seedlings are infected. This infection leads to the decay of seedlings which is known as postemergence damping-off.
3. *Wilts*: SBPF such as *Fusarium* and *Verticillium* could cause severe damage to the vascular system of plants through wilting which results in water loss and turgidity changes which eventually leads to the death of the plant. Wilt is one of the most severe diseases of crop plants. Symptoms of wilt diseases could often resemble that of root rots as well.
4. *Crown rots*: SBPF such as *Fusarium*, *Sclerotium*, etc. could affect the crown or lower stem of crop plants and cause crown rot. Crown rot is characterized by dry rotting at or near the soil line. At severe stages, entire plant may also get affected to make entire plant tan or dark colored, which leads to wilting and death of the plants.
5. *Root rots*: A majority of SBPF including *Fusarium*, *Phytophthora*, *Pythium*, and *Rhizoctonia* cause root rot which is a collective of symptoms from roots to leaves. Root rots are one of the major soilborne diseases which cause severe damage and yield loss in agriculture. The pathogens infect first at roots causing them to die and decay which ultimately leads to wilting and death of the entire plant. Almost all crop plants are susceptible to root rot.
6. *Blights*: Though most common blights are caused by foliar pathogens, SBPF such as *Fusarium*, *Phytophthora*, *Pythium*, and *Rhizoctonia* also causes blight diseases in crop plants.

## 15.3 Management of SBPF

Soilborne diseases especially those caused by the SBPF are responsible for major crop losses worldwide. These diseases are very difficult to manage because of the factors such as:

1. The disease incidence is highly heterogeneous.
2. Pathogens can survive in soil for longer duration.
3. Pathogens can perpetuate by various modes.
4. Pathogens have alternative hosts.

Hence, management strategies for the control of soilborne diseases caused by the SBPF are not centered toward single approach but with multiple options. The various disease management strategies being employed for the management of SBPF are summarized in the following illustration (Fig. 15.1).

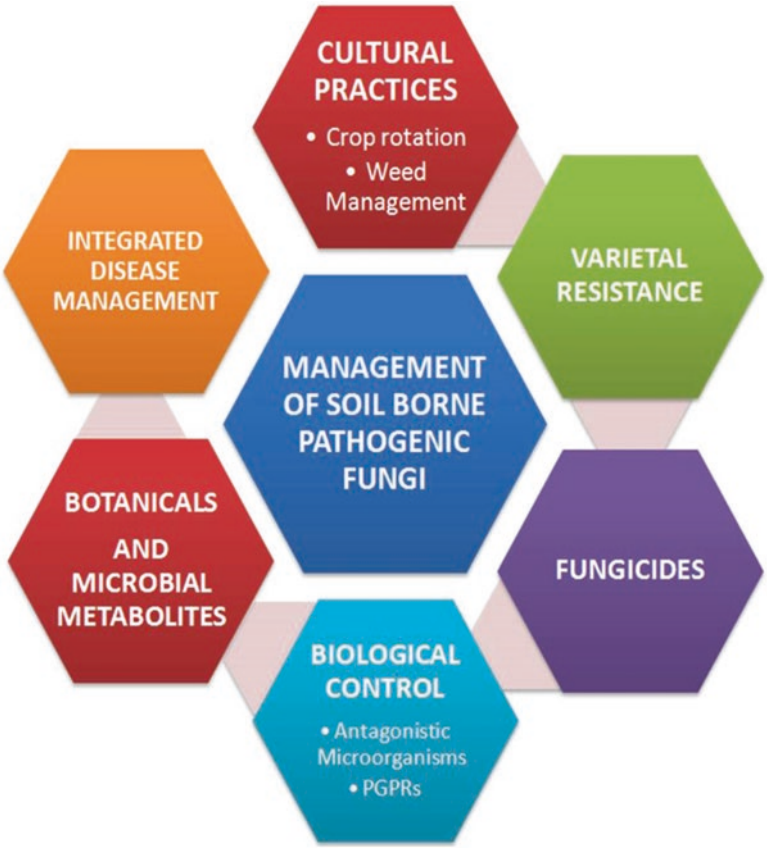


**Fig. 15.1** Various management strategies for soilborne plant pathogenic fungi

The salient features of the various disease management strategies are as follows:

### 15.3.1 Cultural Practices

Cultural practices (CPs) are crop management practices to create an environment which is favorable for the crop and unfavorable for the pathogen. Examples of cultural practices being used for the disease management are flooding, deep plowing, crop rotation, soil solarization, biofumigation, mineral nutrition management, tillage, alteration of soil temperature, etc. (Katan 2010). Though varieties of cultural practices are available as tools for the disease management, they have few disadvantages such as time consuming, requires much skill and knowledge, in-effective towards closed related species and difficulty in assessing the successfulness (Hill 2019).

### 15.3.2 Varietal Resistance

Varietal resistance or host resistance is relatively the most economical and effective way of management of soilborne pathogenic fungi. However, available high yielding cultivars of many economically important crops are not having genetic resistance toward SBPF. Breeding programs for making the susceptible varieties into resistant varieties are the most expensive approach and may also consume a larger time to attain success.

### 15.3.3 The Use of Chemical Fungicides

It is the most effective management strategy for the suppression of SBPF. Several categories of synthetic chemicals with proven antifungal activities were used as fungicides for the control of SBPF for a long time. However, the disadvantages such as persistent residues, relatively higher cost, damage to the environment, evolution of fungicide resistance in pathogens, nontarget effects, etc. outweigh the advantages of the chemical fungicides. It is the prime concern of researchers' world over to find suitable alternative for the chemical pesticides.

### 15.3.4 Biological Control

Several antagonistic PGPR belonging to the genera *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Serratia* have been found to control soilborne pathogenic fungi by various mechanisms and are widely used in disease management practices (Pankhurst and Lynch 2005; Gouda et al. 2018).

### 15.3.5 Botanicals and Microbial Metabolites

Essential oils and plant extracts contain a wide array of bioactive substances that are effective in controlling SBPF. Similarly, instead of whole PGPR, their antagonistic metabolites are characterized for their efficiency in controlling plant diseases, especially soilborne plant disease.

### 15.3.6 Integrated Disease Management

Management of soilborne diseases by a combination of disease control strategies such as host resistance, biological control, and botanicals in an integrated manner and by involving other nonchemical methods of disease management is the current need (Naseri and Hemmati 2017).

## 15.4 Metabolites of PGPR in Plant Disease Management

Plant growth-promoting rhizobacteria (PGPR) are important soil microbial communities which reside on the roots and/or associated with plant roots (Kloepper and Schroth 1978; Ahemad and Kibret 2014). Rhizosphere is by far the most competitive microbial ecosystem comprising an integrated network of plant roots, soil, and microbial communities in which the role of PGPR is enormous (Raaijmakers et al. 2009; Ahkami et al. 2017). Because of the high competitiveness, ability to produce an array of metabolites, affinity toward root system, and direct plant growth promotion activities, PGPRs receive considerable attention worldwide. PGPR have been found to enhance the plant growth by direct mechanisms such as mobilization of mineral resources and production of phytohormones and also through indirect mechanisms such as decreasing the inhibitory effects of various pathogens on plant growth and development. Various studies and recent reviews have documented the increased health and productivity of different plant species by the application of plant growth-promoting rhizobacteria under both normal and stressed conditions (Raaijmakers et al. 2009; Ahemad and Khan 2012; Bhattacharyya and Jha 2012; Prathap and Ranjitha 2015; Islam et al. 2016; Ahkami et al. 2017; Gouda et al. 2018).

The PGPR community used extensively in the biological control of plant diseases has been attributed to have various mechanisms such as microbial siderophores, antibiotics, biosynthesis of surfactants and phytohormones, nutrient and spatial competition, mycoparasitism, induced systemic resistance, quorum quenching, and construction of transgenic lines (Diallo et al. 2011). Though biological control is mediated by different groups of microorganisms, their operational mechanisms fall under some group of mechanisms generally known as antagonism. The antagonism is of three types: competition, antibiosis, and parasitism (Vasudevan et al. 2002; Mathivanan et al. 2006; Ramadan et al. 2016; Jayaprakashvel and Mathivanan 2011). Competition with plant pathogens may be for nutrients and

space. PGPR produce siderophores as a mechanism to sequester limited iron present in the rhizosphere and get a competitive advantage over plant pathogens (Sayyed et al. 2013; Sasirekha and Srividya 2016). Some of the fluorescent pseudomonads have the ability to aggressively colonize in the rhizosphere leaving little space and nutrient for the pathogens and thereby gain competitive advantage and suppress the pathogen growth and disease development (Hibbing et al. 2010; David et al. 2018). Among these mechanisms, antibiosis through the production of bioactive secondary metabolites that are having exceptional antibiotic activity against plant pathogens is found to be the most preferable mechanism in view of developing a rationale for the effective disease management strategies. Antibiosis refers to the inhibition of pathogen by the bioactive secondary metabolites produced by the antagonistic PGPR. The bioactive secondary metabolites include volatile compounds, toxic compounds, and antibiotics, which are deleterious to the growth or metabolic activities of other microorganisms at low concentrations (Fravel 1988; Jayaprakashvel and Mathivanan 2011).

In biological control of plant diseases, the use of PGPR whole microorganisms as bioinoculants has a bottleneck because it requires at least little skill which appears to be cumbersome for the resource-poor farmers in the developing countries. Due to some factors such as unsatisfactory performance of whole cell PGPR in fields, non-availability of viable bioformulations in rural areas, and the fascination among farmers over the immediate cure by the chemical agents instead of the slow-acting BCAs (Jayarakashvel and Mathivanan 2011; Sekar et al. 2016; Tabassum et al. 2017), the use of bioactive secondary metabolites of the PGPR in disease control similar to that of fungicides in the field assumes much significance. Because of their biological origin, these metabolites do not cause any environmental pollution as against their counterparts, fungicides. Hence, in the recent years, the use of secondary metabolites of microbial origin is gaining momentum in crop protection, and such metabolites may be a supplement or an alternative to chemical control (Suzni 1992; Tanaka and Omura 1993; Yamaguchi 1996; Prabavathy et al. 2006; Prabavathy et al. 2008; Mathivanan et al. 2008; Jayaprakashvel and Mathivanan 2011; Jayaprakashvel et al. 2014). Hence, in recent years, the interest in using microbial metabolites for the plant disease control has been renewed because of their greater advantages which are depicted in Fig. 15.2.

## 15.5 Metabolites of PGPR for the Management of SBPF

Strategies for the management of SBPF in the modern systems cannot be a single approach but a multiple of promising disease management strategies. The prospective use of PGPR especially those that produce antimicrobial metabolites against SBPF could be a wise choice for the management of soilborne diseases of crop plants. According to Landa et al. (2013), we are currently far away from being able to understand and exploit the full potential of PGPR as an effective disease management strategy at field scale. PGPR produce a wide array of secondary metabolites such as siderophores, antibiotics, volatile metabolites, and other allelochemicals.

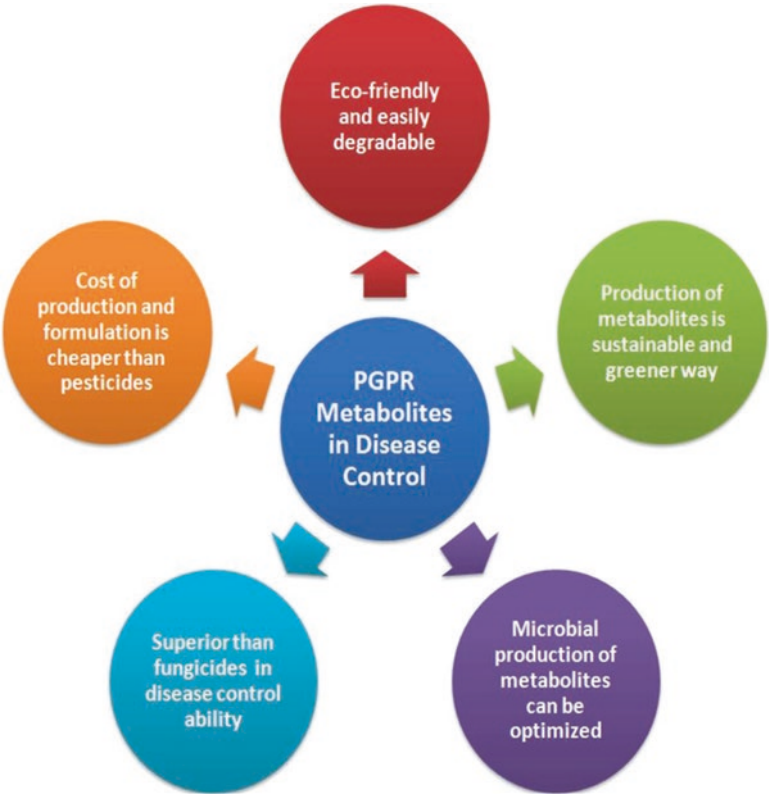


**Fig. 15.2** Advantages of using metabolites of PGPR over fungicides in plant disease control

Their mode of action and molecular mechanisms provide a great awareness for their application for the management of SBPF (Lugtenberg and Kamilova 2009; Saraf et al. 2014).

Antibiosis is considered as one of the most powerful and studied mechanisms of PGPR for combating phytopathogens. Antibiotics encompass a wide and heterogeneous group of low molecular weight organic compounds that are produced by a wide variety of microorganisms. They are deleterious to the growth or metabolic activities of other microorganisms at low concentrations (Fravel 1988; Thomashow 1996). Numerous antibiotics such as 2,4-diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyoluteorin, pyrrolnitrin, oomycinA, viscosinamide, butyrolactones, kanosamine, bacillomycin, iturin A (cyclopeptide), zwittermicin A, aerugine, rhamnolipids, cepaciamide A, ecomycins, pseudomonic acid, azomycin, etc. have been isolated from various PGPR strains representing different bacterial genera (Fernando et al. 2005).

Though a wide number of PGPR genera are reported to produce antibiotic metabolites against SBPF, *Bacillus* and *Pseudomonas* are the two most important genera among the PGPR whose metabolites are intensively studied for their

efficiency in controlling various soilborne diseases caused by SBPF (Jayaprakashvel and Mathivanan 2011; Beneduzi et al. 2012; Prathap and Ranjitha 2015), and hence, the forthcoming sections of this chapter focuses exclusively on the metabolites of *Pseudomonas* and *Bacillus*.

### 15.5.1 Secondary Metabolites of *Pseudomonas*

*Pseudomonas* is a long-studied and comfortably used biocontrol agent in the management of SBPF over several decades. *Pseudomonas* is a versatile organism that is capable of producing an astonishing array of antimicrobial secondary metabolites (Jayaprakashvel and Mathivanan 2011; Mishra and Arora 2018) against SBPF and has greater rhizosphere competence (Adesina et al. 2009; Barret et al. 2011; Schreiter et al. 2018) and ease of bioformulation (Tabassum et al. 2017). Effective antibiotic metabolites such as 2,4-diacetylphloroglucinol, oomycin A, phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyocyanin, anthranilate, pyrrolnitrin, pyoluteorin, hydrogen cyanide, ammonia, viscosinamide, and gluconic acid are frequently reported to control many SBPF in different crop plant world over. Research and comprehensive reviews over the past few decades arguably prove that pseudomonads are the most important soil microbial communities that are having exceptional potential in controlling SBPF (Dowling and O'Gara 1994; Dwivedi and Johri 2003; Chin-A-Woeng et al. 2003; Mathivanan et al. 2005; Shanmugaiah et al. 2006; Jayaprakashvel et al. 2010a, b; Mishra and Arora 2018). Most of the *Pseudomonas* biocontrol strains produce antifungal metabolites (AFMs) such as 2,4-diacetylphloroglucinol (DAPG), pyoluteorin (PLT), pyrrolnitrin (PRN), phenazine-1-carboxylic acid (PCA), 2-hydroxy phenazines, and phenazine-1-carboxamide (PCN) (Bloemberg and Lugtenberg 2001; Shanmugaiah et al. 2010). However, new AFMs belonging to the class of cyclic lipopeptides, such as viscosinamide (Nielsen et al. 1999) and tensin (Nielsen et al. 2000), have been discovered. Having considered the process of root colonization by pseudomonads, the biotic and abiotic factors affecting colonization, bacterial traits and genes contributing to rhizosphere competence, and the mechanisms of pathogen suppression, Weller (2007) has concluded that *Pseudomonas* spp. are well suited as biocontrol agents of soilborne pathogens. The antimicrobial secondary metabolites of several strains of *Pseudomonas* that are reported as responsible for the biological control of soilborne pathogenic fungi in different crops are listed in Table 15.1. The published body of literature evidently concluded that *Pseudomonas* spp. is the single largest bacterial genus that produces an array of secondary metabolites against SBPF.

### 15.5.2 Secondary Metabolites of *Bacillus*

Next to *Pseudomonas*, *Bacillus* is the widely studied PGPR for the biological control of plant diseases especially the soilborne diseases of crop plants. *Bacillus* species are very interesting PGPR that have special characteristics such as formation of

**Table 15.1** Secondary metabolites of pseudomonads characterized as mechanism of biological control of soilborne pathogenic fungi

| Sl. No. | Metabolite | Organism | SBPF/soilborne disease controlled | Host | References |
|---|---|---|---|---|---|
| 1 | 2,4-diacetylphloroglucinol (DAPG) | *Pseudomonas fluorescens* | *Fusarium oxysporum* | Several crops | Schouten et al. (2004), Meyer et al. (2016) and Maurhofer et al. (1995) |
| 2 | 2,4-diacetylphloroglucinol (DAPG) | *Pseudomonas fluorescens* | *Gaeumannomyces graminis* var. *tritici* | Wheat | Kwak et al. (2009) |
| 3 | Pyoluteorin | *Pseudomonas putida* strain NH-50 | Red rot | Sugar cane | Hassan et al. (2011) |
| 4 | Pyoluteorin | *Pseudomonas* sp. | *Rhizoctonia solani* | – | Vinay et al. (2016) |
| 5 | Phenazine-1-carboxylic acid | *Pseudomonas fluorescens* | *Rhizoctonia* root rot | Wheat | Robert et al. (2004) |
| 6 | Phenazine-1-carboxylic acid (PCA) | *Pseudomonas fluorescens* | *Fusarium oxysporum* | – | Upadhyay and Srivastava (2011) |
| 7 | Phenazine-1-carboxamide (PCN) | *Pseudomonas aeruginosa* | *Rhizoctonia solani* | Rice | Shanmugaiah et al. (2010) |
| 8 | Viscosinamide | *Pseudomonas fluorescens* DR54 | *Pythium ultimum* | Sugar beet | Thrane et al. (2000) |
| 9 | Phenazine-1-carboxylic acid (PCA) | *Pseudomonas chlororaphis* (MCC2693) | *Phytophthora* sp. *Fusarium* sp. | – | Jain and Pandey (2016) |
| 10 | Volatile antifungal furanone | *P. chlororaphis* strain 63-28 | *Pythium ultimum*, *Fusarium solani*, *Fusarium oxysporum*, and *Thielaviopsis basicola* | – | Paulitz et al. (2000) |
| 11 | Hydrogen cyanide | *Pseudomonas* sp. | *R. solani* | Rice | Jayaprakashvel et al. (2010a, b) |
| 12 | Ammonia | *Pseudomonas aeruginosa* | *Sclerotium rolfsii* | – | Baligh et al. (1996) |
| 13 | Surfactants | *Pseudomonas fluorescens* | *Phytophthora capsici* | Pepper | Özyilmaz and Benlioglu (2013) |

| | | | | | |
|---|---|---|---|---|---|
| 14 | Massetolide A, cyclic lipopeptide | *Pseudomonas fluorescens* | *Phytophthora infestans* | Tomato | Tran et al. (2007) |
| 15 | Viscosin-like cyclic lipopeptide | *Pseudomonas fluorescens* HC1-07 | *Rhizoctonia* root rot | Wheat | Yang et al. (2014) |

heat- and desiccation-resistant endospores that can be formulated as a stable dry white powder with a long shelf life. Members of the genus *Bacillus* are excellent producers of broad-spectrum antibiotics through which they occupy a pivotal position in the biological control of plant pathogenic bacteria and fungi (Pengnoo et al. 2000; Abanda-Nkpwatt et al. 2006; Prashanth and Mathivanan 2009; Jayaprakashvel and Mathivanan 2011). Awais et al. (2010) have reviewed that almost 167 antibiotics are produced by the genus *Bacillus* of which 66 are derived from *B. subtilis* and 23 from *B. brevis* and the remaining peptide antibiotics are produced by other species of genus *Bacillus.* Because of the broad-spectrum antibiotics, many of the *Bacillus* species have proved to be effective against a broad range of plant pathogens. Similar to metabolically diverse pseudomonads, *Bacillus* spp. are also reported to exhibit plant growth promotion, induce systemic resistance, produce antibiotic secondary metabolites, and exhibit competition for space and nutrients with SBPF (Shafi et al. 2017; Prashanth 2007). *Bacillus* spp. are reported to produce zwittermicin A and amphiphilic cyclic lipopeptides (CLPs) such as iturin, fengycin (or plipastatin), and surfactin (Romero et al. 2007; Shafi et al. 2017). Besides, *Bacillus* spp. are found to produce other antibiotics such as kanosamine, rhizocticin C, and saltavalin, and they are also capable of producing thermostable antimicrobial peptides (Emmert and Handelsman 1999; Kavitha et al. 2005; Jayaprakashvel and Mathivanan 2011). The ability of various *Bacillus* strains to control fungal soilborne, foliar, and post-harvest diseases has been attributed mostly to iturins and fengycins (Ongena and Jacques 2008; Romero et al. 2007; Arrebola et al. 2010). Different groups of antifungal bacillomycin such as bacillomycin Lc, bacillomycin L, bacillomycin D, bacillomycin F, and bacillopeptins that were identified from different strains of *B. subtilis* were effective against fungal pathogens (Fernando et al. 2005). Apart from the production of broad-spectrum antibiotics, members of the genera *Bacillus* are efficient in solubilization and mobilization of mineral nutrients important for plant growth such as phosphate, zinc, and silica (Beneduzi et al. 2012). They are also very efficient in inducing systemic resistance in the crop plants (Akram et al. 2013) and have direct antifungal activity through the production of fungal cell wall lytic enzymes such as chitinase and glucanase (Swain et al. 2008). Various categories of secondary metabolites produced by *Bacillus* spp. which have potential to inhibit SBPF are listed in Table 15.2.

The studies done so far and ongoing throughout the world indicate that there has been a renewed interest among researchers to consider *Bacillus* as the most preferred biological control agents for the management of soilborne diseases due to their ability in producing broad-spectrum antibiotics, survival in adverse environments through endospores, mobilization of plant nutrients, and abundant presence in soil.

### 15.5.3 Secondary Metabolites of Other PGPR

Apart from *Bacillus* and *Pseudomonas*, though many PGPR were found associated with plant growth promotion, a limited number of genera have been studied with

**Table 15.2** Secondary metabolites of *Bacillus* spp. characterized as mechanism of biological control of soilborne pathogenic fungi

| Sl. No. | Metabolite | Organism | SBPF/soilborne disease controlled | Host | References |
|---|---|---|---|---|---|
| 1 | Iturin-like compounds | *B. amyloliquefaciens* strain A1Z | *Sclerotinia* stem rot, charcoal rot, and fusarial wilt | Soybean | Romero et al. (2007) |
| 2 | Zwittermicin A | *B. cereus* UW85 | *Phytophthora medicaginis* | Alfalfa | Stabb et al. (1994) |
| 3 | Bacillomycin D | *Bacillus amyloliquefaciens* FZB42 | *Fusarium graminearum* | Wheat | Gu et al. (2017) |
| 4 | Fengycins | *Bacillus mojavensis* RRC101 | *Fusarium verticillioides* | Maize | Blacutt et al. (2016) |
| 5 | Surfactin, iturin and fengycin | Two strains of *Bacillus velezensis* | *Fusarium oxysporum* | – | Cao et al. (2018) |
| 6 | Zwittermicin A and kanosamine | *Bacillus cereus* | *Pythium* sp. | Tobacco | Shang et al. (1999) |
| 7 | Lantibiotic ericin | *Bacillus velezensis* RC 218 | Fusarium sp. | – | Palazzini et al. (2016) |
| 8 | Fengycin, bacillomycin, bacilysin, surfactin, and iturin A | *B. subtilis* Bs 8B-1 | *Pythium*, *Phytophthora*, *Rhizoctonia* | Cucumber and radish | Khabbaz et al. (2015) |
| 9 | Bacilysin | *Bacillus pumilus* | *Phytophthora* | Potato | Caulier et al. (2018) |
| 10 | Fengycin, mycosubtilin, subtulene | B. subtilis | *F. graminearum*, *R. solani*, *Pythium irregulare* | – | Chen et al. (2008) |

reference to their bioactive metabolites involved in the biological control of soilborne pathogens. *Serratia plymuthica* has received steadily increasing attention as a biological control agent to control both soilborne and foliar pathogens (de Vleesschauwer and Höfte 2007). de Vleesschauwer and Höfte (2007) have made a comprehensive review and emphasized on the use of S. *plymuthica* as biocontrol agent against many soilborne pathogens such as *Rhizoctonia*, *Pythium*, *Verticillium*, etc. Someya et al. (2010) have reported the biocontrol potential of *S. plymuthica* against sheath blight disease in rice. Besides usually reported PGPR, in recent years, actinobacteria are also isolated as rhizobacteria from crop plant roots and associated samples. *Streptomyces* sp. J-2 was found to have biocontrol potential against *Sclerotium rolfsii* damping-off of sugar beet by reducing the disease incidence significantly (Errakhi et al. 2007). *Streptomyces philanthi* RL-1-178 could protect the chili pepper plants from *S. rolfsii* and resulted in 58.75% survival of chili pepper plants against stem and root rot (Boukaew et al. 2011). Jacob et al. (2016) have demonstrated that the cell-free culture filtrates of *Streptomyces* sp. RP1A-12 (capable of producing siderophores) were helpful in managing groundnut stem rot

disease caused by *S. rolfsii*. *Streptomyces* sp. IISRBPAct1, a rhizobacteria, was reported to suppress the growth of *S. rolfsii* and protected the black pepper plant by the production of siderophores. It was also reported to reduce the foot rot incidence up to 80% (Thampi and Bhai 2017). *Streptomyces lydicus* was effectively demonstrated to have biocontrol potential against *S. sclerotiorum* (Zeng et al. 2012). Kunova et al. (2016) have developed a screening protocol for the selection of *Streptomyces* to be used as biocontrol agents against soilborne fungal pathogens. A PGPR, *Chryseobacterium balustinum* CECT 5399 along with a *Bacillus* and *Pseudomonas*, was reported to exhibit a synergistic effect on growth promotion and biocontrol on tomato and pepper against *Fusarium* wilt and *Rhizoctonia* damping-off (Domenech et al. 2006). Though ubiquitous PGPR such as *Bacillus* and *Pseudomonas* are quite extensively studied for their role in biological control of soilborne diseases, attention has been considerably paid over isolating and characterizing newer genera of PGPR for the management of SBPF (Vijayan et al. 2012). Especially, actniobacteria, remarkable producers of antibiotics, which actually dominate the production and diversity of antibiotics for human therapy, are now given due attention. These kinds of alternative PGPR sources may contribute to the isolation of newer antibiotics with newer mechanisms. However, information about the metabolites involved in the biological control of other PGPR is very scanty. More studies are warranted in this area.

## 15.6 Mechanisms of PGPR Metabolites Against Soilborne Pathogenic Fungi (SBPF)

Research and review articles suggest that biological control of SBPF still remains as a hurdle due to three major characteristics of these pathogens (Chet et al. 1991; Alabouvette and Steinberg 2006):

1. Long-term persistence of survival structures
2. Present in soil with high inoculums density
3. Lack of natural resistant sources

Hence, the PGPR to be used for the control of soilborne disease are to be characterized for their ability to overcome the above limitation. While the lack of natural resistant sources totally relies on the host plant, it is worthy to study, either PGPR as a whole or their metabolites for their ability to inhibit the long-time persistence of SBPF by disintegrating their resting structures and reducing their inoculums in soil. A *Streptomyces* strain J-2 has inhibited the germination of *S. rolfsii* and therefore protected the sugar beet from damping-off of sugar beet (Errakhi et al. 2007). Cell-free culture filtrates of *Bacillus* spp. and *Pseudomonas* spp. have completely inhibited the germination of *Rhizoctonia solani and Sclerotium* and provided significant protection against sheath blight disease of rice (Jayaprakashvel 2008). The cell-free culture filtrates of *Streptomyces* sp. RP1A-12 effectively inhibited the *Sclerotium* of *S. rolfsii* through which the groundnut stem rot disease was reduced.

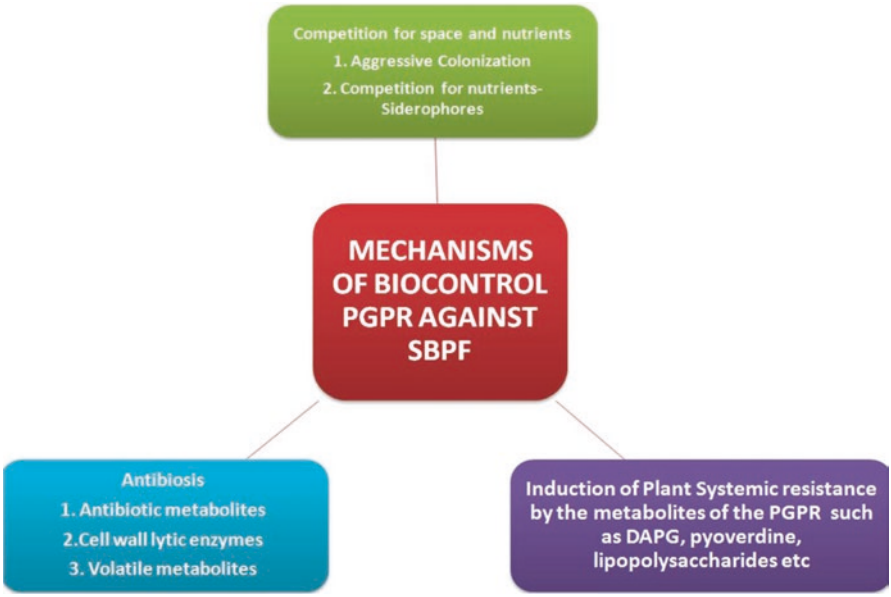


**Fig. 15.3** Mechanisms of PGPR for the biological control of soilborne pathogenic fungi

PGPR used in the biological control of soilborne diseases may adversely affect the population density, dynamics (temporal and spatial), and metabolic activities of soilborne pathogens by exerting three major types of mechanisms such as competition, antagonism, and hyperparasitism (Raaijmakers et al. 2009). Though the metabolites of PGPR contribute for the direct antagonism against SBPF, other mechanisms have also received considerable attention. Though antibiotics of many PGPR were convincingly proved to contribute for the suppression of plant pathogens, their actual role in biocontrol is questioned due to constraints of antibiotic production under natural environmental conditions. Studies on the effect of host plant interactions and other environmental factors on the production of antibiotics by PGPR are less intensive (Fernando et al. 2005). Whereas hyperparasitism operates only in fungal biocontrol agents, induction of systemic resistance of plants against pathogens is considered as one of biocontrol mechanisms of PGPR against SBPF. Figure 15.3 briefly summarizes the three different mechanisms of PGPR while suppressing the SBPF.

## 15.7 Bioprocess Optimization of Metabolites for SBPF Disease Management

The biosynthesis of antibiotic metabolites by PGPR either in planta or in vitro largely depends on the interactions between the pathogen and PGPR, environmental conditions, nutritional requirements, and growth conditions. Pathogen metabolites

have a definite role in modulating the biosynthesis of secondary metabolites in many of the PGPR (Raaijmakers et al. 2007). Pathogen metabolites such as fusaric acid of *Fusarium* spp. repressed the production of antibiotics such as DAPG in pseudomonads (Duffy and Défago 1997). Autoinducers, such as N-acylhomoserine lactones (AHLs), also regulate the production of antibiotics both in culture and in the environments (Zhang and Dong 2004).

By optimizing the glycerol concentration and C/N ratio in the production medium in a bioreactor, cell density and quantity of DAPG and siderophores were increased in a fluorescent pseudomonad strain R81 (Sarma et al. 2010). Physicochemical conditions and inoculum size were optimized using response surface methodology in a *Streptomyces* sp. for the enhanced production of antibiotic secondary metabolites against *R. solani* (Ahsan et al. 2016). A *P. fluorescens* used as a biocontrol agent in strawberry was optimized through response surface methodology by modifying four fermentation parameters to enhance the biomass and production of phenazine antibiotics and siderophores (Haggag and El Soud 2012). Song et al. (2012) have optimized fermentation conditions such as inoculum volume, temperature, and pH for antibiotic production by an actinomycete strain YJ1 against *Sclerotinia sclerotiorum*. An actinomycete strain *Streptomyces lavendulae* Xjy, used as a biocontrol agent for the suppression of two diseases in apple, was optimized for enhanced production of antibiotics by modifying the nutrient and fermentation parameters (Gao et al. 2015). Since a paradigm shift is yet to happen from the use of whole microbial cells to the use of microbial metabolites of the PGPR, studies on optimization of bioprocesses for the production of antifungal metabolites against SBPF by the PGPR are very limited. However, fundamental techniques have been successfully validated in many other productions systems (Singh et al. 2016a, b) which can be very well extrapolated with the biological control agents when necessity arises.

## 15.8 Perspectives of Genetic Modification of PGPR, Plants, and Pathogens for Enhanced Protection Against SBPF

PGPR involved in the biological control of plant disease can be improved further by combining different biocontrol traits in a single organism without affecting its normal function (Glick and Bashan 1997). Having understood the immense significance of induction of systemic resistance in plants by the PGPR, Thomashow (1996) has envisaged that cloning and sequencing of genes involved in the production of microbial metabolites of the PGPR could open new possibilities for improving the performance by modulating the plant resistance mechanisms.

In this context, studies on understanding the genetic and biochemical basis of disease control and the influence of environmental factors on the expression and activity of biocontrol mechanisms have been undertaken by various researchers. The genetic background of important PGPR such as *Pseudomonas* was studied with reference to their rhizosphere competence, and biocontrol traits opened up new avenues for a better exploitation of their plant-beneficial properties for sustainable agriculture. Such genetic analysis would pave way to enhance the nonproducing

biocontrol agents into efficient BCAs due to the transfer of genes responsible for the biosynthesis of antimicrobial secondary metabolites (Couillerot et al. 2009; Robert et al. 2004). Site-directed mutagenesis has been effectively used as a tool to find the biosynthetic genes of gene clusters in PGPRs. Through a combination of genetic (knockout mutagenesis) and chemical techniques (mass spectroscopy), Chowdhury et al. (2015) have identified a total of ten gene clusters involved in the biosynthesis of cyclic lipopeptides, polyketides (three), bacilysin, and plantazolicin and amylocyclicin in *Bacillus amyloliquefaciens*. Thanks to genetic engineering technology, it is now possible to genetically modify all components of the rhizosphere such as plants and microbes to promote enhanced protection against soilborne diseases. Recent approaches suggest the possibilities of overall soil microbial population engineering rather than single strain engineering (Dessaux et al. 2016). However, efforts in these lines are made sporadically, and we have to go a long way to achieve as envisaged in the past and present.

## 15.9 Conclusion

Biological control of soilborne pathogenic fungi by the antimicrobial metabolites of the plant growth-promoting rhizobacteria has achieved significant success, and prospects in the future are enormous. Though many of the PGPR are widely used in the biological control of SBPF, *Bacillus* and *Pseudomonas* are the two most extensively characterized PGPR genera for their metabolites. The metabolites of the PGPR exhibit various mechanisms to control the SBPF. Production of bioactive metabolites by the PGPR could be enhanced through process optimization and genetic improvement. Thus, the metabolites of PGPR could be a potential choice for the effective management of plant diseases caused by soilborne pathogenic fungi.

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# Part III

# Endophytic PGPRs

# 16 Exploring the Beneficial Endophytic Microorganisms for Plant Growth Promotion and Crop Protection: Elucidation of Some Bioactive Secondary Metabolites Involved in Both Effects

Rania Aydi Ben Abdallah, Hayfa Jabnoun-Khiareddine, and Mejda Daami-Remadi

## 16.1 Introduction

The most widely studied group of beneficial microorganisms are the plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere (Kloepper et al. 1999; Singh et al. 2016, 2017). As reviewed by Gray and Smith (2005), some of these PGPR can also enter the inner root tissues and establish endophytic populations. Endophytic microorganisms, as those in the rhizosphere, are conditioned by biotic and abiotic factors, but endophytes could be better protected from biotic and abiotic stresses than rhizospheric microorganisms (Hallmann et al. 1997). Endophytes comprise a large but little explored share of fungal diversity (Perottoab et al. 2013). Despite their different ecological niches, free-living rhizobacteria and endophytes use the same mechanisms to promote plant growth and control phytopathogens (Compant et al. 2005). Using endophytes such as biocontrol and/or biofertilizing agents in various crops has become more and more interesting in organic farming due to their richness in bioactive natural products (Li et al. 2008; Molina et al. 2012). Mutualistic interactions between endophytes and host plants may result in fitness benefits for both partners (Kogel et al. 2006). Endophytes are able to produce a wide range of bioactive compounds with superior biosynthetic capabilities for someone due to their presumable gene recombination with the host while residing and reproducing inside the healthy plant tissues (Li et al. 2005). Searching of new antimicrobial compounds is important to overcome the difficulties related to pathogen resistance (Petersen

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et al. 2004). Thus, endophytic microorganisms have emerged as an alternative source for the production of new antimicrobial agents to inhibit plant pathogenic agents and consequently enhance plant growth (Gaiero et al. 2013).

This chapter focuses on the interaction between endophytes and host plants by elucidating the main mechanisms of action displayed by the beneficial endophytes for the improvement of plant growth and the protection of plant health.

## 16.2 Microorganisms Recovered as Endophytes

### 16.2.1 Definition, Recognition, and Identification

Endophytic microorganisms grow within the healthy tissues of living plants during all or part of their life cycle without causing harmful effects on the host (Hallmann et al. 1997; Sturz et al. 2000; Ray et al. 2017). These microorganisms are often isolated from surface-sterilized tissues or from internal plant tissues. Endophytes either remain localized to their points of entry or spread to other parts of the plant (Hallmann et al. 1997). They occupy the interior of cells, intercellular spaces, or the vascular system of various plant species (Hallmann et al. 1997; Sturz et al. 2000; Rosenblueth and Martínez-Romero 2006). Although the populations of endophytes vary depending on many factors such as microorganism species, host genotypes, host developmental stage, and environmental conditions, bacterial populations are usually larger in roots and lower in stems and leaves (Lamb et al. 1996). Some of them are able to colonize reproductive organs such as flowers, fruits, and seeds (Malfanova et al. 2013).

Endophytic microorganisms may be isolated from surface-disinfected tissues and visualized inside plant tissues (Fig. 16.1) after labeling with green fluorescent protein (*GFP)* (Reinhold-Hurek and Hurek 1998; McDouga et al. 2012) or staining with β-glucuronidase (*GUS*) (Compant et al. 2005; Botta et al. 2013). The two later criteria are not always respected. The use of the term "putative endophytes" was recommended to qualify those that could not be validated microscopically. Endophytes may also be recognized by their ability to colonize disinfected seedlings (Rosenblueth and Martínez-Romero 2006).

Molecular identification of bacterial endophytic may be accomplished through sequencing of the 16S rDNA gene or through marker analysis techniques such as RFLP (restriction fragment length polymorphism) and DGGE (denaturing gradient gel electrophoresis) (Ryan et al. 2008). Fungal endophytes were identified based on their morphological traits and molecular phylogenetic analysis of the internal transcribed spacer (ITS) rDNA and/or 5.8S rDNA sequencing genes (Huang et al. 2009; Yoo and Eom 2012).

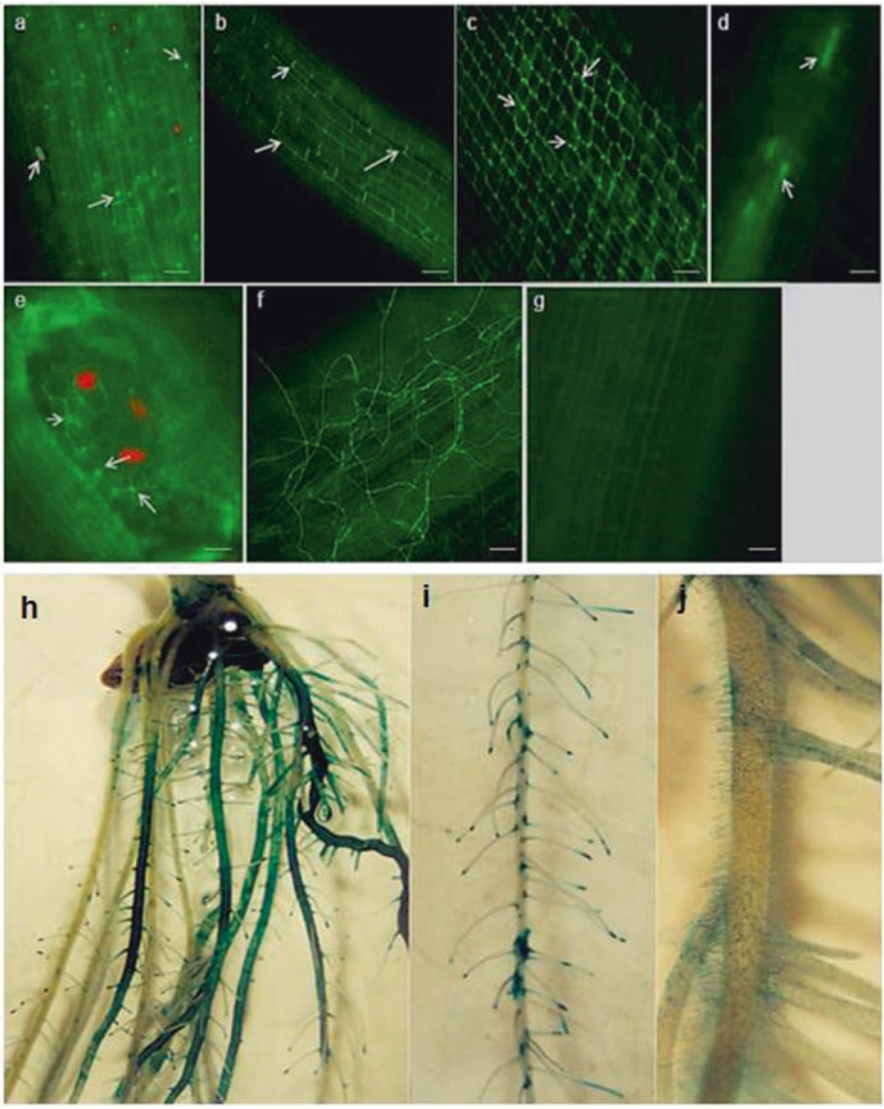


**Fig. 16.1** Colonization of the roots of rice by *GFP*-tagged strain of *Ustilaginoidea virens* and *GUS* gene-tagged strain of *Pantoea agglomerans*. (**a**) Spores of the GFP-tagged strains of *U. virens* after 1 dpi. (**b**) Fungal colonization on the rice root tended to form runner hyphae (*arrow*) (Bar = 100 μm). (**c**) Growing hyphae forming a net-like structure on the root surface 4 dpi (Bar = 100 μm). (**d**) Lesion formed on the adjacent region between the main root and the fibrous root (Bar = 100 μm). (**e**) Lesion formed on the region of the main root and the fungi located on the lesion (Bar = 200 μm). (**f**) Mycelium densely covering the main root after 15 dpi (Bar = 200 μm). (**g**) Control (Bar = 100 μm); 1-week-old seedling was inoculated with *GUS*-tagged strain of *P. agglomerans*, and root system was stained 21 days after inoculation. (**h**) Root system showing extensive colonization with the bacterial strain. (**i**) One of the main roots showing colonization of the root caps and points of emergence of lateral roots. (**j**) Stained root hairs indicating colonization by the *GUS*-tagged strain of *P. agglomerans.* (Verma et al. 2001; Andargie et al. 2015)

### 16.2.2 Diversity and Populations of Endophytic Microorganisms

In general, endophytic populations are at low densities compared to rhizosphere populations (Rosenblueth and Martínez-Romero 2006). Endophytes, colonizing the same host plant, are not limited to a single species, but they can include several genera and species. The density of endophyte populations varied mainly depending on microbial species, host genotypes, development stage of host plant, colonized tissues, and environmental conditions (Tan et al. 2003).

Endophytes harbor all plants (Ryan et al. 2008). They have been isolated from potato tubers (Sturz et al. 2002); tomato (Patel et al. 2012); pepper (Sziderics et al. 2007; Paul et al. 2013); cotton and sweetcorn (Mclnroy and Kloepper 1995); coffee (Vega et al. 2005); sweet potato (Khan and Doty, 2009); sugarcane (Magnani et al. 2010); citrus, alfalfa, and laurel roots (Kalai-Grami et al. 2014); and *Cestrum nocturnum* (Aydi Ben Abdallah et al. 2017a). Microbial endophytes colonize mainly wild species such as *Prosopis strobilifera* (Sgroy et al. 2009), *Huperzia serrata* (Wang et al. 2010), *Suaeda maritima*, *Carex scabrifolia*, and *Elymus mollis* (Bibi et al. 2012) and wild *Solanaceae* species such as *Nicotiana attenuata*, *N. glauca*, *Solanum trilobatum*, *S. melongena*, *S. torvum*, *S. nigrum*, *S. elaeagnifolium*, *Datura stramonium*, and *D. metel* (Nimal et al. 2012; Bhuvaneswari et al. 2013; Izhaki et al. 2013; Achari and Ramesh 2014; Kuriakose et al. 2014; Mahdi et al. 2014; Santhanam et al. 2014; Aydi Ben Abdallah et al. 2017b).

Since the first published reports on the isolation of endophytic bacteria from surface-sterilized plant tissues (Mundt and Hinkle 1976), more than 200 genera of bacteria have been reported as endophytes. These endophytic bacteria include culturable and non-culturable bacteria (Berg and Hallmann 2006; Manter et al. 2010; Sessitsch et al. 2012). The most widely studied endophytic bacteria belong to three major branches including *Actinobacteria*, *Proteobacteria*, and *Firmicutes* including the genera of *Azoarcus* (Krause et al. 2011), *Acetobacter* (renamed *Gluconobacter*) (Bertalan et al. 2009), *Bacillus* (Deng et al. 2011), *Enterobacter* (Taghavi et al. 2010), *Burkholderia* (Weilharter et al. 2011), *Herbaspirillum* (Pedrosa et al. 2011), *Pseudomonas* (Taghavi et al. 2009), *Serratia* (Taghavi et al. 2009), *Stenotrophomonas* (Ryan et al. 2009), *Alcaligenes* (Castro et al. 2014), and *Streptomyces* (Suzuki et al. 2005). Concerning fungi, there are at least 1 million species of endophytic fungi (Ganley et al. 2004). The mostly known genera of endophytic fungi are *Aspergillus*, *Curvularia*, *Emericella*, *Chaetomium* (Mahdi et al. 2014), *Alternaria*, *Colletotrichum*, *Phomopsis*, *Xylaria* (Huang et al. 2009), *Beaveria*, *Trichoderma*, *Phoma*, and *Acremonium* (Orole and Adejumo 2009). Some of these endophytes exhibit host and tissue specificity (Table 16.1).

Species of these genera are ubiquitous in the rhizosphere that represents the main source of endophytes (Berg and Hallmann 2006). Other possible sources of endophytes include phyllosphere through the stomata as demonstrated for *Gluconobacter diazotrophicus* recovered from sugarcane (James et al. 2001), *Streptomyces galbus* associated with rhododendron (Suzuki et al. 2005), and *Lophodermium conigenum* and *Septoria pini-thunbergii* isolated from coniferous trees (Yoo and Eom 2012).

**Table 16.1** Representative list of endophytes in host tissues

| Endophyte | Plant species | Plant part | References |
|---|---|---|---|
| *Pseudomonas* sp. | *Brassica napus* L. | Roots | Misko and Germida (2002) |
| *Pseudomonas* sp. | *Glycine max* L. | Leaves, stems, roots | Kuklinsky-Sobral et al. (2005) |
| *Pseudomonas* sp. | *Oryza sativa* L. | Stems, roots | Adhikari et al. (2001) |
| *Pseudomonas* sp. | *Vitis vinifera* L. | Xylem sap | Bell et al. (1995) |
| *Pseudomonas* sp. | *Pisum sativum* L. | Stems | Elvira-Recuenco and Van Vuurde (2000) |
| *Pseudomonas* sp. | *Datura metel* | Roots | Aydi Ben Abdallah et al. (2016c) |
| *P. aeruginosa* | *Solanum lycopersicum* | Roots and stems | Patel et al. (2012) |
| *Stenotrophomonas maltophilia* | *Oryza sativa* L. | Roots | Zhu et al. (2012) |
| *S. maltophilia* | *Zea mays* L. | Roots and stems | McInroy and Kloepper (1995) |
| *S. maltophilia* | *Coffea* L. | Seeds | Zhang and Yuen (1999) |
| *S. maltophilia* | *Datura stramonium* | Stems | Aydi Ben Abdallah et al. (2016a) |
| *S. maltophilia* | *D. metel* | Stems | Aydi Ben Abdallah et al. (2016c) |
| *Bacillus amyloliquefaciens* | *S. lycopersicum* | Stems | Nawangsih et al. (2011) |
| *B. endophyticus* | *Nicotiana glauca* | Leaves | Izhaki et al. (2013) |
| *B. megaterium* | *N. glauca* | Leaves | Izhaki et al. (2013) |
| *B. niacini* | *N. glauca* | Leaves | Izhaki et al. (2013) |
| *B. simplex* | *N. glauca* | Leaves | Izhaki et al. (2013) |
| *B. stratosphericus* | *N. glauca* | Leaves | Izhaki et al. (2013) |
| *B. cereus* | *N. glauca* | Stems | Aydi Ben Abdallah et al. (2016e) |
| *B. tequilensis* | *Solanum elaeagnifolium* | Stems | Aydi Ben Abdallah et al. (2016b) |
| *Alcaligenes faecalis* | *Tabernaemontana divaricata* | Leaves | Pradeepa and Jennifer (2013) |
| *A. faecalis* | *Withania somnifera* | Fruits | Aydi Ben Abdallah et al. (2016d) |
| *A. faecalis* | *Nicotiana glauca* | Stems | Aydi Ben Abdallah et al. (2016e) |
| *Serratia* sp. | *Cestrum nocturnum* | Leaves | Aydi Ben Abdallah et al. (2017a) |
| *Streptomyces* sp. | *Solanum nigrum* | Roots | Goudjal et al. (2013) |
| *Aspergillus pulvinus* | *D. stramonium* | Stems | Mahdi et al. (2014) |
| *A. terreus* | *D. stramonium* | Stems | Mahdi et al. (2014) |
| *A. flavus* | *D. stramonium* | Stems | Mahdi et al. (2014) |
| *Curvularia* sp. | *D. stramonium* | Stems | Mahdi et al. (2014) |
| *Fusarium tricinctum* | *S. nigrum* | Leaves | Khan et al. (2015) |

(continued)

**Table 16.1** (continued)

| Endophyte | Plant species | Plant part | References |
|---|---|---|---|
| *Alternaria alternata* | *S. nigrum* | Leaves | Khan et al. (2015) |
| *Emericella* sp. | *Moringa oleifera* | Stems and leaves | Mahdi et al. (2014) |
| *Aspergillus tamari* | *M. oleifera* | Stems and leaves | Mahdi et al. (2014) |
| *A. parasiticus* | *M. oleifera* | Stems and leaves | Mahdi et al. (2014) |
| *Emericella rugulosa* | *Prosopis chilensis* | Stems | Mahdi et al. (2014) |
| *E. nidulans* | *P. chilensis* | Stems | Mahdi et al. (2014) |
| *Aspergillus niger* | *P. chilensis* | Stems | Mahdi et al. (2014) |
| *Alternaria alternata* | *Artemisia capillaris* | Inflorescences | Huang et al. (2009) |
| *Colletotrichum gloeosporioides* | *Artemisia indica* | Leaves | Huang et al. (2009) |
| *C. gloeosporioides* | *Artemisia lactiflora* | Leaves, stems | Huang et al. (2009) |
| *Phomopsis* | *A. indica* | Stems | Huang et al. (2009) |
| *bougainvilleicola* | *A. indica* | Stems | Huang et al. (2009) |
| *Lophodermium conigenum* | *Pinus densiflora* | Leaves | Yoo and Eom (2012) |

## 16.3 Plant Colonization

### 16.3.1 Establishment in the Rhizosphere

Colonization of plant tissues by microorganisms begins usually by their establishment in the rhizosphere. Climatic and edaphic factors can be equally important in influencing endophyte microbiome community structure that can inhabit the bulk soil (Fig. 16.2). For example, *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* associated with *Osmorhiza depauperata* roots were more abundant at sites with higher precipitation and annual temperature, while *Paenibacillus* strains were more common at sites with higher latitudes and lower precipitation (Li et al. 2012). The diversity of *Frankia* spp. communities was found to be highest in plants grown in intermediate soil moisture compared to those growing in arid and saturated environments (Benson and Dawson 2007). Soil pH is a major determinant of bacterial species composition in bulk soil (Barker et al. 2005) and therefore influences the pool of potential endophytes available for plant recruitment. Indeed, endophyte communities recovered from roots of *N. attenuata* were more diverse in organic soils than in mineral soils (Long et al. 2010). These factors can also influence plant-endophyte interactions (Fuentes-Ramirez et al. 1999). Furthermore, soil type can interact with plant species. The endophytic microbial community is highly dependent on the field where the plant was grown (Dunfield and Germida 2001). Fertilizer and pesticide application and soil tillage can also influence the composition of the endophytic community (Gaiero et al. 2013).

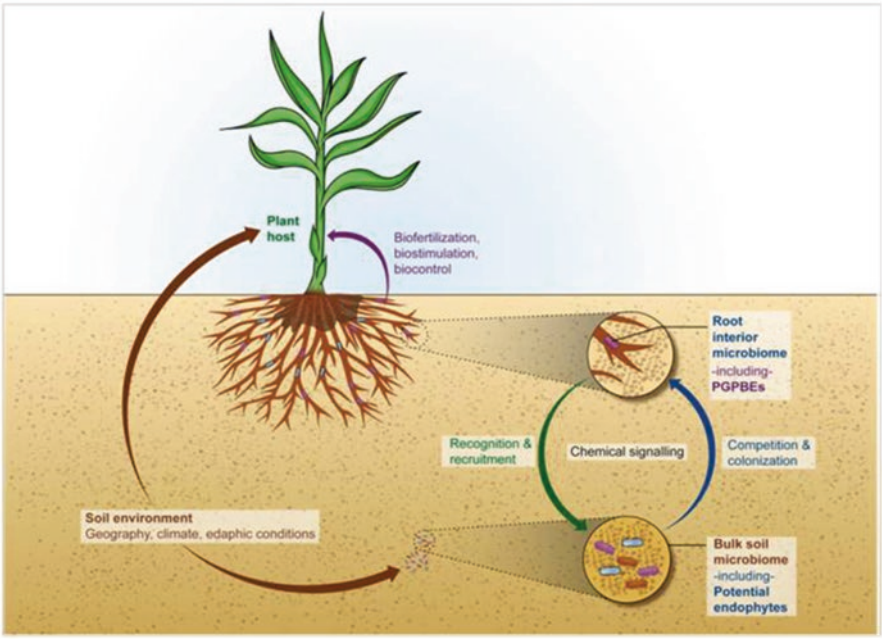


**Fig. 16.2** Establishment of microbial community in the soil, recognition and colonization of plant roots, and their beneficial effects in the plant growth. (Brown arrows) Soil environment factors influenced the composition of the bulk soil microbiome and the plant physiology, (green arrow) biochemical interactions between plant roots and the soil microbiome and selection of potential endophytes via root architecture differences and chemical signaling in root exudates, (blue arrow) cooperation of potential endophytes and competition for invasion sites on the root, and (purple arrow) plant growth-promoting ability by some endophytes when established inside the plant root (Gaiero et al. 2013)

### 16.3.2 Colonization of the Rhizoplane

The early events of this process such as recognition and chemotaxis have been widely reviewed by Lugtenberg et al. (2001) and Lugtenberg and Kamilova (2009). Composition of root exudates induced chemotaxis responses for endophytes to earlier recognition and colonization of plant tissues (Fig. 16.2) (Bacilio-Jiménez et al. 2003). Chemotaxis through root exudates such as malic acid and citric acid is also crucial for the colonization of tomato roots by *Pseudomonas* (De Weert et al. 2007). Yuan et al. (2015) demonstrated the role of banana root exudates especially oxalic, malic, and fumaric acids in the colonization of *B. amyloliquefaciens* NJN-6. Bacilio-Jiménez et al. (2003) revealed that amino acids and carbohydrates, present in the root exudates of rice plants, facilitated its colonization by *Corynebacterium flavescens* and *B. pumilus*. These allelochemicals may be also involved in the promotion of plant growth (Kamilova et al. 2006).

A number of mutation studies demonstrated that the attachment of bacterial cells at the root is a crucial step for subsequent endophytic establishment. Several

bacterial surface components are involved in the fixation process. Indeed, for *Azoarcus* sp. BH72, an endophytic diazotroph of rice, type IV pili cells are required for attachment to the root surface (Dörr et al. 1998). Fixation of another endophyte diazotroph, *Herbaspirillum seropedicae*, to surface of maize roots depends on the nature of liposaccharide (LPS) (Balsanelli et al. 2010). A similar study showed that exopolysaccharides are necessary for rhizoplane fixation and endophytic colonization of rice plants by *Gluconobacter diazotrophicus* (Meneses et al. 2011).

### 16.3.3 Entry of Endophytes Inside Plant Tissues

The entry sites of endophytes inside the plant are essentially the apical root zone with the thin-walled surface root layer such as the cell elongation and the root hair zone (zone of active penetration). Bacteria can also enter the plant through the basal root zone with small cracks caused by the emergence of lateral roots (zone of passive penetration) (Malfanova et al. 2013).

During active penetration, endophytes must be well equipped with hydrolytic enzymes such as pectinases and cellulases to penetrate into and persist in the host plant (Hallmann et al. 1997; Reinhold-Hurek and Hurek 1998). It should be also mentioned that pectinolytic enzymes act normally as virulence factors for plant pathogenic microbial agents but in case of endophytic microorganisms, they might play a role in invasion of host plants by endophytes as demonstrated for *Enterobacter asburiae* JM22 (Quadt-Hallmann and Kloepper 1996); *Bacillus cereus*, *B. subtilis*, and *B. stearothermophilus* (Torimiro and Okonji 2013), and *Stenotrophomonas maltophilia* (Garbeva et al. 2001). In our recent studies, beneficial *Alcaligenes faecalis* S18, *B. cereus* S42, *B. mojavensis* S40, *S. maltophilia* S37, *Pseudomonas* sp. S85, and *Serratia* sp. C4, exhibiting endophytic colonization ability on tomato plants, were found to be pectinase-producing agents (Aydi Ben Abdallah et al. 2016a, c, e, 2017a).

Indeed, endoglucanases and polygalacturonases are involved in the colonization of *Vitis vinifera* by *Burkholderia* sp. (Compant et al. 2005). Pectate lyase seems to be also necessary for the entry of *Klebsiella oxytoca* into wheat roots.

Induction of this path usually results in the stimulation of plant growth thus resulting in an increase in the biomass of wheat treated with this bacterium (Kovtunovych et al. 1999). Bacterial cell wall-degrading enzymes are also known to be involved in the elicitation of defense pathways in plants (Norman-Setterblad et al. 2000) and/or in decreasing the spread of pathogens inside the plants (Iniguez et al. 2005).

The passive penetration of endophytes via the natural cracks in the lateral root differentiation zone (often combined with the active penetration) has been suggested for *Azoarcus* sp. BH72 (Reinhold-Hurek and Hurek 1998), *Burkholderia vietnamiensis* (Govindarajan et al. 2008), and *Herbaspirillum seropedicae* Z67 (James et al. 2002) in rice, *Burkholderia phytofirmans* PsJN in grape (Compant et al. 2005), *B. cepacia* Lu10-1 in mulberry (Ji et al. 2010), and *Gluconacetobacter diazotrophicus* Pal5 in sugarcane (James et al. 1994).

### 16.3.4 Colonization of the Cortex

Once microorganism cells have crossed the exodermal barrier, they may remain at the site entry as demonstrated for *Paenibacillus polymyxa* in *Arabidopsis* (Timmusk et al. 2005) or move to inside and occupy the intercellular space of the cortex as proven for *Burkholderia* sp. PsJN in grape and *Serratia marcescens* IRNG500 in rice (Compant et al. 2005; Gasser et al. 2011).

### 16.3.5 Colonization of the Xylem

Only few microorganisms can penetrate the endodermal barrier and invade xylem vessels (Compant et al. 2005; Gasser et al. 2011). The transport of these endophytes to the aerial parts of the plant is probably ensured through the transpiration process (Compant et al. 2005). Although the concentrations of available nutrients are relatively low, they are sufficient for the growth of endophytes (Bacon and Hinton 2006). The ability of a microorganism to use certain plant metabolites could be a decisive condition for its successful endophytic behavior (Malfanova et al. 2013). Thus, intercellular spaces and xylem vessels are the most sites colonized by endophytic bacteria as demonstrated for *Pseudomonas veronii* VM 1449, *P. asplenii* VM 1450, and *P. putida* VM 1453 in poplar tree (Reinhold-Hurek and Hurek 1998; Germaine et al. 2004).

### 16.3.6 Colonization of the Reproductive Organs

The concentration of nutrients available in the xylem decreases along the plant axis; this may explain the decrease in diversity and density of endophytic microorganism populations with the increase of the distance from the roots. Indeed, only a small number of endophytes reach the upper parts of the leaves and reproductive organs such as flowers, fruits, and seeds as demonstrated for *Burkholderia phytofirmans* PsJN at the berry grape (Compant et al. 2010). In various plants, roots contain the large number of endophytes compared to other plant tissues (Rosenblueth and Martínez-Romero 2004).

## 16.4 Plant Growth Promotion by Endophytes

Once established in the plant, beneficial endophytic microorganisms can influence positively the growth of the plant through three interdependent mechanisms (Fig. 16.2), i.e., phytostimulation, biofertilization, and indirectly via the biocontrol of phytopathogenic agents (Bloemberg and Lugtenberg 2001). These mechanisms involve several active metabolites (Fig. 16.3 and Table 16.2) and are briefly described below.

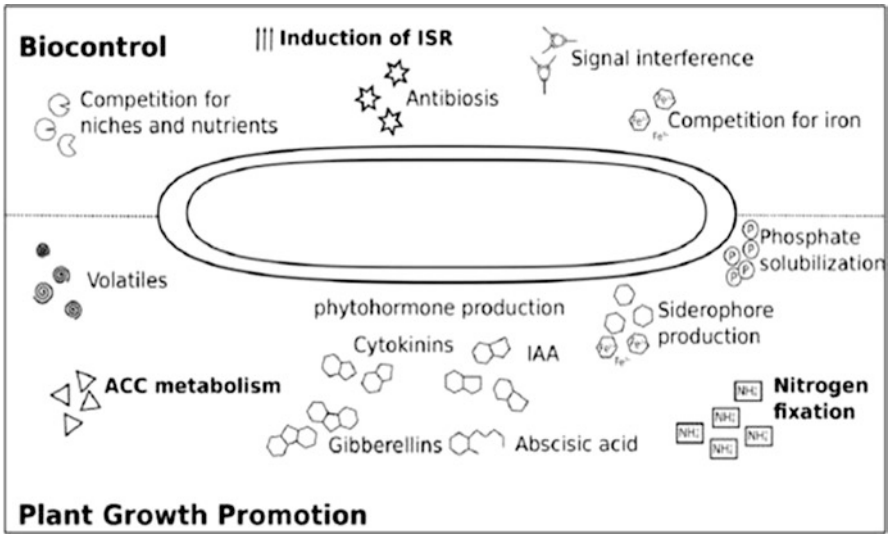


**Fig. 16.3** Mechanisms and metabolites involved in plant growth-promoting ability and biocontrol potential of endophytic plant-beneficial microorganisms. (Malfanova et al. 2013)

### 16.4.1 Phytostimulation

Phytostimulation is the direct stimulation of plant growth via the production and/or the regulation of phytohormones (Bloemberg and Lugtenberg 2001). Indole-3-acetic acid (IAA), jasmonates, cytokinins, and gibberellins are frequently produced by endophytic bacteria such as *B. subtilis*, *B. pumilus*, *Methylobacterium extorquens*, *Alcaligenes* sp., and *Achromobacter xylosoxidans* isolated from *Heracleum mantegazzianum*, *Pinus sylvestris*, and *Helianthus annuus*. These metabolites are known to be involved in the stimulation of plant growth (Pirttilä et al. 2004; Forchetti et al. 2007; Malfanova et al. 2011). In our recent studies, bacterial isolates recovered from wild *Solanaceous* plants and belonging to the genera of *Serratia*, *Alcaligenes*, *Stenotrophomonas*, *Pseudomonas*, and *Bacillus* were shown able to enhance tomato growth and to produce IAA. Indeed, Abdallah et al. (2017a) demonstrated that the IAA amount released by *Serratia* sp. C4 (29.52 µg/mL, after 48 h of incubation) is interestingly higher when compared to 11.1 µg/mL produced by *S. marcescens* SRM isolated from *Cucurbita pepo* flowers (Selvakumar et al. 2008b). Our isolates of *A. faecalis* S18 and *A. faecalis* subsp. *faecalis* S8, recovered from *Nicotiana glauca* and *Withania somnifera*, produced 17.73 and 33.91 µg IAA /mL (Aydi Ben Abdallah et al. 2016d, e). These amounts are higher than those produced by *A. piechaudii* (16.4 µg/mL) according to Barazani and Friedman (1999) study. Furthermore, IAA production ability of *S. maltophilia* S37 and *S. maltophilia* S33 recovered from *Datura* species was estimated at 21 and 29 µg/mL, respectively (Abdallah et al. 2016a, b, c, d, e, f), which is higher than that secreted by the endophytic *S. maltophilia* TEM56 isolated from *Amaranthus hybridus* and

**Table 16.2** Secondary metabolites produced by endophytic microorganisms and involved in plant growth-promoting (PGP) ability and biocontrol (BC) potential

| | | PGP | | | | BC | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Endophyte | Host plant | IAA | P | $N_2$ | Sd | Lps | HCN | Chit | Prot | Pect | SA | |
| *Streptomyces* sp. | *Panicum turgidum* | + | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | Goudjal et al. (2013) |
| *Serratia* sp. | *Cestrum nocturnum* | + | + | n.t | n.t | n.t | – | + | + | + | n.t | Aydi Ben Abdallah et al. (2017a) |
| *S. marcescens* | *Cucurbita pepo* | + | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | Selvakumar et al. (2008b) |
| *S. marcescens* | *Pueraria thunbergiana* | n.t | + | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | Selvakumar et al. (2008a) |
| *Alcaligenes faecalis* subsp. *faecalis* | *Withania somnifera* | + | + | n.t | n.t | n.t | + | + | + | + | n.t | Aydi Ben Abdallah et al. (2016d) |
| *Stenotrophomonas maltophilia* | *Amaranthus hybridus* | + | + | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | Ngoma et al. (2013) |
| *S. maltophilia* | *C. maxima* | + | + | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | Ngoma et al. (2013) |
| *Stenotrophomonas* sp. | *Datura metel* | + | + | n.t | n.t | n.t | – | + | + | + | n.t | Aydi Ben Abdallah et al. (2016c) |
| *Pseudomonas* sp. | *D. metel* | + | + | n.t | n.t | n.t | – | + | + | + | n.t | Aydi Ben Abdallah et al. (2016c) |
| *Bacillus tequilensis* | *Solanum elaeagnifolium* | + | + | n.t | + | + (*Sfp*) (*FenD*) | – | + | + | + | + | Aydi Ben Abdallah et al. (2016b, 2017b) |
| *B. cereus* | *N. glauca* | + | + | n.t | + | – | – | + | + | + | + | Aydi Ben Abdallah et al. (2016e, f) |
| *B. methylotrophicus* | *Citrus*, *Medicago*, and *Laurus* | – | – | n.t | + | + (*FenD*) (*ItuC*) | n.t | – | + | + | n.t | Kalai-Grami et al. (2014) |
| *B. mojavensis* | *Citrus*, *Medicago*, and *Laurus* | – | + | n.t | + | + (*Sfp*) (*FenD*) | n.t | – | + | + | n.t | Kalai-Grami et al. (2014) |
| *B. velezensis* | *Citrus*, *Medicago*, and *Laurus* | – | + | n.t | + | + (*BamC*) | n.t | – | + | + | n.t | Kalai-Grami et al. (2014) |
| *Achromobacter xylosoxidans* | *Helianthus annuus* | n.t | + | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | Forchetti et al. (2007) |

(continued)

**Table 16.2** (continued)

| Endophyte | Host plant | PGP | | | | BC | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IAA | P | $N_2$ | Sd | Lps | HCN | Chit | Prot | Pect | SA | |
| *Pseudomonas* sp. | *Solanum lycopersicum* | + | n.t | n.t | + | n.t | + | n.t | n.t | n.t | + | Nandhini et al. (2012) |
| *Klebsiella* sp. | *S. lycopersicum* | + | n.t | n.t | + | n.t | + | n.t | n.t | n.t | + | Nandhini et al. (2012) |
| *Citrobacter s*p. | *S. lycopersicum* | + | n.t | n.t | + | n.t | + | n.t | n.t | n.t | + | Nandhini et al. (2012) |
| *Bacillus* sp. | *S. lycopersicum* | + | n.t | n.t | + | n.t | + | n.t | n.t | n.t | + | Nandhini et al. (2012) |
| *Flavimonas oryzihabitans* | *Musa* sp. | n.t | + | + | + | n.t | n.t | n.t | n.t | n.t | n.t | Ngamau et al. (2012) |
| *Serratia glossinae* | *Musa* sp. | n.t | + | + | – | n.t | n.t | n.t | n.t | n.t | n.t | Ngamau et al. (2012) |
| *Serratia plymuthica* | *Musa* sp. | n.t | – | + | – | n.t | n.t | n.t | n.t | n.t | n.t | Ngamau et al. (2012) |
| *Rahnella aquatilis* | *Musa* sp. | n.t | + | + | – | n.t | n.t | n.t | n.t | n.t | n.t | Ngamau et al. (2012) |
| *Enterobacter asburiae* | *Musa* sp. | n.t | + | + | + | n.t | n.t | n.t | n.t | n.t | n.t | Ngamau et al. (2012) |
| *Fusarium tricinctum* and *Alternaria alternata* | *Solanum nigrum* | + | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | n.t | Khan et al. (2015) |

*IAA* indole-3-acetic acid, *P* phosphate solubilization, $N_2$ nitrogen fixation, *Sd* siderophores, *Lps* lipopeptide antibiotics, *HCN* hydrogen cyanide, *Chit* chitinase, *Prot* protease, *Pect* pectinase, *SA* salicylic acid

*S. maltophilia* PM22 isolated from *Cucurbita maxima* (0.32 and 0.49 mg/L, respectively) (Ngoma et al. 2013). Aydi Ben Abdallah et al. (2016c) showed that the endophytic *Pseudomonas* sp. S85 recovered from *D. metel*, shown able to enhance tomato growth, was also found to be an IAA-producing agent. Similar result was reported for three isolates of endophytic *Pseudomonas* sp. (JDB3, JDB5, and JDB6) isolated from soybean plants (Dalal and Kulkarni 2013). Plant growth-promoting bacteria (PGPBs) belonging to *Bacillus* genus (*B. mojavensis* S40, *B. cereus* S42, *B. tequilensis* SV104, and *Bacillus* sp. SV101) and associated with *D. stramonium*, *N. glauca*, and *Solanum elaeagnifolium* are shown able to produce IAA at 7–26 µg/mL (Aydi Ben Abdallah et al. 2016a, b, e). Endophytic fungi *Fusarium tricinctum* RSF-4 L and *Alternaria alternata* RSF-6 L isolated from *Solanum nigrum* are able to produce 54 and 30 µg/mL IAA, respectively (Khan et al. 2015). Furthermore, gibberellins were detected in the culture filtrate of an endophytic *Penicillium* sp. Sj-2-2 recovered from a halophyte plant and shown able to promote growth of rice seedlings (You et al. 2012).

Endophytic bacteria such as *Arthrobacter* spp. and *Bacillus* spp. stimulated the growth of pepper plants and are found able to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Sziderics et al. 2007). This is also the case of *Pseudomonas putida* and *Rhodococcus* spp. endophytes of pea (Belimov et al. 2001). The production of ACC deaminase can alleviate abiotic and/or biotic stress by decreasing levels of production of ethylene by the plant, because high levels of ethylene inhibit cell division, DNA synthesis, and root and hypocotyl growth (Gaiero et al. 2013).

Volatile substances such as 2,3-butanediol (or butane-2,3-diol) and acetoin, produced by two isolates of *B. subtilis*, are also involved in the promotion of *Arabidopsis thaliana* growth (Ryu et al. 2003).

Other substances such as adenine derivatives, which can be used as precursors in the biosynthesis of cytokinin, are produced by an endophytic bacteria *M. extorquens* and an endophytic fungus *Rhodotorula minuta*, recovered from the meristematic tissues of pine buds (Pirttilä et al. 2004). Endophytes produce adenine ribosides that stimulate growth and mitigate the browning of pine tissues (Pirttilä et al. 2004).

### 16.4.2 Biofertilization

The biofertilizing action provided by endophytes is mainly attributed to their ability to make major nutrients more accessible to plants thus promoting their growth (Gaiero et al. 2013).

A well-studied form of biofertilization is the fixation of nitrogen via the conversion of nitrogen atmospheric to ammonia (Bloemberg and Lugtenberg 2001). Several PGPBs are widely studied for their ability to fix nitrogen such as *Pantoea agglomerans* (Verma et al. 2004), *Azoarcus* spp. (Hurek et al. 2002), *B. subtilis* subsp. *subtilis*, *Pseudomonas protegens*, *P. moraviensis*, *Serratia glossinae*, *S. plymuthica*, *Enterobacter amnigenus*, *Klebsiella granulomatis*, *Rahnella aquatilis*, and *Flavimonas oryzihabitans* (Ngamau et al. 2012).

Some PGPBs may increase the availability of phosphorus to the plant due to their capacity to solubilize phosphorus. Indeed, the release of organic acids with low molecular weight in soil either from microbial cells or from root exudates promotes the release of phosphorus by making it so more accessible to the plant (Kpomblekou-A and Tabatabai 2003). This ability to solubilize the phosphate has been demonstrated in endophytic bacteria *Achromobacter xylosoxidans* and *B. pumilus* associated with sunflower (*Helianthus annuus*) (Forchetti et al. 2007); *B. velezensis*, *B. mojavensis*, and *B. methylotrophicus* isolated from roots of *Citrus*, *Medicago*, and *Laurus* from Tunisia (Kalai-Grami et al. 2014); and *S. marcescens* KR-4 recovered from *Pueraria thunbergiana* (Selvakumar et al. 2008a). Yazdani and Bahmanyar (2009) demonstrated that PGPBs used for maize fertilization (*Zea mays*) reduced the contribution of phosphorus by 50% without loss of yield. In our previous findings, we demonstrated that the application of endophytic bacteria showing a phosphatase activity such as *Bacillus* sp. SV101, *B. cereus* S42, *B. mojavensis* S40, *Stenotrophomonas* sp. S33, *S. maltophilia* S37, *Pseudomonas* sp. S85, *A. faecalis* subsp. *faecalis* S8, and *Serratia* sp. C4 as biofertilizers led to increase in growth parameters in tomato plants compared to the untreated control (Aydi Ben Abdallah et al. 2016b, c, d, e, 2017a). The phosphate solubilization potential of endophytic bacteria such as *Pseudomonas* spp., *Serratia* spp., *Enterobacter asburiae* J1v1r, *Rahnella aquatilis* ME 19V2c and ME 18V2c, *Ewingella americana* K32V2c and *Yokenella regensburgei* J4V1c, and *S. maltophilia* PM22 were already reported in Ngamau et al. (2012) and Ngoma et al. (2013).

### 16.4.3 Indirect Promotion via the Suppression of Plant Pathogens

The protection of plants against attacks of phytopathogenic agents may lead indirectly to the promotion of plant growth (Gaiero et al. 2013). Similarly, authors reported that disease-suppressive ability exhibited by endophytic bacteria was shown to be associated with the promotion of tomato growth (Aydi Ben Abdallah et al. 2017b).

Several secondary metabolites may be involved in this effect such as the production of siderophores, antibiotics, and/or elicitors such as salicylic acid (Gaiero et al. 2013). The production of hydrocyanic acid (HCN) and siderophores was demonstrated by Nandhini et al. (2012) for four endophytic isolates recovered from roots and stems of tomato and belonging to the genera of *Bacillus*, *Pseudomonas*, *Klebsiella*, and *Citrobacter*. Indeed, the competition for iron is involved in the suppression of Fusarium wilt disease by some *P. fluorescens* isolates via the production of siderophores and mainly pyoverdine and pyochelin (Lepoivre 2003).

## 16.5 Plant Protection by Endophytes

Biocontrol of phytopathogens is essentially based on three mechanisms of action including competition for nutrients and sites of infection, antibiosis, and induction of systemic resistance (ISR) in the plant (Malfanova et al. 2013) which involved several antimicrobial metabolites and/or elicitors (Fig. 16.3 and Table 16.2).

### 16.5.1 Competition

The competition between endophytes and phytopathogenic agents for carbon, nitrogen, and iron is a crucial way to limit disease incidence and severity since these nutrients are necessary for successful germination, penetration, and infection of host cells by pathogens (Viterbo et al. 2007). However, microorganisms that can compete for infection sites and colonize root tissues are not all able to reduce the severity of diseases (Pliego et al. 2008).

Antagonist activity through the competition for nutrients was widely reported in endophytes. Indeed, bacteria isolated from banana plants and belonging to the genera *Enterobacter*, *Pseudomonas*, *Flavimonas*, and *Serratia* are able to produce siderophores that are iron chelators which eliminate and reduce iron availability to the other microorganisms (Ngamau et al. 2012; Lugtenberg et al. 2013). In addition, bacteria associated with banana belonging to the genera *Serratia*, *Pseudomonas*, *Raoultella*, *Enterobacter*, *Rahnella*, *Yokenella*, *Bacillus*, *Klebsiella*, and *Ewingella* are able to fix nitrogen making it, thus, unavailable to the other microorganisms (Ngamau et al. 2012). Moreover, siderophores producing *Bacillus* spp. (*B. methylotrophicus*, *B. mojavensis*, *B. velezensis*, *B. amyloliquefaciens*) have not only survival capacity into plant cells by competing for iron supply but also outcompete ability with other pathogens during their progress within host tissues (Kalai-Grami et al. 2014). In Aydi Ben Abdallah et al. (2016b) study, a siderophore-producing bacterium, *B. tequilensis* SV104 recovered from *S. elaeagnifolium* stems, was shown able to reduce Fusarium wilt severity in tomato plants compared to the inoculated and untreated control. This metabolite displayed antifungal property and is also involved in the plant growth-promoting process (Bar-Ness et al. 1992).

Root exudates are necessary for the germination of microconidia of the pathogen (*P. fluorescens*) (Kamilova et al. 2008) and for root fixation and colonization via chemotaxis (De Weert et al. 2007). Thus, a reduction in the availability of these exudates for pathogen limits its development and its ability to penetrate into host plant cells. Kamilova et al. (2008) demonstrate that the competition for root exudates from tomato plants displayed by *P. fluorescens* WCS365 led to decrease in *F. oxysporum* f. sp. *radicis-lycopersici* growth by inhibiting the activity, the multiplication, the germination, the sporulation, and the invasion of plants by the pathogen.

### 16.5.2 Antibiosis

Antibiosis has been widely used by endophytes against phytopathogenic agents (Sessitsch et al. 2004; Nandhini et al. 2012; Vethavalli and Sudha 2012). This effect occurs through the release of antibiotics, hydrolytic enzymes, and/or other antimicrobial metabolites synthesized by these microorganisms in the area of their interaction with target pathogens.

### 16.5.3 Production of Antibiotics

Lipopeptide antibiotics, of low molecular weight, are usually produced by *Bacillus* spp. (Cai et al. 2013; Ramyabharathi and Raguchander 2014; Gond et al. 2015). Three families of lipopeptides are known as iturins, fengycins, and surfactins. Fengycins and iturins are known for their antifungal effect against filamentous fungi by acting on sterols, phospholipids, and oleic acids of fungal membranes (Romero et al. 2007; Alvarez et al. 2012). In our recent study, lipopeptide antibiotics were confirmed in the genome of four of the endophytic *Bacillus* spp. recovered from wild *Solanaceous* plants and showing interesting phytoprotective activity against *F. oxysporum* f. sp. *lycopersici*. In particular, surfactin (*Sfp* gene) was detected in *B. tequilensis* SV39 and *B. tequilensis* SV104 and fengycin (*FenD*) gene in *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. tequilensis* SV104, while the bacillomycin (*Bam C*) gene was detected in *B. methylotrophicus* SV44 (Aydi Ben Abdallah et al. 2017b). Lipopeptides, such as bacillomycin D, surfactin, iturin A, and fengycin D, were synthesized by endophytic isolates of *B. velezensis*, *B. methylotrophicus*, *B. mojavensis*, and *B. amyloliquefaciens* associated with *Citrus reticulata*, *Citrus sinensis*, *Citrus limon*, *Medicago truncatula*, and *Laurus nobilis* (Kalai-Grami et al. 2014). The secretion of these antibiotics can take place inside plant tissues and/or on their surfaces to protect them from fungal infections (Gond et al. 2015). Other lipopeptides are detected in various *Bacillus* species. Indeed, kurstakins, maltacins, and polymyxins were produced by *B. thuringiensis*, *B. subtilis*, *B. polymyxa*, and *B. amyloliquefaciens* (Storm et al. 1977; Hathout et al. 2000; Lee et al. 2007; Hagelin et al. 2007).

Other types of antibiotics have been produced by beneficial endophytes such as hydrogen cyanide (HCN). This metabolite is a volatile antibiotic secreted by Gram-negative bacteria (Lugtenberg et al. 2013). HCN was produced by *P. fluorescens*, *P. aeruginosa*, and *Chromobacterium violaceum* (Askeland and Morrison 1983; Haas and Défago 2005) and showed antifungal activity against *Sclerotium rolfsii* (Rakh et al. 2011) and *Rhizoctonia solani* (Nagarajkumar et al. 2004). In our current investigations, endophytic bacteria shown able to successfully limit the Fusarium wilt severity in tomato such as *A. faecalis* S18, *A. faecalis* subsp. *faecalis* S8, and *S. maltophilia* S37 recovered from *N. glauca*, *W. somnifera*, and *D. stramonium* were potentially able to produce HCN (Aydi Ben Abdallah et al. 2016a, d, e), whereas

*Stenotrophomonas* sp. S33 and *Pseudomonas* sp. S85, recovered from *D. metel*, were not HCN-producing agents (Aydi Ben Abdallah et al. 2016c). This allelochemical acts through the inhibition of cytochrome oxidase of several microorganisms, and the bacteria that can produce it usually possess a HCN-resistant cytochrome oxidase (Voisard et al. 1989).

Other antibiotics are also known in Gram-negative bacteria as is the case of 2,4-diacetylphloroglucinol, phenazin, pyoluteorin, and pyrrolnitrin (Lugtenberg et al. 2013). The antibiotic 2,4-diacetylphloroglucinol is produced by *P. fluorescens* and involved in the biocontrol of *Fusarium oxysporum* f. sp. *lycopersici* (Fakhouri and Buchenauer 2002). Maltophilins and xanthobaccins are also produced by *S. maltophilia* and showed antifungal activity against *Pythium ultimum*, *Botrytis cinerea*, *F. solani*, *R. solani*, *Rhodotorula solani*, *Penicillium variotii* and *P. notatum* (Jakobi et al. 1996; Nakayama et al. 1999).

### 16.5.4 Production of Hydrolytic Enzymes

Synthesis of hydrolytic enzymes such as chitinases, β-1,3-glucanases, proteases, pectinases, cellulases, lipases, esterases, amylases, and endoglucanases involved in the degradation of cells of pathogens has been reported in several genera of endophytic bacteria such as *Bacillus*, *Micrococcus*, *Microbacterium*, *Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, *Alcaligenes*, *Burkholderia*, *Enterobacter*, *Serratia*, *Pantoea*, *Sphingopyxis*, *Curtobacterium*, *Brevundimonas*, and *Chryseobacterium* (Alström 2001; Berg et al. 2005; Bibi et al. 2012; Castro et al. 2014; Kalai-Grami et al. 2014). An endophytic bacterium, *B. cereus* 65 isolated from *Sinapis*, was shown able to produce chitinase with a molecular weight of 36 KDa and active at pH ranging between 4.5 and 7.5. The direct application of this bacterium in the soil has significantly protected cotton plants from root rot disease caused by *R. solani* (Pleban et al. 1997). Two endophytic bacteria, *B. pumilus* and *Brevibacterium halotolerans* recovered from *Prosopis strombulifera*, exhibited proteolytic activity and successfully inhibited the mycelial growth of *Alternaria* sp. (Sgroy et al. 2009). The protease activity was also detected in *S. maltophilia* which is bioactive against *Pythium ultimum* (Dunne et al. 1997). Four isolates of *P. fluorescens*, able to reduce the severity of tomato Fusarium wilt and to inhibit the mycelial growth of pathogen, have the ability to produce various metabolites including chitinases and proteases (Fakhouri and Buchenauer 2002). Aydi Ben Abdallah et al. (2016a, b, c, d, e, 2017a) suggest that the antifungal activity of endophytic bacteria may be due in part to the production of extracellular hydrolytic enzymes and/or to the synthesis of secondary metabolites active against *F. oxysporum* f. sp. *lycopersici*. Indeed, *B. cereus* S42, *B. tequilensis* SV104, *B. mojavensis* S40, *Stenotrophomonas* sp. S33, *S. maltophilia* S37, *Pseudomonas* sp. S85, *A. faecalis* subsp. *faecalis* S8, and *Serratia* sp. C4 were found to be chitinase- and protease-producing strains, respectively. *Bacillus* sp. SV101 did not produce both enzymes, and *A. faecalis* S18 did not produce protease despite their antifungal

potential toward tomato Fusarium wilt pathogen (Aydi Ben Abdallah et al. 2016b, e).

The use of *ChiA* probe for detection of chitinase genes in *B. circulans*, *B. megaterium*, *B. subtilis*, and *B. amyloliquefaciens* was developed in Ramaiah et al. (2000) and Solanki et al. (2012) studies. The *ChiA* sequence is highly conserved and allows identifying the *ChiA* gene in a wide range of bacteria (Cretoiu et al. 2012). In Aydi Ben Abdallah et al. (2016f, 2017b) studies, *ChiA* gene was expressed in endophytic *Bacillus* spp. (*B. tequilensis* SV39, *B. subtilis* SV41, *B. methylotrophicus* SV44, *B. amyloliquefaciens* subsp. *plantarum* SV65, *B. tequilensis* SV104, and *B. cereus* S42).

### 16.5.5 Production of Biochemical Secondary Metabolites

Endophytes are also able to produce biochemical substances showing antifungal and antibacterial activities. Among these endophytes, *B. subtilis* EPC016 associated with cotton plants and *B. cereus* NRL2 isolated from *Azadirachta indica* are able to produce bioactive phthalic acids exhibiting antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (Ramyabharathi and Raguchander 2014) and antibacterial potential toward *Staphylococcus aureus* (Kumar et al. 2015). In Aydi Ben Abdallah et al. (2016f) study, major compounds identified using GC-MS analysis in the bioactive chloroform extract from *B. cereus* S42 toward *F. oxysporum* f. sp. *lycopersici* belonged to the family of phthalic acids. The other compounds identified are phenol 3,5-dimethoxy, benzoic acid 3,5-dihydroxy, 2-hydroxy-1-isoindolinone, 3-isobutylhexahydropyrrolo [1,2-a] pyrazine-1,4-dione, 3-Keto-1-aza-2,3-dihydrobenzopyran, 3-(4-pyridyl) acrylic acid, 9-octadecenoic acid (Z)-methyl ester, and dioctyl hexanedioate. Other chemical metabolites belonging to the family of aldehydes, ketones, and benzenes are produced by *B. amyloliquefaciens* and are found to be active against *F. oxysporum* f. sp. *cubense* (Yuan et al. 2012).

The dibutyl phthalate, detected in extracts from the endophytic marine fungus *Varicosporina ramulosa* is also biologically active against *F. solani* (Mabrouk et al. 2008). The phthalic acid, bis(2-ethylhexyl), was also produced by *Tsukamurella inchonensis* and *Corynebacterium nitrilophilus* exhibiting antifungal potential toward *Alternaria solani*, *F. oxysporum*, and *Penicillium digitatum* (El-Mehalawy et al. 2008). An endophytic fungus *Alternaria* sp. recovered from *Tabebuia argentea* was found able to produce phthalic acid that showed antimicrobial and antioxidant activity (Govindappa et al. 2014). Furthermore, the phthalic acid, mono(2-ethylhexyl) ester, was excreted by an endophytic fungus *Aspergillus flavipes* which displayed antifungal activity against *Sclerotinia sclerotiorum* (Verma et al. 2014).

### 16.5.6 Induced Systemic Resistance (ISR)

Induced systemic resistance (ISR) in the plant is largely activated by root-colonizing bacteria (Kloepper et al. 2004; van Loon et al. 2006; van Wees et al. 2008; Pieterse et al. 2009). ISR is mainly dependent on the signaling pathways of jasmonic acid and/or ethylene rather than salicylic acid (Pieterse et al. 2009). However, some ISR inducers seem to activate the dependent pathway of salicylic acid which indicates that multiple signaling pathways may be cooperated when the ISR mechanism is triggered (Niu et al. 2011). The significantly enhanced expression of the *LOXD* and especially acidic *PR-1* and *PR-3* genes in plants treated with *B. amyloliquefaciens* subsp. *plantarum* SV65 and those uninoculated or inoculated with *F. oxysporum* f. sp. *lycopersici* suggested that the ISR induced by *Bacillus* strain SV65 in tomato against this fungus is dependent on the jasmonic acid and salicylic acid signaling pathways (Aydi Ben Abdallah et al. 2017b). In contrast, jasmonic acid is not essential for the mode of action used by an endophyte fungus, *F. solani* Fs-K, recovered from tomato seeds, to confer resistance to tomato plants inoculated with *F. oxysporum* f. sp. *radicis-lycopersici*, where conversely ethylene is required for the mediation of biocontrol activity of this strain (Kavroulakis et al. 2007).

Substances of low molecular weights resulting from the degradation of cells of microbial agents are exogenous elicitors for ISR (van Loon 2000). Various secondary metabolites are involved in ISR such as siderophores (pyocyanin and pyochelin) (Audenaert et al. 2002); 2,3-butanediol (Ryu et al. 2003); eugenol, 3-methoxybutyl acetate, pentachloroaniline, and phthalic acid methyl ester (Akram et al. 2015); phloroglucinol (Iavicoli et al. 2003); and lipopeptides (Ongena et al. 2007).

Applied on cotton plants inoculated with *Verticillium dahliae*, the iturin produced by an endophytic bacterium, *B. amyloliquefaciens* 41B-1 isolated from cotton roots, has induced the expression of defense genes such as chitinase, peroxidase, lipoxygenase, and PR-1. In addition, iturin has improved $H_2O_2$ accumulation and leaf callose deposition in plants (Han et al. 2015). However, Gond et al. (2015) study reported that treatment with lipopeptides produced by *B. subtilis*, isolated from corn seeds, did not stimulate the defense genes (PR-1 and PR-4), while treatment with the bacterial suspension of this endophytic bacterium has induced the expression of PR-1 and PR-4 genes.

Dimethyl disulfide, synthesized by *B. cereus* C1L, acts as an elicitor by inducing defense responses on tobacco and maize plants toward *Botrytis cinerea* and *Cochliobolus heterostrophus* when applied as soil drench (Huang 2012).

Induction of synthesis of defense-related proteins such as peroxidases, chitinases, and ß-1,3-glucanases was demonstrated in Fishal et al. (2010) study for *Pseudomonas* sp. and *Burkholderia* sp. used as biocontrol agents against *F. oxysporum* f. sp. *cubense*. In addition, a significant interaction between the antifungal potential of *P. fluorescens* towards *R. solani* and its ability to produce β-1,3-glucanase, salicylic acid, and hydrogen cyanide acid was noted (Nagarajkumar et al. 2004). Among the most commonly tested elicitors, salicylic acid plays an important role in the expression of both local resistance, controlled by major genes, and ISR developed after an initial pathogen attack (Hammerschmidt and Smith-Becker

2000). In our recent study, five bioactive *Bacillus* spp. showing Fusarium wilt suppression ability were found to produce salicylic acid, with the greatest production recorded for *B. subtilis* SV41. Accordingly, SV41-based treatments displayed a slight upregulation in acidic *PR-1* and *PR-3* expression genes in tomato plants inoculated or not with *F. oxysporum* f. sp. *lycopersici* (Aydi Ben Abdallah et al. 2017b).

## 16.6 Application of Beneficial Endophytes as Biofertilizing and Biocontrol Agents

### 16.6.1 Endophytes as Biocontrol Agents

Endophytes are increasingly used in plant protection against phytopathogens. Indeed, endophytic microorganisms associated with potato plants showed antagonistic activity against fungi (Sessitsch et al. 2004; Mejdoub-Trabelsi et al. 2016) and bacterial pathogens such as *Erwinia* and *Xanthomonas* (Sessitsch et al. 2004). The endophytic bacteria *Curtobacterium flaccumfaciens* decreased *Xylella fastidiosa* infections in citrus plants (Araújo et al. 2002). Endophytic actinomycetes successfully protected wheat plants from the pathogenic fungus *Gaeumannomyces graminis* (Coombs and Franco 2003), and several endophytes had successfully protected cotton seedlings from root rot disease caused by *R. solani* (Pleban et al. 1997). Furthermore, bacterial endophytes are capable to suppress nematode proliferation in the soil (Sturz and Kimpinski 2004). *Stenotrophomonas maltophilia* recovered from potato and rice roots (Garbeva et al. 2001) showed antifungal potential against plant pathogenic fungi, bacteria *(Ralstonia solanacearum*), and the plant-parasitic nematode *Meloidogyne incognita* (Krechel et al. 2002; Messiha et al. 2007). This species was also isolated from coffee seeds and was successfully used for the control of leaf spot caused by *Bipolaris sorokiniana* on tall fescue (Zhang and Yuen 1999). The endophytes *Herbaspirillum seropedicae* and *Clavibacter xyli* were genetically modified to produce the δ-endotoxin of *Bacillus thuringiensis* to control insect pests (Downing et al. 2000; Turner et al. 1991).

Field trials performed for the assessment of the effectiveness of three endophytic fungi *Colletotrichum gloeosporioides*, *Clonostachys rosea*, and *Botryosphaeria ribis* recovered from healthy *Theobroma cacao* tissues to control pod loss due to *Moniliophthora roreri* and *Phytophthora palmivora* and *Phytophthora perniciosa* revealed the higher efficiency of *C. gloeosporioides* (Mejía et al. 2008). Brum et al. (2012) demonstrated the efficacy of fungal endophyte community associated with *Vitis labrusca* in protecting the host plant against pathogenic *Fusarium* species and selected *C. gloeosporioides* and *Flavodon flavus* as the most efficient agents against *F. oxysporum* f. sp. *herbemontis*. Furthermore, naturally occurring potato-associated fungi (*Aspergillus* spp. and *Penicillium* spp.) and their extracellular metabolites can suppress in vitro and in vivo growth of *Fusarium* species infecting potato tubers (Mejdoub-Trabelsi et al. 2016). Vinale et al. (2017) demonstrated the insecticidal activity of secondary metabolites produced by an endophytic fungus *Talaromyces pinophilus* isolated from *Arbutus unedo* where its organic extracts revealed the

presence of three bioactive metabolites, namely, the siderophore ferrirubin, the platelet-aggregation inhibitor herquline B, and the antibiotic 3-O-methylfunicone. The latter one was the major metabolite produced by this strain and displayed toxic effects against the pea aphid *Acyrthosiphon pisum* (*Homoptera aphidiidae)*.

In our recent investigations, several endophytic bacteria belonging to the genera *Bacillus*, *Alcaligenes*, *Serratia*, *Pseudomonas*, and *Stenotrophomonas* isolated from wild *Solanaceous* plants successfully suppressed tomato Fusarium wilt disease compared to the inoculated control. Indeed, *A. faecalis* S18 and *B. cereus* S42 from *N. glauca* (Aydi Ben Abdallah et al. 2016e), *Stenotrophomonas* sp. S33 and *Pseudomonas* sp. S85 from *D. metel* (Aydi Ben Abdallah et al. 2016c), *Bacillus* sp. SV101 and *B. tequilensis* SV104 from *S. elaeagnifolium* (Aydi Ben Abdallah et al. 2016b), *A. faecalis* subsp. *faecalis* S8 from *W. somnifera* (Aydi Ben Abdallah et al. 2016d), and *Serratia* sp. C4 from *C. nocturnum* (Aydi Ben Abdallah et al. 2017a) were found to be the most effective in decreasing yellowing and wilt symptoms by 77–94% and the vascular browning extent by 76–97.5% relative to the inoculated and untreated control. Furthermore, the extracellular metabolites from six endophytic *Bacillus* spp., recovered from wild *Solanaceae*, were assessed for their ability to control tomato Fusarium wilt. Results showed a significant decrease in Fusarium wilt severity by 87–100% as compared to the inoculated and untreated control (Aydi Ben Abdallah et al. 2016f, 2017b). Both the cell-free culture filtrates and whole-cell suspensions of *Bacillus* spp. tested had the same suppressive effect toward tomato Fusarium wilt, indicating that living cells are not required for disease control. This may be useful for the implementation of a biocontrol scheme involving these biocontrol strains. Using only extracellular metabolites may be more cost-effective for the production of a biopesticide. On the other hand, the application of whole bacteria suspension may improve soil fertility and biodiversity and act in multiple and indirect ways in promoting plant protection and growth (Aydi Ben Abdallah et al. 2017b).

### 16.6.2 Endophytes as Biofertilizing Agents

The plant growth-promoting potential expressed by endophytes associated with *Prosopis stormbulifera* roots (Sgroy et al. 2009) and *Zingiber officinale* rhizomes (Jasim et al. 2014) was previously reported. Moreover, *Burkholderia caribensis*, *Kosakonia oryzae*, *Pectobacterium* sp., *Enterobacter asburiae*, *E. radicincitans*, *Pseudomonas fluorescens*, and *E. cloacae*, recovered from sugar cane roots and stems, were shown able to enhance the growth of this plant (Marcos et al. 2016). Also, four endophytic bacteria, namely, *Azospirillum brasilense*, *Burkholderia ambifaria*, *Gluconacetobacter diazotrophicus*, and *Herbaspirillum seropedicae*, were shown able to colonize root, stem, and leaf tissues of *S. lycopersicum* var. *lycopersicum* and to stimulate its growth (Botta et al. 2013). An endophytic bacterium, *Klebsiella pneumoniae*, isolated from the maize and coffee roots enhanced the growth of *Triticum* and *Arabidopsis* (Chelius and Triplett 2000; Dong et al. 2003). Furthermore, an endophytic bacterium *B. amyloliquefaciens* JK-SD002

recovered from tomato stems also improved the height of inoculated tomato seedlings (Nawangsih et al. 2011). Algam et al. (2005) also found that *Brevibacillus brevis* B2 and *B. subtilis*, initially isolated from the rhizosphere of tomato and showing endophytic behavior, successfully stimulated tomato growth and controlled bacterial wilt caused by *Ralstonia solanacearum*. Furthermore, *Pseudomonas* spp. (*P. aeruginosa* HR7 and *Pseudomonas* sp.), isolated from roots and stems of healthy tomato plants, were able to stimulate the development of this *Solanaceae* as indicated in Nandhini et al. (2012) and Patel et al. (2012) studies. *P. geniculata* IC-76 recovered from nodules of cultivated chickpea showed a plant growth-promoting ability when applied either separately or in combination with five *Streptomyces* sp. isolates (Gopalakrishnan et al. 2015). *A. faecalis* AF3, associated with corn, showed a growth-promoting ability on maize plants even under drought stress (Naseem and Bano 2014). Gyaneshwar et al. (2001) reported that *S. marcescens*, associated with rice plants, significantly stimulated the root length and dry weight of the plant compared to the untreated one. In addition, growth parameters such as plant height, plant fresh weight, leaf dry weight, and fruit number per plant were improved in tomato plants treated by rhizospheric and/or endophytic bacteria such as *Pseudomonas putida*, *P. fluorescens*, *S. marcescens*, *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* (Almaghrabi et al. 2013).

Disease-suppressive ability exhibited by endophytic *Bacillus* spp., *A. faecalis*, *Pseudomonas* sp., *Serratia* sp., and *S. maltophilia* and/or their extracellular metabolites was accompanied by a plant growth-promoting effect observed both on tomato plants inoculated or not with *F. oxysporum* f. sp. *lycopersici* (Fig. 16.4) (Aydi Ben Abdallah et al. 2016a, c, d, e, 2017a, b). Similar growth enhancements were also observed on *B. mojavensis*-treated maize plants grown in the presence of pathogenic isolates of *F. verticillioides* (Kalai-Grami et al. 2014). Ramyabharathi and Raguchander (2014) findings reported that disease-suppressive effect displayed by an endophytic bacterium *B. subtilis* EPC016 isolated from cotton plants was also associated with an enhancement of plant growth and fruit yield in treated tomato plants as compared to control.

*Fusarium tricinctum* RSF-4 L and *Alternaria alternata* RSF-6 L recovered from leaves of *S. nigrum* significantly enhanced the plant growth attributes examined using the chlorophyll content, the root-shoot length, and the biomass production (Khan et al. 2015). Khan et al. (2008) concluded that a major proportion of endophytic fungi inhabited in the sand dune plants produce metabolites leading to increased growth and development of the host plant. Furthermore, growth of *Carex kobomugi* was also increased using culture filtrate from the endophytic fungus *Penicillium citrinum* KACC43900 isolated from *Ixeris repens* L. roots which showed ability to produce gibberellins. Treatment using culture filtrate of *P. citrinum* increased the leaf blade length, the contents of chlorophyll a and chlorophyll b, the total chlorophyll, and the carotenoid content in leaf blades of *Carex kobomugi*. The extracellular metabolites from *P. citrinum* KACC43900 also increased the photosynthetic rate, the transpiration rate, the carboxylation efficiency, and the water-use efficiency. Also, soil respiration rates were higher in the site treated with the culture filtrate of *P. citrinum* compared to control (Hwang et al. 2011).

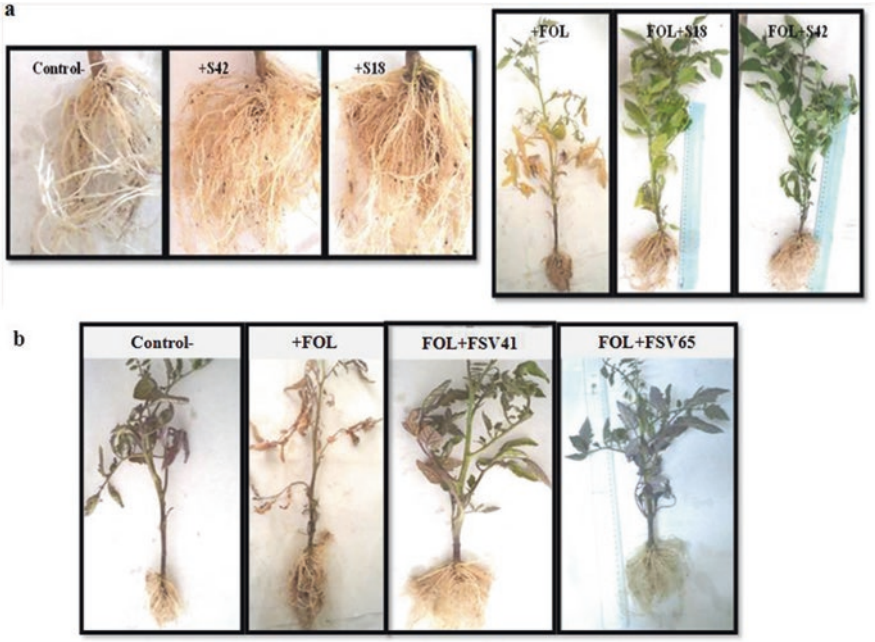


**Fig. 16.4** Effect of endophytic bacteria *Bacillus cereus* S42 and *Alcaligenes faecalis* S18 recovered from *Nicotiana glauca* (**a**) and the extracellular metabolites of *Bacillus subtilis* SV41 and *Bacillus amyloliquefaciens* subsp. *plantarum* SV65, isolated from *Datura metel* and *Solanum nigrum*, respectively, on Fusarium wilt severity and growth promotion of tomato cv. Rio Grande plants compared to the controls noted 60 days post-inoculation with the pathogen. Control: Uninoculated with the pathogen and untreated control. +FOL: Inoculated with *Fusarium oxysporum* f. sp. *lycopersici* and untreated control. FSV41 and FSV65: Cell-free culture filtrates from *B. subtilis* SV41 and *B. amyloliquefaciens* subsp. *plantarum* SV65. (Aydi Ben Abdallah et al. 2016e, 2017b)

## 16.7 Conclusion

A success in endophytic behavior begins from the soil and needs favorable environmental and edaphic conditions. The colonization of plants by endophytes in roots involved several mechanisms and metabolites such as chemotaxis and the production of hydrolytic enzymes. Once established inside plant tissues, endophytes have a great influence on plant health and growth and are an important source of bioactive natural compounds.

Using living cells and only bioactive substances is momentarily beneficial for improving growth or inducing resistance in plants, while the use of the whole-cell suspensions plays an important role in the diversity of soil microbial community which influences soil fertility and productivity thereafter which is interesting for a sustainable agriculture.

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# 17 Bioprocessing of Endophytes for Production of High-Value Biochemicals

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## 17.1 Introduction

The term endophyte literally translates into 'within a plant' and was initially coined to refer to organisms living inside a plant (Chaichanan et al. 2014). However, it is currently used in the context of mutualistic fungi and bacteria living inside plants. Endophytes have been found in plants belonging to every plant family (Ray et al. 2017; Singh et al. 2017). There are several hypotheses regarding endophyte-plant relationship, and it is believed that plants harbouring endophytes are healthier than their endophyte-free counterparts (Martinez-Klimova et al. 2017). The symbiotic relationship seems beneficial to the endophyte as nutrients for growth are available from the plant. Endophytes promote plant growth by fixing nitrogen, helping in the uptake of mineral nutrients such as phosphorus and iron (Thiry and Cingolani 2002). Endophytes are also said to modulate the levels of phytohormones (Santoyo et al. 2016). Endophytes also defend the plants against pathogens and insects by producing secondary metabolites. Many metabolites isolated from endophytes are found to exhibit antimicrobial (Golinska et al. 2015) and antifungal (Ola et al. 2013) activity. Another important hypothesis is that endophytes compete with pathogens in colonizing plant tissues and therefore help in minimizing damages caused by pathogens. Endophytes have also evolved to overcome plant defences and thrive inside their host plant. Endophytes are of special interest because they have been found to synthesize chemical compounds that are also known to be produced by their host plant, such as taxol and camptothecin (Thiry and Cingolani 2002). Apart from host-identical compounds, several other compounds such as antibiotics and bioactive peptides that are of commercial interest are also produced by endophytes (Castillo et al. 2002; Ezra et al. 2004). Further, the advances in analytical

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techniques, such as gas/liquid chromatography coupled with mass spectrometry and liquid chromatography coupled with nuclear magnetic resonance, have led to recent interest in bioprospecting of endophytes for characterization and identification of known and novel bioactive compounds with relative ease, for prospective commercial applications. In addition the biochemical production from endophyte can be improved through various strategies. Bioprocess condition optimization can help in enhancing the productivity (Singh et al. 2016). Exogenous addition of elicitors helps in stress-induced production of metabolites, and precursor addition helps in driving the biosynthetic pathway towards metabolite formation. Also strain improvements through genetic modifications can help in overcoming their drawback of unsustainability. Diverse methods and developments towards bioprocessing and bioprospecting are discussed below in this chapter.

## 17.2 Endophytes: Discovery and Terminology

Until the nineteenth century, it was believed that healthy growing plants are sterile and devoid of microbiota as hypothesized by Pasteur (Compant et al. 2012). Endophytes were first described by the German botanist H.F. Link in 1809 (Link 1809), and over the next few years, endophytes were defined in numerous ways. Béchamp referred to microorganisms living in plants as microzymas (Béchamp 1866). A few years later, Galippe reported the occurrence of microorganisms – fungi and bacteria – in the interior parts of vegetable plants (Galippe 1887). The initial assumption that all microbes living inside plant hosts are parasitic in nature was disproved by the Dutch microbiologist Martinus Willem Beijerinck (Beijerinck 1888). His discovery of rhizobium bacteria present in root nodules of leguminous plants, which help in fixing atmospheric nitrogen, was a major breakthrough. Another important discovery was the symbiosis between roots of trees and underground fungi which was reported by Albert Bernhard Frank who coined the term 'mycorrhiza' (Frank 1885). A number of other studies confirmed the occurrence of microbes in plants with reports ranging from parasitic organisms to beneficial ones. Currently, it is a renowned fact that many types of microbial endophytes, including bacteria, fungi, archaea and protists such as algae (Trémouillaux-Guiller et al. 2002) and amoebae (Müller and Döring 2009), utilize plants as their host for living. In the 1990s, endophytes were defined as all organisms residing within plants at some time in their life cycle, colonizing their internal tissues without creating any evident harm to the hosts (Petrini 1991). However, the definition has undergone numerous transitions with time. While some microbes may be living as dormant pathogens in plants and turn out to be pathogenic under particular environments (Kloepper et al. 2013), others may be beneficial and growth promoting to a specific plant species and pathogenic to another plant. Thus, establishing a crystal clear definition for endophytes has been an arduous task. Microbial numbers, genotype of plant and microbes, environmental circumstances and quorum sensing are important factors to be considered while distinguishing between pathogenic and non-pathogenic endophytes. As the word suggests, *endo* (inside) and *phyte* (plant), the term could be

used to refer to only the habitat that all microbes live inside the plant host for a part or all of their lifespan regardless of the function. As of today, endophytes have been identified to be dwelling in every plant family.

## 17.3 Plant-Endophyte Relationship

The presence of endophytic fungi has been traced back to 400 million-year-old Devonian Rhynie chert deposits from fossil records of plants which lacked a rooting system. Fungi and Peronosporomycetes (organisms similar to fungi) were ubiquitous and spread out widely on the Earth before the first appearance of land plants during the Silurian period of the Palaeozoic era (Taylor and Taylor 1993). Initial land plants lacked proper leaves or roots in them until the Devonian period, during which they developed prominent rooting system and leaves (Beerling et al. 2001; Raven and Edwards 2001). However, even the most ancient preserved land plants, which are deficient of distinct leaves, roots and shoots, had fungal endophytes present in them (Krings et al. 2012). This clearly states that plants have evolved along with fungi and other microbes, which were present on the Earth before them, and plants had adapted to exist on Earth along with the endophytes in them during their period of evolution.

The nature of plant-endophyte interactions ranges from mutualism to pathogenicity depending on numerous biotic and abiotic factors including genotypes of the plant and the microbe, environmental conditions and dynamic interactions within the plant biome. Endophytes promote plant growth by fixing nitrogen, helping in the uptake of mineral nutrients such as phosphorus and iron (Alvin et al. 2014). Endophytes also modulate the levels of phytohormones such as auxin, cytokinin, gibberellin and ethylene in plants (Martinez-Klimova et al. 2017). Endophytic fungi are also known to help the plants in which they reside by assisting them in acclimatization towards various stress factors (heat, salinity, drought, diseases, herbivores, etc.) (Rodriguez et al. 2009). *Curvularia* sp., isolated from *Dichanthelium lanuginosum*, has portrayed improved heat resistance on the host plant. Similarly, *Fusarium culmorum* is found to increase the tolerance against salinity in the host plant *Leymus mollis* (Rodriguez et al. 2009).

Endophytes can remain in plant tissues throughout their lifespan. When the plant parts, like leaves, fall off, they can continue to survive in the fallen leaves of host plants by converting into saprophytes and help in degradation (Korkama-Rajala et al. 2008; Voriskova and Baldrian 2013; Prakash et al. 2015). Endophytes undergo up-regulation of several genes in order to support this conversion to saprophytes (Zuccaro et al. 2011).

Though the mechanism and role of endophytes in plants are still under study, there are various hypothesis proposed on the endophyte's properties (Kusari et al. 2012a). One of those is mosaic theory according to which the endophytes create a chemical environment in the host plant tissue which prevents them from phytophagous and pathogens (Carroll 1991). In another parallel theory, endophytes are addressed as acquired immune systems for the plant in which they reside (Arnold

et al. 2003). An even more topical hypothesis called xenohormesis (Howitz and Sinclair 2008) states that evolutionarily certain microbes might have attained the potential to sense stress-induced signalling molecules from plants and also the competence to synthesize the bioactive compounds, due to selection pressure. However, with time, the heterotrophs might have lost the potential to synthesize the compounds, or the genes responsible for synthesis might have got silenced, and they only retain their sensing ability (Kusari et al. 2012a). Recently, several natural products which were believed to be restricted only in plants are found to be synthesized by microbes and animals. For example, morphine which was earlier reported only from plants (*Papaver somniferum*) was discovered even in mammals (Grobe et al. 2010). Similarly, several metabolites produced by natural plants were reported to be produced by the endophytes as well. In fact, there is also a possibility that some of the metabolites produced by the natural plants are the byproducts of the endophytes residing in the plants (Kusari et al. 2012a).

## 17.4 Production of High-Value Plant Secondary Metabolites

Plants produce certain bioactive compounds which are not essential for their growth but are defence response towards the environmental stress factors. These compounds are generally termed as 'secondary metabolites' which have various medicinal applications. Secondary metabolites produced by plants include alkaloids, terpenoids, flavonoids, steroids, peptides, quinols, phenols and polyketones, which have several medicinal properties like anticancer, antimicrobial, immunesuppressive, anti-inflammatory and antioxidant (Korkina 2007).

From the statistical point of view, it is clear that plants play a vital role in the worldwide drug market with 25% of the approved drugs being originated from plants, and among the 252 generic drugs acknowledged by the WHO, 11% are plant-based drugs (Dubey et al. 2012). A minimum of 120 plant-based active compounds are in regular practice in most countries (Taylor 2005). Besides, ~47% of the anticancer drugs actively being used worldwide are plant-derived natural products (Newman and Cragg 2007). WHO has reported recently that nearly 60,000 plant species across the world have been estimated to be used for their medicinal properties, leading to 500,000 tons of the plant material being traded annually worldwide with a market value of USD 2.5 billion (Dushenkov 2016). Increased trading has reduced most plant population drastically, with only 1.4% remaining on the Earth's surface (Dushenkov 2016). The tropical rainforests which have the largest diversity of plant species are plunging at swift rate from 14% to a meagre 6% with not even 1% of them being focused towards novel drug discoveries, which eventually may result in several species getting extinct without even studying them for valuable metabolites (Taylor 2005). Hence, there is a severe need to reduce the dependence on the plants for their metabolites by shifting towards alternate and sustainable sources of such metabolites.

Though in vitro plant cell culture techniques are visualized as commercial alternatives for plant secondary metabolite production, they suffer from limitations

including scale-up difficulties, low productivities, contamination risk, need of expensive phytohormones and genomic instability (Howat et al. 2014). Hence, production of secondary metabolites using plant cell cultures on a commercial scale is not much successful except in few cases such as ginseng, shikonin, berberine and taxol (Linden 2006). In case of camptothecin, though there are reports on plant cell culture production, they are not yet commercially successful (Kai et al. 2015). Apart from this, chemical synthesis is also looked forward as potential substitute for such metabolite production. However, commercial trials on total chemical synthesis of complex plant secondary metabolites have mostly resulted in failure, except for a few simple structured compounds like vanillin, whose demands have been widely substituted with synthetic vanillin (Koeller and Wong 2001). Chemical synthesis of compounds like morphine is uneconomical owing to complications in their sterical structure with five chiral centres. Similarly for chemical synthesis of paclitaxel, 40 steps of processing are required which finally results in a low product yield of less than 5% (Holton et al. 1994a, b). Camptothecin when attempted to be synthesized chemically also resulted in a low yield of 14% with losses in many intermediate steps (Yu et al. 2012).

Another method of production is heterologous expression of genes involved in the biosynthesis pathway. In taxol, a number of steps in the pathway are catalysed by cytochrome P450 (cP450) acyltransferases and oxygenases (Howat et al. 2014). Functional expression of these cP450s in microbial systems such as *Escherichia coli* and *Saccharomyces cerevisiae* has been the bottleneck in taxol synthesis using heterologous microbial hosts, as cP450s fold incorrectly and are inserted into the cell membrane in these systems (Howat et al. 2014). Further, expression of taxol biosynthesis genes in a plant host, *Arabidopsis thaliana*, led to growth retardation (Besumbes et al. 2004)**.** Heterologous production using microbial host could not be achieved for metabolites like camptothecin, since the complete biosynthetic pathway has not been elucidated (Kai et al. 2015). This is the case for many other similar metabolites like podophyllotoxin, vincristine and vinblastine.

## 17.5 Secondary Metabolite Production by Endophytes: An Alternate Route?

Endophytes, which reside in the plants throughout the plants' lifetime, have attracted researchers from around the globe for their potential to produce the same secondary metabolites as that of the host plant. The first such reported endophytic fungus, *Taxomyces andreanae*, producing taxol was isolated from *Taxus brevifolia* during the early 1990s (Stierle et al. 1993). Over the past decade, reports on endophytes producing plant secondary metabolites have increased by more than tenfolds. Certain commercially significant metabolites produced by the endophytes and their host plants are illustrated in Fig. 17.1.

Cultivation of endophytes under in vitro conditions is more economical in comparison to plant cell culture due to low-cost substrate and other nutrient requirements for microbial fermentation. Unlike plant cell cultures, endophytes do not

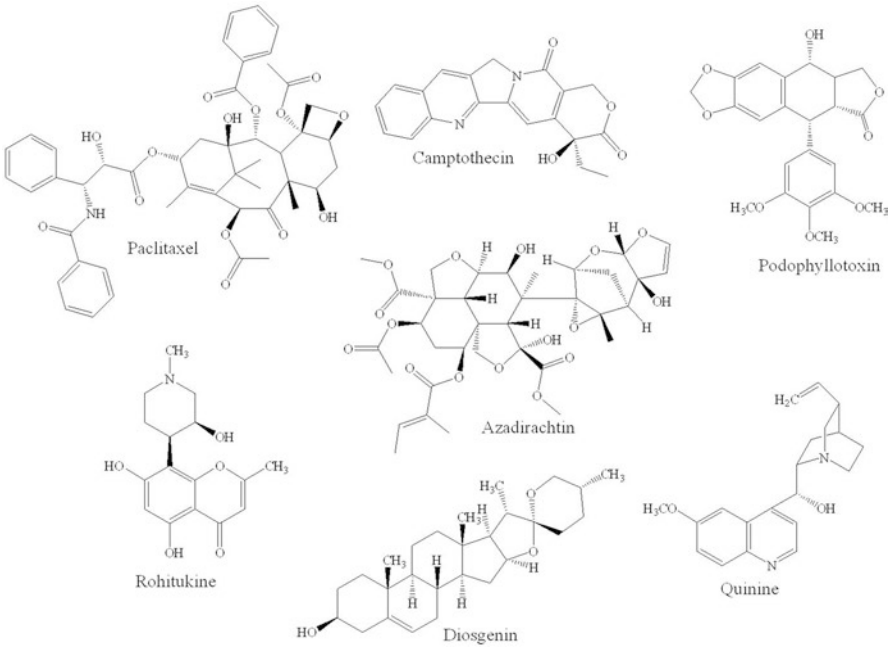


**Fig. 17.1** Structures of selected commercially important secondary metabolites produced from plants and also the endophytes isolated from them

have hormonal requirements for growth. Production on industrial scale can be done using waste products such as molasses or whey liquid, which can make the process even more simple and cost-effective (Venugopalan and Srivastava 2015). It is difficult to maintain sterility for longer cultivation period in plant cells due to slow growth rates in comparison to microbes. In case of camptothecin production, plant cells were incubated for a period of 3 weeks (van Hengel et al. 1992; Karwasara and Dixit 2013), whereas endophytes were incubated only for 4 days (Shweta et al. 2010) to 7 days (Puri et al. 2005). Fermentation condition optimization and scale-up process are simpler in case of microbes over plant cell cultivations. Also, implementation of yield improvement techniques such as addition of elicitor and supply of precursor is easily adaptable (Zhao et al. 2010). Endophytic production of metabolites can prevent over-exploitation of the natural plant sources and are also a sustainable source in comparison to plants which vary in their yield depending on developmental stages and seasonal variation (Vance et al. 1994; Liu et al. 1998; Pai et al. 2013).

Though metabolites extracted from endophytes demonstrate a wide range of commercial applications, a major deterrent to commercial exploitation of endophytes has been the widely reported problem of product yield attenuation with subculture (Table 17.1), which can make them a non-sustainable and non-reliable source at large scale. However, it is reported that these disadvantages can be surmounted through optimization of bioprocess parameters and by triggering the

**Table 17.1** Attenuation in the metabolite yield by endophytes

| Endophytic fungus | Host plant | Metabolite produced | Yield of different generations | | | | | | | | References |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th | 8th | |
| *Periconia* sp. 2026 | *Torreya grandifolia* | Taxol | 350 ng $L^{-1}$ | 325 ng $L^{-1}$ | 290 ng $L^{-1}$ | 200 ng $L^{-1}$ | 118 ng $L^{-1}$ | | | | Li et al. (1998) |
| *Fusarium solani* INFU/CA/KF/3 | *C. acuminata* | Camptothecin | ~6 μg $g^{-1}$ | ~5.5 μg $g^{-1}$ | ~0.5 μg $g^{-1}$ | ~1 μg $g^{-1}$ | ~1 μg $g^{-1}$ | ~0.5 μg $g^{-1}$ | ~0.4 μg $g^{-1}$ | | Kusari et al. (2009b) |
| *Phomopsis* sp. UAS014 | *N. nimmoniana* | Camptothecin | + | 21.7 μg $g^{-1}$ | 11.4 μg $g^{-1}$ | 6.6 μg $g^{-1}$ | | | | | Gurudatt et al. (2010) |
| *Aspergillus* sp. LY341 | *C. acuminata* | Camptothecin | 7.93 μg $l^{-1}$ | <LOD | | | | | | | Pu et al. (2013) |
| *Aspergillus* sp. LY355 | *C. acuminata* | Camptothecin | 42.92 μg $l^{-1}$ | 4.06 μg $l^{-1}$ | <LOD | | | | | | |
| *T. atroviride* LY357 | *C. acuminata* | Camptothecin | 197.82 μg $l^{-1}$ | 5.33 μg $l^{-1}$ | 2.57 μg $l^{-1}$ | 2.47 μg $l^{-1}$ | 3.69 μg $l^{-1}$ | 2.15 μg $l^{-1}$ | 1.90 μg $l^{-1}$ | 1.83 μg $l^{-1}$ | |
| *F. oxysporum* MTCC-11383 | *Dysoxylum binectariferum* | Rohitukine | ~1.9 μg $g^{-1}$ | ~1 μg $g^{-1}$ | ~0.8 μg $g^{-1}$ | ~0.6 μg $g^{-1}$ | | | | | Kumara et al. (2014) |
| *G. fujikuroi* MTCC-11382 | *Amoora rohituka* | Rohitukine | ~1.8 μg $g^{-1}$ | ~1.3 μg $g^{-1}$ | ~1.1 μg $g^{-1}$ | ~0.8 μg $g^{-1}$ | | | | | |

cryptic pathways for metabolite synthesis in the endophytes (Venugopalan and Srivastava 2015). Table 17.1 lists some of the reports, which show product yield attenuation with subculture in the axenic cultures of endophytes.

## 17.5.1 Antimicrobial and Anticancer Compounds Produced by Endophytes

### 17.5.1.1 Antimicrobials

In the past century, antimicrobial compounds such as antibiotics have proven indispensable in combating microbial infections not only in humans but also in other areas such as agriculture. However, this has also led to the evolution of antibiotic-resistant strains. It was estimated that nearly 25,000 people died in Europe in 2009 due to infections caused by multiple drug-resistant bacteria (Freire-Moran et al. 2011). Hence, the need of the hour is the development of novel antimicrobial compounds to combat multidrug-resistant bacteria.

Endophytes produce a wide range of antimicrobial compounds, presumably to compete with the other microorganisms residing in the plant tissues and prevent their colonization. Therefore, bioprospecting of endophytes can be a promising alternative for discovery of novel antimicrobial compounds. The antibiotic compounds reported from endophytic fungi majorly belong to the phylum *Ascomycota* and that from the endophytic bacteria are from the phylum *Actinobacteria* (Martinez-Klimova et al. 2017). Antibiotic-producing endophytes have been isolated from a diverse variety of plants, globally (Martinez-Klimova et al. 2017). Methanol, ethyl acetate and hexane extracts from *Colletotrichum gloeosporioides*, an endophyte isolated from *Vitex negundo* by Arivudainambi et al. (2011), showed inhibitory activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Further, the extracts showed a synergistic effect when used with common antibiotics such as penicillin and methicillin, opening up the avenues for new means of combating microbial infections. Rani et al. (2017) isolated 20 different fungal endophytes from the medicinal plant *Calotropis procera*, out of which 7 showed antimicrobial activity against various species of bacteria belonging to the genus *Salmonella*. There has been an increase in the number of studies that show endophytes from medicinal plants being a source of antimicrobial metabolites (Dar et al. 2017). Table 17.2 lists few examples of antimicrobial compounds identified from isolated endophytes in literature.

### 17.5.1.2 Anticancer Agents

The scientific pursuit of using plant-derived metabolites as anticancer agents started with vinblastine and vincristine in 1950 (Chandra 2012). Thereafter, several compounds from plants have been used for the production of clinically useful anticancer drugs. Major compounds on that list include taxol, camptothecin, vinblastine, vincristine and podophyllotoxin.

**Table 17.2** Bioactive metabolites produced by some isolated endophytes

| Metabolite | Bioactivity | Endophyte | Host plant | Yield | References |
|---|---|---|---|---|---|
| Azadirachtin | Biopesticide | *Eupenicillium parvum* | *Azadirachta indica* | 0.4 µg/100 gm 43 µg/L | Kusari et al. (2012b) |
| Camptothecin | Anticancer | *Entrophospora infrequens* | *Nothapodytes foetida* | 49.6 µg/gm | Amna et al. (2006) |
| | | *Fusarium solani* (MTCC 9667) | *Apodytes dimidiata* | 0.37 µg/gm | Shweta et al. (2010) |
| | | *Fusarium solani* (MTCC 9668) | *Apodytes dimidiata* | 0.53 µg/gm | |
| | | *Nodulisporium* sp. | *Nothapodytes foetida* | 5.5 µg/gm | Rehman et al. (2008) |
| Deoxypodophyllotoxin | Anticancer | *Aspergillus fumigatus* (INFU/Jc/KF/6) | *Juniperus communis* | 3 µg/L | Kusari et al. (2009a) |
| Diosgenin | Progesterone precursor, cholesterol lowering activity | *Cephalosporium* sp. (84) | *Paris polyphylla* var. *yunnanensis* | (+) | Zhou et al. (2004) and Xiao-dong et al. (2007) |
| Huperzine A | Neurodegenerative disease treatment | *Acremonium* sp. (2F09P03B) | *Huperzia serrata* | (+) | Li et al. (2007) |
| | | *Blastomyces* sp. (HA15) | *Phlegmariurus cryptomerianus* | (+) | Ju et al. (2009) |
| | | *Botrytis* sp. (HA23) | *Phlegmariurus cryptomerianus* | (+) | |
| | | *Penicillium chrysogenum* (SHB) | *Lycopodium serratum* | (+) | Zhou et al. (2009) |
| Hypericin | Antidepressant, anti-inflammatory, antimicrobial, antioxidant, antiviral | *Chaetomium globosum* (INFU/Hp/KF/34B) | *Hypericum perforatum* | (+) | Kusari et al. (2008) |
| Podophyllotoxin | Anticancer | *Alternaria neesex* (Ty) | *Sinopodophyllum hexandrum* | 2.4 µg/L | Cao et al. (2007) |
| | | *Fusarium oxysporum* (JRE1) | *Juniperus communis* | 28 µg/g | Kour et al. (2008) |
| | | *Phialocephala fortinii* (PPE5, PPE7) | *Sinopodophyllum peltatum* | 0.5–189 µg/L | Eyberger et al. (2006) |
| | | *Trametes hirsuta* | *Sinopodophyllum hexandrum* | 30 µg/gm | Puri et al. (2006) |

(continued)

**Table 17.2** (continued)

| Metabolite | Bioactivity | Endophyte | Host plant | Yield | References |
|---|---|---|---|---|---|
| Quinine | Antimalarial | *Diaporthe* sp. | *Cinchona ledgeriana* | 30 µg/l | Maehara et al. (2011) |
| | | *Arthrinium* sp. | *Cinchona ledgeriana* | 50 µg/l | Maehara et al. (2013) |
| | | *F. solani* | *Cinchona calisaya* | 0.9 mg/l | Hidayat et al. (2015) |
| | | *F. oxysporum* | | 0.9 mg/l | |
| | | *F. incarnatum* | | 0.8 mg/l | |
| Paclitaxel | Anticancer, antiviral | *Alternaria alternate* (TPF6) | *Taxus chinensis* var. *mairei* | 84.5 µg/l | Tian et al. (2005) |
| | | *Aspergillus fumigatus*(EPTP-1) | *Podocarpus* sp. | 557.8 µg/l | Sun et al. (2008) |
| | | *Cladosporium cladosporioides* (MD2) | *Taxus media* | 800 µg/l | Zhang et al. (2009) |
| | | *Fusarium solani* (Tax-3) | *Taxus chinensis* | 163.35 µg/l | Deng et al. (2009) |
| | | *Metarhizium anisopliae* (H-27) | *Taxus chinensis* | 846.1 µg/l | Liu et al. (2009) |
| | | *Pestalotiopsis neglecta* (BSL045) | *Taxus cuspidata* | 375 µg/l | Kumaran et al. (2010) |
| | | *Pestalotiopsis versicolor* (BSL038) | *Taxus cuspidata* | 478 µg/l | |
| | | *Tubercularia* sp.(TF5) | *Taxus chinensis var. mairei* | 185.4 µg/l | Wang et al. (2000) |
| Rohitukine | Anti-inflammatory, immuno-modulatory, anticancer | *Fusarium proliferatum* (MTCC 9690) | *Dysoxylum binectariferum* | 1.9 µg/gm | Kumara et al. (2014) |
| Toosendanin | anti-botulismic agent; agricultural insecticide | Unidentified (O-L-5, O-SC II-4, O-RC-3) | *Melia azedarach* | (+) | Wang et al. (2007a) |
| Vincamine | Vasodilator, cerebral stimulant | Unidentified (Vm-J2) | *Vinca minor* | 0.1 mg/L | Yin and Sun (2011) |
| Vinblastine | Anticancer | *Alternaria* sp. (97CG1) | *Catharanthus roseus* | (+) | Guo et al. (1998) |
| Vincristine | Anticancer | *Fusarium oxysporum* (97CG3) | *Catharanthus roseus* | (+) | Zhang et al. (2000) |
| | | Unidentified (97CG1) | *Catharanthus roseus* | 0.205 µg/L | Yang et al. (2004) |

### Taxol

Taxol was initially isolated from the bark of yew tree, *Taxus brevifolia* (Wani et al. 1971). Several other species from the genus *Taxus* were later reported to produce taxol. It is widely used for the treatment of ovarian, breast, lung, head, neck, renal, prostate, colon, cervix, gastric and pancreatic cancers (Zhou et al. 2010). The taxol-producing trees are not abundantly found in nature, and they also grow very slowly. The compound is found only in trace amounts (Zhou et al. 2010), as low as 0.01% dry weight of the bark (Zhou et al. 2010). Increasing demand for the drug has led to indiscriminate exploitation of taxol-producing trees, and it has become important to seek alternative, sustainable methods of producing taxol.

After the discovery of first taxol-producing endophyte in 1993, several similar endophytes with varying yields have been isolated. Interestingly taxol-producing endophytes have been isolated not only from taxol-producing plants but also from other plants such as chilli (*Capsicum annuum*) (Kumaran et al. 2011) and hazel (*Corylus avellana*) (Yang et al. 2014). Kumaran et al. (2011) reported a yield of 687 μg/L from the endophyte *Colletotrichum capsici* they isolated from the chilli plant, which is higher than what is usually seen in the case of endophytes isolated from taxol-producing plant species. Yang et al. (2014) further sequenced the entire genome of the taxol-producing endophyte *Penicillium aurantiogriseum* NRRL 62431 which they had isolated from the hazel plant and detected candidate gene sequences that could be involved in taxol biosynthesis. By comparison of these genes with taxol biosynthesis genes from plants, it seems unlikely that the genes were horizontally transferred to this fungus from a plant host. Apart from isolation of taxol-producing endophytes, several bioprocess strategies have also been applied to achieve yield enhancements, and they are discussed later in the chapter. At this moment, one may say that taxol is the most popularly sought after product with research in bioprospecting and bioprocessing of endophytes.

### Camptothecin

Camptothecin is a pentacyclic quinoline alkaloid used as a potent anticancer agent. Camptothecin and its derivatives find applications in the treatment of lung, breast, cervical and uterine cancers (Chandra 2012). Wall et al. (1966) first isolated camptothecin from the wood of the tree, *Camptotheca acuminata*. Other plants reported to contain camptothecin include *Nothapodytes nimmoniana*, *Ophiorrhiza*, *Ervatamia heyneana* and *Merrilliodendron megacarpum* (Chandra 2012). The scenario in camptothecin production is very similar to that of taxol, with the yield from natural sources being very low and the increasing demand leading to exploitation of the natural sources of the compound. The first report of a camptothecin-producing endophyte was by Puri et al. (2005). The fungus was isolated from the host plant, *Nothapodytes nimmoniana* and identified as *Entrophospora infrequens*. Reports on isolation of camptothecin-producing endophytes have been tabulated in Table 17.2. The major bottleneck in scaling up camptothecin production using endophytes is yield attenuation. In a study by Pu et al. (2013) on a camptothecin-producing endophyte *Trichoderma atroviride* LY357, yield attenuation was observed on repeated subculturing. The yield decreased but however was detectable even after eight

generations of subculturing and increased by 50-fold when optimization strategies were applied. This suggests that endophytes lose their biosynthetic capability in the absence of stimulus and regain their capability when appropriate stimuli are applied externally.

Even bacterial endophytes have been reported to produce camptothecin with anticancer activity (Shweta et al. 2013; Soujanya et al. 2017). In the latter case, the production of camptothecin by an endophytic strain of *Bacillus subtilis* attenuated on subculturing and completely ceased when it was cured of a plasmid it harboured. It is therefore possible that the plasmid contained key genes involved in camptothecin biosynthesis. Apart from camptothecin, endophytes have been shown to produce even derivatives of camptothecin such as 10-hydroxycamptothecin (Liu et al. 2010; Shweta et al. 2010) and 9-methoxycamptothecin (Shweta et al. 2013).

### Podophyllotoxin

Podophyllotoxin is a pharmaceutically active lignan compound, reported to occur in both gymnosperm and angiosperm plants belonging to the families Cupressaceae, Berberidaceae, Polygalaceae, Lamiaceae and Linaceae (Chandra 2012). *Podophyllum hexandrum* is now declared as 'critically endangered', and also agricultural production of podophyllotoxin by cultivation of *Podophyllum* plants has been unsuccessful due to unsuitable climatic conditions (Chandra 2012). The first report of a podophyllotoxin-producing endophyte was by Yang et al. (2003). Subsequently, several fungal endophytes belonging to the genera *Alternaria*, *Trametes*, *Phialocephala*, *Fusarium* and *Aspergillus* have been reported to produce podophyllotoxin. Yields as high as 189 μg/L have been reported for podophyllotoxin from endophytes (with the endophyte *Phialocephala fortinii* isolated from *Podophyllum peltatum*) (Eyberger et al. 2006). In another study Nadeem et al. (2012) isolated a strain of *Fusarium solani* from the roots of *Podophyllum hexandrum* that could yield 29.0 μg/g of podophyllotoxin. The maximum yield was obtained on the 8th day of cultivation, and application of bioprocess optimization strategies could increase the yield further. A few more examples of such species, along with their host plants, yield and reference, have been listed in Table 17.2.

### Vincristine and Vinblastine

Vincristine and vinblastine are alkaloids obtained from the plant *Catharanthus roseus*, commonly known as the Madagascar periwinkle. They can lower the number of white blood cells (Chandra 2012) and are hence used in the treatment of lymphoma and leukaemia. Though *Catharanthus roseus* is not endangered and can be easily cultivated in agricultural fields, the yield of vincristine and vinblastine from these plants is very low. To produce 1 g of vincristine, about 500 kg of *C. roseus* leaves are required (Yue et al. 2016). As the worldwide demand for vincristine and vinblastine is largely met by agricultural cultivation of *Catharanthus roseus,* not much of research has been focused on endophytes for producing these alkaloids. However, endophytes may offer a significantly cost-effective alternative in the future. The first report on a vinblastine-producing endophyte was by Guo et al. (1998) and that on vincristine was by Zhang et al. (2000). Palem et al. (2015) isolated 22 fungal

endophytes with the goal of discovering endophytes that produce vincristine and vinblastine. They tested them for anti-proliferative activity using HeLa cells. They also screened the fungi for the presence of the tryptophan decarboxylase (TDC) gene, which is a key gene involved in the synthesis of terpene indole alkaloids, the class to which vincristine and vinblastine belong to. *Talaromyces radicus* showed the highest anti-proliferative activity and was the only isolated species which contained the TDC gene. On further analysis, it was found that this species indeed could produce vincristine (670 μg/L) and vinblastine (70 μg/L). Endophytes that are known to produce vinblastine and vincristine are listed in Table 17.2.

Apart from antibiotics and anticancer agents, compounds isolated from endophytes show potential use as antidiabetics (Uzor et al. 2017), anti-inflammatory (Gao et al. 2008), antiviral (Zhao et al. 2010), antidepressants (Zhao et al. 2010) and antioxidants (Zhao et al. 2010).

## 17.6 Bioprospecting of Endophytes for Identification of Useful Metabolites

### 17.6.1 Isolation of Endophytes from the Host Plant

Isolation of endophytes is the initial process towards bioprospecting endophytes for metabolite production. A host plant contains a wide range of endophytes distributed throughout the plant. Hence, the endophytes isolated from any natural plant may vary with the type of plant tissue selected, the environmental factors and the developmental stage of the plant (Fisher et al. 1993; Collado et al. 1999).

Followed by the selection of explants, surface sterilization of the selected explants is carried out to get rid of various epiphytes and surface contaminants. It is necessary to carry out the surface sterilization on fresh explants so that the microorganisms inside the plant tissue are viable. In case if it is impossible to perform surface sterilization immediately, then it is mandatory to refrigerate the explants to restrain the microorganisms from death (Golinska et al. 2015). Surface sterilization process is a critical step as it decides the fate of the isolated microorganism, if it is an endophyte or an epiphyte (Verma et al. 2009). Examples of some of the surface sterilization protocols from literature are compiled and listed in the Table 17.3. Exposing the tissue to highly concentrated sterilizing reagents or longer exposure time to the reagents might result in destruction of the microorganisms residing within the tissues, and hence additional care should be taken while performing this step (Golinska et al. 2015). Post surface sterilization treatment, adequate washing of the explant is done with sterile distilled water to prevent any harmful effects caused by residual amount of surface-sterilizing agents. Further, to check the effectiveness of surface sterilization, water used for final wash of the explants is streaked onto a suitable agar plate and observed for any visible growth of microorganisms. Alternatively, the imprint of the explants can be taken on a suitable agar plate and observed for any visible growth of microorganisms. After surface sterilization, the explants are wounded and placed on suitable agar plates (Golinska et al. 2015).

**Table 17.3** Various surface sterilization protocols for isolation of plant endophytes

| Explant | Surface sterilization | Time of exposure | Excision | Medium | Brief description | References |
|---|---|---|---|---|---|---|
| Leaf and outer stem bark | Distilled water | Thorough wash | Random (0.5 $cm^2$) | Initially aqueous agar | When hyphae emerged from cut region, single hyphal tips were isolated and subcultured on rich medium PDA (28+/−2 °C). Brought to pure by serial subculturing | Shweta et al. (2010) |
| | 70% ethanol | 1 min | | Later with rich medium (PDA) | | |
| | 5% NaOCl | 5 min | | | | |
| | Sterile water | 3 times | | | | |
| Leaves (~2 gm/tree) | Distilled water | Thorough wash | 6 fragments per leaf (6 mm dia) | 1.5% MEA | 48-well plates were used. 1 ml of MEA in each well. One fragment placed on each well. Incubated at daylight at 20–22 °C for 4 weeks. The leaf fragments, grouped according to their macroscopic appearance (e.g. senescence, discolouration) and morphological-anatomical characters of their fungal colonies, such as sporulation, colony colour and diameter | Unterseher and Schnittler (2009) |
| | 70% ethanol | 2 min | Base with middle vein, centre left of middle vein, centre right of middle vein, margin right, margin left, tip | | | |
| | 1% NaOCl | 5 min | | | | |
| | 70% ethanol | 1 min | | | | |
| | Sterile water | | | | | |
| Yew bark (0.5 × 0.5 × 0.5 cm) | 70% ethanol, sterile water | | Outer bark removed | Small pieces of inner bark placed on PDA | Incubated at 25 °C for at least 2 weeks. Pure cultures transferred to another agar plate by the hyphal tip method. Identification methods were based on the morphology of the fungal culture, the mechanism of spore production and the characteristics of the spores | Wang et al. (2000) |
| Bark | 70% ethanol | 3 min | Cut into pieces | 0.1 ml bark paste added to 15 ml | Growth was observed. Individual hyphal tips of the various fungi were removed from the agar plates and placed on new PDA medium and incubated at 25 °C for at least 2 weeks. Checked for purity | Huang et al. (2001) |
| | Sterile water | Several times | 0.5 gm bark pieces ground into paste with 2 ml sterilized water | PDA medium in a Petri plate and cultured at 25 °C | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Leaf (2 × 2 mm from leaf blade and 2 mm long from petiole) | 70% ethanol | 1 min | | 2% MEA plates. Incubated for 1 month at 20 °C | Fungi grown from the segments were recognized as endophytes and isolated for morphological identification | Hata and Sone (2008) |
| | 15% hydrogen peroxide | 15 min | | | | |
| | 70% ethanol | 1 min | | | | |
| Leaf (6 mm dia. disc of veins and interveins), petiole (6 mm disc from 3–4 cm long segments) | 75% ethanol | 1 min | | Surface dried and placed on 90 mm Petri dish containing 2% MEA, supplemented with 1 mg/ml streptomycin sulphate and 0 ± 03 mg/ml rose bengal | Incubated at 25 °C for 2 months. Developed colonies transferred to new plates with MEA. Subcultures done with PDA, CMA, TWA | Guo et al. (2000) |
| | 65% commercial Chlorox (3.25% aqueous sodium hypochlorite) | 10 min | | | | |
| | 75% ethanol | 30 sec | | | | |
| Leaves, stems and fruits (0.5 $cm^2$) | Running tap water | Thorough wash | | Nutrient agar | Incubated at 23–25 °C for 10 days. Bacteria emerging from tissue were purified and cultured on NA plates | Shweta et al. (2013) |
| | 70% (v/v) ethanol | 3 min | | | | |
| | 4% sodium hypochlorite solution | 3 min | | | | |
| | Sterile water | 3 times | | | | |
| Leaves, branch pieces | Running water | Thorough wash | | 1.5% oxoid malt extract agar (MEA) supplemented with 250 mg/L Terramycin to suppress bacterial growth | Incubated at 20 ± 2 °C for 5–14 days. Isolation was by transfer of mycelium, conidia or ascospores to 2% MEA plates | Fisher et al. (1993) |
| | 75% ethanol | 1 min | | | | |
| | 0.93–1.3 M sodium hypochlorite | 3 min | | | | |
| | 75% ethanol | 0.5 min | | | | |

To selectively isolate endophytes of interest, i.e. either bacteria or fungi, growth inhibitory compounds are added to the isolation medium which preferentially permits the growth of only the organism of interest while restricting the unwanted organisms. For example, nalidixic acid and nystatin are supplemented in the isolation media to selectively isolate actinomycetes (Gohain et al. 2015). Similarly, antibacterials such as chloramphenicol can be added to facilitate growth of only endophytic fungi, by avoiding endophytic bacteria (Melo et al. 2014), while streptomycin can be used to isolate fungi with slower growth rate (Miller et al. 2012a, b). Morphologically distinct colonies obtained from the explants incubated on an isolation medium are further isolated and purified to obtain pure culture. Further, the pure cultures are screened for their ability to produce various metabolites or bioactive compounds. Various surface sterilization protocol and the specially formulated medium used for the isolation of endophytes from literature are provided in Table 17.3.

### 17.6.2 Screening of Endophytes for Valuable Metabolite Production

This step involves screening of the endophytes based on their ability to produce diverse bioactive compounds. Many endophytes are known to produce growth inhibitory compounds (such as antibiotic, antibacterial, antifungal) which can be of commercial interest. Such endophytes can be screened by their ability to inhibit test strains grown on the same agar plate. Alternatively, the spent medium, i.e. the fermentation broth used to grow the endophyte, can be used to check for its growth inhibitory potential using a test organism. For example, endophytic isolates (*Streptomyces* sp., *Streptosporangium* sp. and *Nocardia* sp.) from *Azadirachta indica* showed inhibitory effect on *Pseudomonas fluorescens*, *E. coli*, *S. aureus*, *B. subtilis*, *C. albicans*, *Microsporum* sp., *Phytophthora* sp., *Trichophyton* sp., *Aspergillus* sp. and *Pythium* sp. Methanolic extracts of the isolates' spent media were infused on paper discs, and the assay was performed by using Bauer-Kirby method with slight modifications (Verma et al. 2009). Similarly, methanolic extract from the spent media of *Colletotrichum gloeosporioides* isolated from *Vitex negundo* showed inhibitory effect on *B. subtilis* MTCC 619, *P. aeruginosa* MTCC 2488, *S. aureus* MTCC 3160, *E. coli* MTCC 4296 and *C. albicans* MTCC 3018, when used individually. Interestingly, the same extract if used in synergistic combination with antibiotics (vancomycin and penicillin) showed better inhibitory effect on the multidrug-resistant *S. aureus* strain 6 (Arivudainambi et al. 2011).

The antiparasitic activity of the extracts can be tested by performing gGAPDH and APRT assays. For example, *Diaporthe phaseolorum* isolated from *Viguiera arenaria* showed inhibition of GAPDH enzyme and adenine phosphoribosyltransferase (APRT) enzyme. The fermentation broth of the endophyte was extracted with ethyl

acetate, and it was found to inhibit gGAPDH enzyme of *Trypanosoma cruzi* by 95% and APRT enzyme of *Leishmania tarentolae* by 60.7% (Guimarães et al. 2008).

Similarly, the ability of endophytes to produce industrially relevant enzymes is screened by plating them on agar plates with suitable substrates. For example, skimmed milk agar plates are used to evaluate protease activity, carboxymethylcellulose (CMC) agar plates are used to determine cellulase activity, and chitin agar (CA) plates are utilized to evaluate the chitinase activity of the endophytes by measuring their zone of inhibition (Zheng et al. 2011). These methods of screening are generally used when the endophytes are screened for untargeted compounds. Few examples of similar activity screenings reported earlier have been listed in Table 17.4.

### 17.6.3 Extraction of Metabolites from Endophytes

Isolation of endophytic fungi is relatively a simple process; however screening them for the presence of metabolite is often complicated, especially in case of discovery of novel compounds which is quite challenging process. It is often straightforward to identify a class of compound but difficult to narrow down to a precise one. A wide range of the solvents have been employed in literature to extract out the metabolite of interest from the endophytes. It should be considered that a metabolite can be extracellular or intracellular. In few cases, metabolites are seen to be observed both in the culture medium and the cell pellet. *Gibberella fujikuroi* MTCC 11382 isolated from *Amoora rohituka* bark produced 1.93 μg/gm of rohitukine from the mycelia and 0.72 μg/mL rohitukine from broth (Kumara et al. 2014). Extracellular metabolites are generally present in the medium and can be extracted by simple liquid-liquid extraction method. Rohitukine was extracted from the spent media twice by using equal volume of n-butanol in a separating funnel (Kumara et al. 2014). On the other hand, intracellular metabolite requires cell disruption techniques to bring the metabolites from the cells into the solvent. Various cell disruption techniques such as homogenizer and sonicator are conventionally used. In a recent report, camptothecin was extracted from *Fusarium solani* MTCC 9668 by sonicating the dried biomass suspended in water using an ultrasonicator (Venugopalan et al. 2016). Similarly in another report, homogenization using a mortar and pestle was employed for disruption of cell wall (Shweta et al. 2013). Along with the conventional methods, microwave-assisted extraction has also been employed for camptothecin and is found to give better product yield when compared with the conventional methods (Fulzele and Satdive 2005). However, for purification of the product from crude mixture, solvent extraction plays a major role, which is mainly selected based on the polarity of the compound of interest. Solvents should have optimum polarity to dissolve both polar and non-polar compounds. Therefore, usage of very alkaline or acidic and extremely polar solvent should be evaded (Milne et al. 2013).

**Table 17.4** Representative list of bioactivity of endophytes against various test strains

| Endophytes | Activity | Host plant | Type of extract | Test strain | References |
|---|---|---|---|---|---|
| *Bacillus tequilensis*, *Chryseobacterium indologenes*, *Pseudomonas entomophila* and *Bacillus aerophilus* | Antibacterial; antifungal | *Aloe vera* | Crude and ethyl acetate | *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus pyogenes* and *Candida albicans* | Akinsanya et al. (2015) |
| *Streptomyces* sp., *Streptosporangium* sp. and *Nocardia* sp. | Antibacterial; antifungal | *Azadirachta indica* | Methanol | *Pseudomonas fluorescens*, *S. aureus*, *E. coli*, *B. subtilis*, *C. albicans*, *Trichophyton* sp., *Microsporum* sp., *Aspergillus* sp., *Pythium* sp. and *Phytophthora* sp. | Verma et al. (2009) |
| *Colletotrichum* sp., *Fusarium* sp., *Guignardia* sp., *Phomopsis* sp., *Phoma* sp. and *Microdochium* sp. | Antibacterial | *Tradescantia spathacea* | Ethyl acetate | *P. aeruginosa*, *S. aureus* and *E. coli* | Alvin et al. (2016) |
| *Diaporthe phaseolorum* | Antiprotozoan | *Viguiera arenaria* | Ethyl acetate | *Trypanosoma cruzi*, *Leishmania tarentolae* | Guimarães et al. (2008) |
| *Macrophomina phaseolina* | Antifungal | *Ocimum sanctum* | Hexane | *Sclerotinia sclerotiorum* | Chowdhary and Kaushik (2015) |
| *Botryosphaeria dothidea*, *Fusarium proliferatum*, *Rhizopus* sp. and *Aschersonia* sp. | Antibacterial; antifungal | *Camptotheca acuminata* | Supernatants | *B. subtilis*, *E. coli*, *Fusarium solani* and *Verticillium dahliae* | Machavariani et al. (2014) |
| *Nocardia caishijiensis* | Antibacterial; antifungal | *Sonchus oleraceus* | Crude | *S. aureus*, *E. coli*, *K. pneumoniae*, *S. aureus* and *Candida tropicalis* | Tanvir et al. (2016) |
| *Colletotrichum gloeosporioides* | Antibacterial; antifungal | *Vitex negundo* | Methanol | *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans* | Arivudainambi et al. (2011) |
| *Streptomyces* sp. | Antibacterial; antifungal | *Polygonum cuspidatum* | Ethyl acetate | *E. coli*, *Salmonella* sp., *B. subtilis*, *Enterococcus faecium*, *S. aureus* and *C. albicans* | Wang et al. (2016) |
| *Phoma* sp. | Antifungal | *Eleusine coracana* | Methanol | *Fusarium graminearum*, *Fusarium lateritium*, *Fusarium sporotrichioides*, *Fusarium avenaceum*, *Trichoderma longibrachiatum*, *Aspergillus flavus* and *Alternaria alternata* | Mousa et al. (2015) |
| *Pseudonocardia carboxydivorans* | Antibacterial; antifungal | *Ageratum conyzoides* | Crude | *B. subtilis*, *C. tropicalis*, *S. aureus* and *E. coli* | Tanvir et al. (2016) |

### 17.6.4 Identification and Confirmation of Metabolites from Endophytes

Preliminary investigation to test the presence of metabolites in the crude extracts from the culture broth of the endophytes involves techniques such as TLC (thin-layer chromatography), HPTLC (high-performance thin-layer chromatography) or HPLC (high-performance liquid chromatography) that gives information for the presence of a compound by matching with their standards.

Thin-layer chromatography (TLC) is a rapid technique using which multiple samples can be screened for the presence of metabolites. Crude extracts of multiple samples can be spotted on the silica plates along with the standards and drawn up using suitable solvents via capillary action. The plates are then visualized under UV with the presence of appropriate indicators, if needed. HPTLC was reported to quantify taxol content from 20 endophytic fungi samples by comparing with standard taxol using chloroform: methanol (9:1) as the solvent system. The samples exhibited many spots on the plate with one of them corresponding to standard taxol indicating its presence in the test sample (Gangadevi and Muthumary 2008). In another report, TLC and HPTLC were used to detect camptothecin content in the endophytic extract. The extracts along with the standard are spotted on the silica gel plates and developed using chloroform and ethyl acetate in the ratio 1:1 and analysed using TLC scanner and Win CATS 1.4.4.6337 software at a wavelength of 254 nm (Bhalkar et al. 2015, 2016).

HPLC is yet another most widely used technique for the identification and quantification of secondary metabolites. HPLCs are well known for their high reliability, accuracy, reproducibility and precision in data measurement. Small amount of the samples are separated on the column packed with 2–50 μm particles as stationary phase based on the difference in their physicochemical interactions and partition coefficients between the stationary and the mobile phase. Reduced flow rate and smaller pore-sized packing facilitate better separation with high precision and accuracy. The retention times of the analytes are compared with that of their standard retention time and quantified using the standard correlations built using X-Y plots of area under the curve versus known concentrations of the standard. HPLC-based quantification has been employed widely in literature for various secondary metabolites produced by endophytes like paclitaxel (Jianfeng et al. 1999; Pan et al. 2004; Renpeng et al. 2006; Sun et al. 2008; Deng et al. 2009; Liu et al. 2009; Zhang et al. 2009; Kumaran et al. 2010), camptothecin (Amna et al. 2006; Rehman et al. 2008; Kusari et al. 2009b; Gurudatt et al. 2010; Shweta et al. 2010; Pu et al. 2013), vinca alkaloids (Guo et al. 1998; Yang et al. 2004; Yin and Sun 2011), azadirachtin (Kusari et al. 2012b), podophyllotoxin (Eyberger et al. 2006; Puri et al. 2006; Cao et al. 2007; Kour et al. 2008), rohitukine (Kumara et al. 2014), etc.

However, the above said methods do not confirm for the presence of the compound when standards are not available. However, mass spectrometry is a tool for identification of known and unknown compounds and for confirmation of specific targeted compounds. Coupling of mass spectrometry with liquid and gas chromatography is a powerful technique for detection and identification of low-volume known and novel compounds in crude extracts.

The endophyte screened for the presence of bioactivity can be further subjected to gas chromatography-mass spectrometry (GC-MS) to identify the array of compounds present in the extract which can be responsible for the bioactivity. GC-MS approach helps us in identifying the compounds by performing a library search from the databases (Schauer et al. 2005). The mass of the compounds corresponding to each peak of the chromatogram is further fragmented into $MS^2$ and compared with the library to predict and identify the compound. Stoppacher et al. (2010) reported identification of 25 different microbial volatile organic compounds (2-heptanone, 1-octen-3-ol, 3-octanone, 2-pentyl furan, 3-octanol, α-phellandrene, α-terpinene, β-phellandrene, γ-terpinene, α-terpinolene, 2-nonanone, phenylethyl alcohol, 2-n-heptylfuran, p-menth-2-en-7-ol, 2-undecanone, α-bergamotene, β-farnesene, 6-pentyl-α-pyrone, γ-curcumene, α-curcumene, α-zingiberene, α-farnesene, β-bisabolene, β-sesquiphellandrene, nerolidol) from the extract of *Trichoderma* sp. by coupling solid-phase extraction with GC-MS. Similarly, extract of an endophyte *Colletotrichum gloeosporioides* isolated from *Lannea corammendalica*, when subjected to GC-MS analysis, displayed the presence of compounds such as 9-octadecenamide, hexadecanamide, diethyl pythalate, 2-methyl-3-methyl-3-hexene and 3-ethyl-2,4-dimethyi-pentane and exhibited antimicrobial activity. Another strain of *C. gloeosporioides* isolated from *Phlogacanthus thyrsiflorus* revealed the presence of phenol, 2,4-bis (1,1-dimethylethyl), 1-hexadecene, 1-hexadecanol, hexadecanoic acid, octadecanoic acid methyl ester and 1-nonadecene upon GC-MS analysis (Rabha et al. 2015). It is to be noted that gas chromatography can be employed only for compounds which could be volatile and thermostable.

Liquid chromatography-mass spectrometry (LC-MS) approach is majorly used for specific metabolite confirmation since they lack inbuilt library search as in the case of GC-MS but has higher resolution and sensitivity. Recent advanced versions of LC-MS instrument with high accuracy have the capability to display $[M + H]^+$ value up to 4 decimals with an error of less than 5 ppm. Additionally, fragmentation of specific *m/z* values results in MS/MS ion formation, which can be compared with literature for further confirmation. Few $[M + H]^+$ values and their MS/MS fragments of known metabolites are shown below in Table 17.5. There are also several online search tools or databases such as the METLIN database (Smith 2005), the Madison Metabolomics Consortium Database (MMDB) (Cui et al. 2008) and the Human Metabolome Database (HMDB) (Wishart et al. 2009). These databases help in identification by comparing the spectral data with those available from the databases for metabolite search. However these databases are yet to be updated with many compounds which are still unreported (Vasundhara et al. 2016). Though mass spectrometry confirms our metabolite at molecular weight level, isomers which have varying structures cannot be clearly differentiated with this technique.

Nuclear magnetic resonance (NMR) is an approach which helps in structure prediction of known or novel compounds and also confirmation of known compounds by analysing the proton ($^1H$) or carbon ($^{13}C$) magnetic resonance. For example, presence of vincristine and vinblastine in the endophytic extract was confirmed by analysing the $^1H$ NMR spectra and chemical shift of the endophytic vincristine and vinblastine in comparison with the standards (Kumar et al. 2013). Similarly,

**Table 17.5** m/z values of selective metabolites and their fragmentation pattern.

| Compound | $[M + H]^+$ | $MS^2$ fragments | References |
|---|---|---|---|
| Azadirachtin | 663 | 645, 627, 609, 545, 527 | Kusari et al. (2012b) |
| Camptothecin | 349.1 | 305, 447.3, 284.2, 149.0 | Ramesha et al. (2008) and Shweta et al. (2010) |
| 9-Methoxy camptothecin | 379.1 | 335.2, 516.4, 474.3, 305, 379.2 | |
| 10-Hydroxy camptothecin | 365.1 | 303, 305 | |
| Diacetoxy-camptothecin | 431.1 | 349.1, 303, 149 | Ramesha et al. (2008) |
| Diacetoxy-9-methoxy camptothecin | 461.2 | 379.1, 333.1, 415.2 | |
| Acetoxy-camptothecin-glycoside | 511.1 | 469.2, 365.1, 289.0, 307.1, 349.1, 149, 189 | |
| 9-Methoxy-mappacine-20-β-glucopyranoside | 499.2 | 337.1 | |
| Mappicine-20-β-glucopyranoside | 469.2 | 289, 307, 365.1, 207, 349, 319 | |
| Rohitukine | 306.1 | 288, 245 | Kumara et al. (2014) |
| Rohitukine N-oxide | 322.12 | 304, 276 | |
| Piperine | 286.1 | 135, 143, 171, 201 | Chithra et al. (2014) |
| Paclitaxel | 854.3 | 286, 367, 395, 464, 509, 545, 551, 568, 587 | Das et al. (2017) |
| Vinblastine | 811 | 355, 522, 542, 733, 751, 793 | Kumar et al. (2013) |
| Vincristine | 825 | 766, 807 | |

withanolide from the endophyte *Talaromyces pinophilus* isolated from *Withania somnifera* was structurally confirmed using NMR (Sathiyabama and Parthasarathy 2017). However, convectional NMR requires metabolite to be in the pure form for structure prediction. Recent development such as coupling an LC with NMR has made that task even simpler, where separation can be made by the LC and the fractions can be analysed simultaneously in NMR. Though LC-NMR helps in analysis of each peak of the chromatogram using a stop flow valve, it is difficult to analyse crude extract, with complex and closely eluting compounds (Wolfender et al. 2001). An overall representation of various techniques used for identification and quantification of targeted and untargeted metabolites is given below (Fig. 17.2).

## 17.7 Bioprocess Optimization Strategies for Enhanced Metabolite Production by Endophytes

### 17.7.1 Culture Condition Optimization

Fermentation parameters such as temperature, pH, medium composition, agitation, inoculum concentration and photoperiod are known to significantly affect the yield of secondary metabolites in fermentation processes (Thiry and Cingolani 2002). A

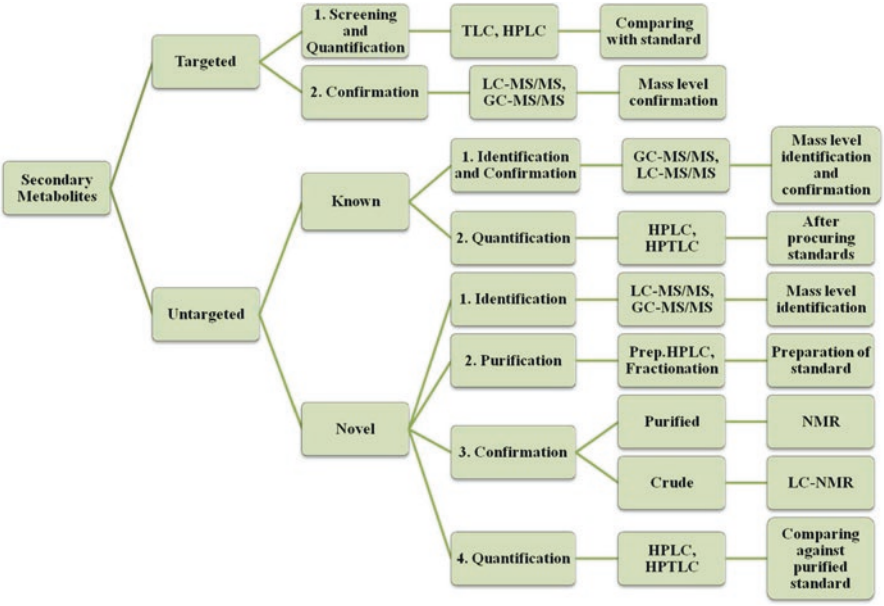


**Fig. 17.2** Schematic representation of the steps which can be involved in the identification and quantification of known and novel metabolites produced in endophytic fermentation

straightforward approach to culture condition optimization is single-factor optimization, where each factor is separately optimized while keeping all other factors constant. However, statistical optimization gives us the advantage of understanding interactions between different factors with minimum number of experiments.

In the case of endophytic fungi, statistical as well as single-factor optimization has been explored. An eightfold increase was observed in the yield of zofimarin (antifungal compound) from the endophyte *Xylaria* sp. Acra L38 after statistical optimization of carbon and nitrogen sources (Chaichanan et al. 2014). Similarly, a single-factor medium optimization study resulted in 10.3-fold enhancement in the production of mycoepoxydiene from the endophyte *Phomopsis* sp. Hant25 (Thammajaruk et al. 2011). Optimization of initial pH along with carbon and nitrogen sources resulted in 1.27-fold enhancement in beauvericin production from *Fusarium redolens* Dzf12 (Xu et al. 2010). In another study optimization of temperature and medium composition gave 77% enhanced sipeimine yield from *Fritillaria ussuriensis* Fu7 (Yin and Chen 2011).

## 17.7.2 Exogenous Additions

### 17.7.2.1 Elicitors

Elicitors are molecules that are involved in signalling under different stress conditions such as pathogenesis and hypersensitivity and are known to induce and regulate many genes. Elicitors may be classified as biotic and abiotic, and abiotic

elicitors may be further classified as physical and chemical. Biotic elicitors include materials of biological origin such as chitin, polysaccharides, glycoproteins, etc.

Elicitors such as salicylic acid and methyl jasmonate are signalling molecules in plant systems and have been widely used for yield enhancement even in endophytic systems. Among other chemicals used as elicitors, metal ions are involved in metabolism indirectly as enzyme cofactors or directly by means of redox reactions with other metabolites. Hence, metal ions can be added as a means to enhance the yield of target metabolites. Liu et al. (2010) optimized the production of 10-hydroxycamptothecin in *Xylaria* sp. isolated from *Camptotheca acuminata* by adding various elicitors, which included metal ions such as $Ce^{3+}$, $Cr^{3+}$, $La^{3+}$, $Cu^{2+}$, $Fe^{2+}$, $Se^{5+}$, $Mn^{2+}$, $Ca^{2+}$ and $Li^{+}$. Among them $Mn^{2+}$ and $Li^{+}$ produced a yield of 5 mg/l compared to the control (2 mg/l).

Somjaipeng et al. (2016) studied the effect of seven different chemical elicitors (salicylic acid, jasmonic acid, phenylalanine, serine, silver nitrate, sodium acetate and ammonium acetate) on taxol yield from the endophytes *Paraconiothyrium variabile* and *Epicoccum nigrum*. They also studied the synergistic effects of the elicitor and the pH of the growth medium using response surface models, which is one of the statistical optimization methods commonly used in bioprocess optimization. Serine was found to be the best elicitor for *E. nigrum*, resulting in an increase of taxol yield up to 29.6-fold.

In another study by Qiao et al. (2017), taxol yield from the endophytic fungus *Aspergillus aculeatinus* Tax-6 isolated from the tree *Taxus chinensis* was improved from 335 μg/L to 1338 μg/L after addition of sodium acetate, salicylic acid and copper sulphate. Copper ions are said to increase the activity of oxidases involved in taxol biosynthesis, and salicylic acid is a well-known signalling molecule that acts as an elicitor. The amounts of the elicitors added were further optimized using response surface methodology.

#### 17.7.2.2 Precursors

Another strategy to improve the product yield is by exogenously adding its biosynthetic precursors in the culture medium. Adding intermediates of the desired metabolite synthesis pathway can increase the reaction flux towards the desired metabolite leading to its enhanced production. Such intermediates may be readily available and hence this technique is useful. The amount of precursor added must be optimized such that there is an increase in yield without causing toxicity to cells (Gaosheng and Jingming 2012). Amna et al. (2012) reported the stimulation of camptothecin production from the endophytic fungus *Entrophospora infrequens* RJMEF001 using various precursors such as tryptophan, tryptamine and leucine.

Apart from elicitors and precursors, enzyme inhibitors such as 5-azacytidine, which blocks DNA methyltransferase, have been added exogenously to sustain the production of secondary metabolites. As DNA methylation was hypothesized to attenuate camptothecin production in the endophyte by silencing the genes involved in camptothecin biosynthesis, this enzyme inhibitor was used to enhance camptothecin production in the attenuated cultures of the endophyte *Botryosphaeria rhodina* isolated from *Camptotheca acuminata* (Vasanthakumari et al. 2015). Also, multiple

strategies mentioned above may be combined to produce a synergistic effect as observed by Pu et al. (2013) in the case of camptothecin production from the endophyte *Trichoderma atroviride* isolated from *Camptotheca acuminata.* A combination of different optimization strategies involving culture conditions (media composition, pH, temperature, agitation, incubation time) and elicitation led to a 50-fold enhancement in the yield of camptothecin.

### 17.7.3 Co-cultivation

Several strategies in the case of optimization of secondary metabolite production seek to simulate the natural environment of the endophyte. The regulation of biosynthetic genes is tightly linked to environmental parameters, and hence secondary metabolites are produced only when required by the cells. Co-cultivation of endophytes with other endophytes or cells/tissues from the host plants is one such strategy that seeks to simulate the natural environmental conditions. In several cases, a significant yield enhancement has been reported with the use of co-cultivation strategy.

The parameters such as inoculum ratio, environmental parameters, medium components and reactor design can be optimized during co-cultivation to maximize the production of the desired metabolite (Venugopalan and Srivastava 2015).

#### 17.7.3.1 Microbial Co-culture Systems

In the natural environment of the endophyte, it also interacts with other endophytes and invading microorganisms which may also affect the metabolite production by the endophyte. It is hence worthwhile to experiment with co-cultures of different endophytes to enhance the production of secondary metabolites.

Soliman and Raizada (2013) worked with the taxol-producing endophyte *Paraconiothyrium* SSM001 and reported that co-culturing the endophyte with another endophyte *Alternaria* sp. resulted in a threefold increase in taxol yield. Further, adding another endophyte *Phomopsis* sp. to this co-culture system resulted in a net eightfold increase in taxol production. They hypothesize that *Paraconiothyrium* SSM001 produces more taxol in response to other fungi that invade the plant, so as to benefit the plant and survive in symbiosis with the plant.

Ola et al. (2013) reported a 78-fold increase in the yield of enniatin A1 from the endophyte *Fusarium tricinctum* when co-cultured with *Bacillus subtilis* in comparison to axenic culture. They also observed that some metabolites that were not detected in the axenic culture were found to be above the detectable limits in the co-culture system. Though there have been no reactor level studies reporting co-culture of an endophyte with another endophyte, it is possible to culture several strains in a bioreactor. For example, Hernández et al. (2018) cultivated up to four strains of fungi together in a batch process, for cellulase production. Similar approaches could be used to co-cultivate endophytic fungi for maximizing the yields of desired metabolites.

#### 17.7.3.2 Plant-Endophyte Co-culture Systems

In nature, the endophytic fungi adapt to grow inside their host plants, and hence the profile of the metabolites can significantly change under axenic culture conditions possibly due to loss in in planta selection pressure and stimulus, thereby also affecting its biosynthetic potential. Therefore, one of the ways to simulate the natural environment under in vitro conditions can be by co-cultivation of plant cells/tissues with endophytes which may mutually benefit the two organism's (i.e. plant and microbe) biosynthetic capabilities. Ding et al. (2017) isolated three endophytic fungal strains, *Aspergillus* sp., *Fusarium* sp. and *Ramularia* sp., from the plant *Rumex gmelini* Turcz (RGT). All three strains were capable of producing bioactive metabolites that were produced by their host plant. They reported an increase in the production of the bioactive secondary metabolites, chrysophaein, resveratrol, chrysophanol, emodin and physcion in the seedlings of the plant when co-cultured with the endophytic fungi. In another co-culture study by Baldi et al. (2008), it was found that co-culturing podophyllotoxin-producing plant cells from *Linum album* showed an increased production of podophyllotoxin and 6-methoxypodophyllotoxin when co-cultivated with arbuscular-mycorrhiza like fungi. Co-culturing of *Linum album* plant cells with *Piriformospora indica* resulted in a yield enhancement of 3.6 times for podophyllotoxin and 7.4 times for 6-methoxypodophyllotoxin. Similarly, the same plant cells when co-cultivated with *Sebacina vermifera* resulted in an yield enhancement of 3.9 times for podophyllotoxin and 7.6 times for 6-methoxypodophyllotoxin These findings highlight that co-cultivation of endophytic fungi with plant cells when either or both of them are capable of producing the target metabolite can be a promising yield enhancement strategy.

Bioreactors for co-culturing plant cells and fungal cells have also been designed and reported in literature. Such bioreactors usually consist of two divisions, one each for plant and fungal cells, separated by a semipermeable membrane (Fig. 17.3). The semipermeable membrane serves for the exchange of metabolites between the plant cells and fungal cells, without having to place them in direct contact with each other. Li et al. (2009) co-cultured *Taxus chinensis* plant cells and the endophytic fungus *Fusarium mairei* isolated from the same plant in a specially designed bioreactor. The bioreactor consisted of two tanks, one each for the plant cell suspension and the fungus, separated by a membrane to allow only exchanges between metabolites (Fig. 17.3). A 38-fold increase in the pacilitaxel yield could be achieved in comparison to monocultures possibly due to exchange of metabolites (including biosynthetic intermediates) during the co-cultivation period.

### 17.7.4 Genetic Modifications

Genetic transformations can play a key role in commercial exploitation of endophytes, by enabling research on the genetics of endophytes as well as insertion of biosynthetic genes and regulatory elements of interest for yield enhancement of the target metabolite. One of the earliest methods used for transformation of fungal endophytes is protoplast transformation. PEG-mediated transformation and *Agrobacterium tumefaciens*-mediated transformations were also developed later.

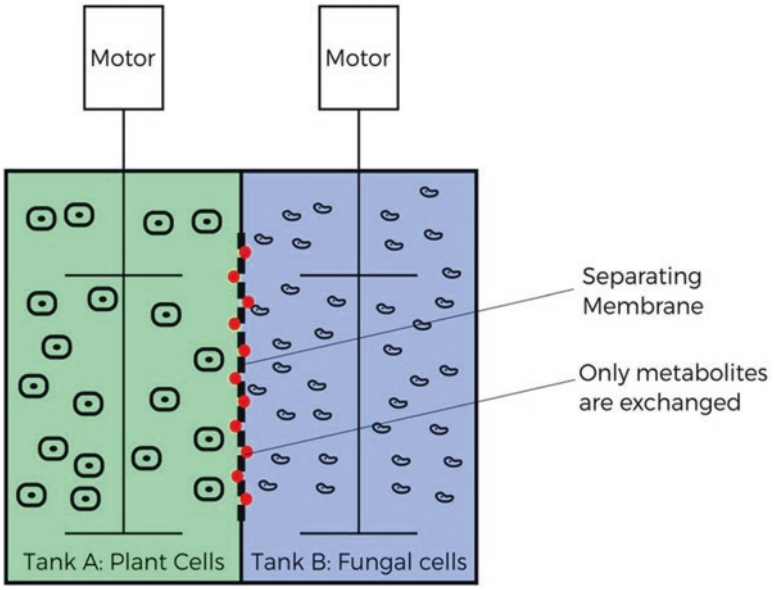


**Fig. 17.3** Plant-microbe co-cultivation bioreactor set-up (adapted from Narayani and Srivastava 2017)

#### 17.7.4.1 Protoplast Transformation

Protoplasts are cells in which the cell wall has been removed. Once the cell wall is removed, it is easier for the cells to take up exogenously added DNA. Protoplast transformation on endophytic fungi was first demonstrated by Long et al. (1998). A filamentous fungus, *Pestalotiopsis microspora*, isolated from the inner bark of the taxol-yielding Himalayan yew tree was used in the experiments. A gene-encoding hygromycin resistance was expressed using regulatory sequences from *Aspergillus*.

#### 17.7.4.2 PEG-Mediated Protoplast Transformation

While using protoplast transformation, adding PEG (polyethylene glycol) increases the rate of uptake of DNA into cells and is hence used in transformation techniques. Wei et al. (2010) established a PEG-mediated transformation protocol for the endophytic fungal strain *Ozonium* sp. EFY-21. The strain is known to produce taxol. A gene conferring resistance to hygromycin was expressed the trpC promoter from *Aspergillus nidulans* to verify successful transformation. This protocol was used by the same group (Wei et al. 2012) to overexpress the taxadiene synthase gene in the same strain, *Ozonium* sp. EFY-21,which resulted in an increase of up to 3.77-fold in the taxol yield.

#### 17.7.4.3 *Agrobacterium tumefaciens*-Mediated Transformation

*Agrobacterium tumefaciens* is commonly used for transformation of plant cells. Interestingly, Bundock et al. (1995) reported that *A. tumefaciens* is able to transfer its T-DNA to a fungal species, *Saccharomyces cerevisiae*. Several other reports on

using *A. tumefaciens* for transforming different types of fungi subsequently came out (Aimi et al. 2005; Michielse et al. 2005; Betts et al. 2007).

Liu et al. (2013) successfully used this method of transformation on the above-stated taxol-producing endophytic strain of *Ozonium* sp. EFY-21. The transformation efficiency was higher compared to the PEG-mediated transformation method. An *Agrobacterium*-mediated transformation protocol was used by Soliman et al. (2017) to integrate geranylgeranyl diphosphate synthase gene into the genome of the taxol-producing fungus *Paraconiothyrium* SSM001. Geranylgeranyl diphosphate is a precursor in taxol synthesis, and a threefold increase in taxol yield was observed when this precursor was overproduced by the modified fungal cells.

#### 17.7.4.4 Nuclease-Based Methods: REMI and CRISPR

REMI (restriction enzyme-mediated integration) is a method of integrating DNA fragments into the host genome using restriction enzymes introduced into the cells. It was first demonstrated by Schiestl and Petes (1991). The taxol-producing endophytic strain *Ozonium* sp. BT2 was transformed using this method by Wang et al. (2007b) which was proven to have increased transformation efficiency when compared with conventional PEG-mediated protoplast transformation (Bölker et al. 1995). However, there are not many reports demonstrating the use of REMI on endophytes.

The CRISPR/Cas9 system from the bacterial adaptive defence system (Barrangou et al. 2007) has been adapted into a tool for a genome editing (Doudna and Charpentier 2014). There is a considerable potential for the use of CRISPR/Cas9-based genome editing in endophytes as the system offers simple customizability with regard to the target sequences and precision in editing. Though CRISPR/Cas9 has not been directly demonstrated on an endophyte after isolation from a plant system, a report by Chen et al. (2017) demonstrates the use of CRISPR/Cas9-based genome editing in the fungus *Beauveria bassiana*, which is capable of growing as a plant endophyte (Parsa et al. 2013). Apart from introducing biosynthetic genes into endophytes, CRISPR/Cas9 system may also be used to edit regulatory sequences and control the expression of biosynthetic genes that are not expressed under axenic conditions. Hence, we can expect CRISPR/Cas9-based genome editing to be used for genetic modification of endophytes in the future.

## 17.8 Conclusion and Future Directions

Endophytes continue to be a promising alternative source for production of plant-based secondary metabolites. Literature suggests that one of the major reasons for product yield attenuation in endophytes under axenic conditions could be the absence of genetic and epigenetic stimulus provided by the natural environment and lack of biosynthetic intermediates. Implementation of bioprocess optimization strategies has resulted in yield enhancement of secondary metabolites during endophytic fermentations. The product yield retrieval and enhancement even in the attenuated strains of endophytes via bioprocess optimization strategies demonstrate

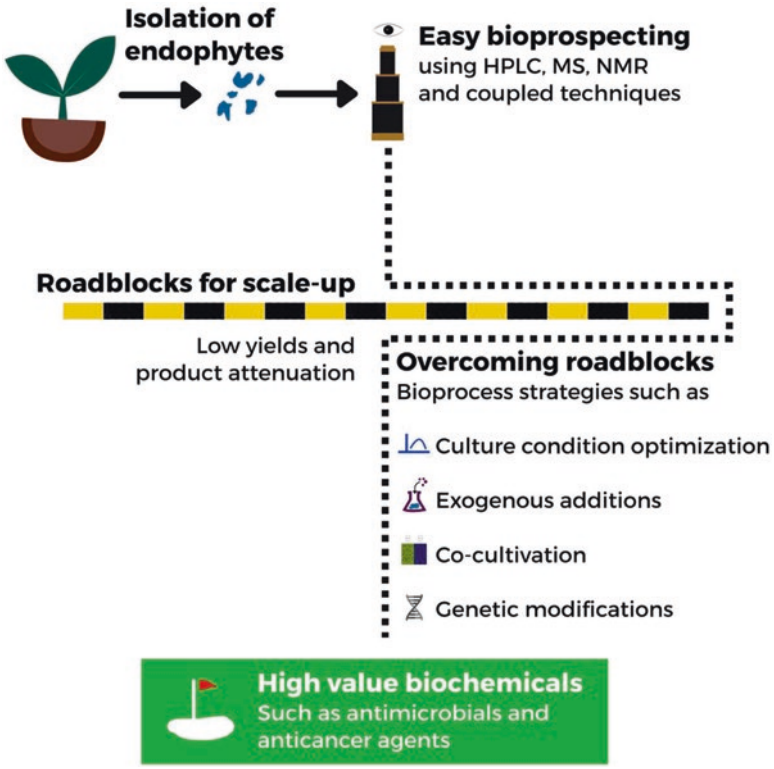


**Fig. 17.4** Schematic summary of endophyte-based bioprocess development for in vitro production of high-value biochemicals

that endophytes are capable of metabolite production even under in vitro conditions if provided an optimum environment. Hence, mimicking the natural environment under in vitro condition and activation of silent genes through genetic modification in combination with the most optimum fermentation conditions (Fig. 17.4) can help us overcome the current limitation of low product yield and attenuation in endophyte fermentations.

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# 18

# Synthesis and Application of Hydroxamic Acid: A Key Secondary Metabolite of *Piriformospora indica*

Bansh Narayan Singh, Akash Hidangmayum, Ankita Singh, Shailendra Singh Shera, and Padmanabh Dwivedi

## Abbreviations

| | |
|---|---|
| 6MPTOX | 6-Methoxy podophyllotoxin |
| DIBOA | Hydroxamic acids 2,4-dihydroxy-1,4-benzoxazin-3-one |
| DIMBOA | 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one |
| HDAC | Histone deacetylase |
| MeJA | Methyl jasmonate |
| NCED | 9-cis-epoxycarotenoid dioxygenase |
| PTOX | Podophyllotoxin |
| SAHA | Suberoylanilide hydroxamic acid |

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## 18.1 Introduction

*Piriformospora indica* is a root endophytic fungus which belongs to the group Basidiomycota having growth-promoting effects in several hosts (Cordoba et al. 2009). In plant species, this fungus can be seen growing both inter- and intracellularly by formation of pear shaped chlamydospores. It doesn't enter endodermis and aerial parts of the plants (Cordoba et al. 2009; McGarvey and Croteau 1995). Colonization of *P. indica* with roots of plant enhanced growth and development of host plant, disease resistance against biotic and abiotic stresses as well as phosphorus and nitrogen assimilation (Humphrey et al. 2006; Kumar et al. 2012). Fungal spore and culture filtrate of *P. indica* have beneficial effects on plant growth suggesting better nutrient uptake or hormonal signaling by the fungus. Colonized plants of *P. indica* show morphological changes in the root and physiology suggesting the induction of regulatory pathways (Yuan et al. 2007). *P. indica* can be cultured axenically and has the capability to grow on a number of complex and semisynthetic media (Zuccaro et al. 2011). These multifaceted attributes of *P. indica* led researchers to investigate its symbiotic association with a wide range of host plants and study the association on molecular basis. Association of *P. indica* with medicinal plants is reported to enhance secondary metabolites production in plants. Commercially important bioactive compounds can be enhanced by the use of plant-fungus interaction. This symbiotic association of plant-fungus can pave way for an alternative way of enhancing the accumulation of secondary metabolites. Molecular mechanisms responsible for increasing secondary metabolite content in plants associated with *P. indica* are presently unknown. The possible reason for enhanced accumulation of metabolite could be better nutrient uptake by the host and activation of defense-related pathways and associated signaling networks. This chapter reviews the most recent literature focus on plant growth promotion, defense mechanisms and accumulation of plant bioactive compounds in a diverse variety of crops associated with *P. indica.* Both nutritional and non-nutritional factors have been taken into account to suggest the biomass enhancement and accumulation of plant secondary metabolites upon association with *P. indica*.

Metabolomic analysis by using high-throughput, gas-chromatography-based mass spectrometry observed that 549 metabolites out of 1126 total compounds were produced in colonized and uncolonized Chinese cabbage roots having hyphae of *P. indica* (Hua et al. 2017). HPLC analysis of *P. indica* culture supernatant showed seven peaks in the hyphae and one main peak in the culture filtrate. Major peak was identified as benzoic acid, but the function is still not clear. The nature of the stimulatory effect of *P. indica* is yet to be known (Adya et al. 2012). Several evidences have highlighted that *P. indica* hyphae secrete many secondary metabolites such as hydroxamic acid, indoleacetic acid (IAA), chlorohydroxamic acid, etc. In this review, we focus on the role of hydroxamic acid of *P. indica* in plant growth promotion and defense mechanism.

## 18.2 Mechanism of Enzymatic Synthesis of Hydroxamic Acid

Amidase has broad substrate specificity which converts amides to the corresponding carboxylic acids and ammonia. Amidase exhibits "Bi-bi Ping-pong" mechanism for acyl transfer activity. First the amides react with the enzyme to give acyl-enzyme complexes (E-S complexes) which form carboxylic acids. If hydroxylamine is present instead of water (in case of acyl transfer activity) which is a strong nucleophilic agent, then its interaction with E-S complex results in the production of hydroxamic acids (Fig. 18.1). The enzyme retains its original state after the formation of the product and is ready to convert another molecule of amide and hydroxylamine to hydroxamic acid (Haron et al. 2011; Pandey et al. 2011; Sharma et al. 2012).

## 18.3 Levels and Effects of Hydroxamic Acid in Plants

Patanun et al. (2017) reported that histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA), which is a derivative of hydroxamic acid, can alleviate salt stress by decreasing sodium ion concentration in stems and increase survival rates under high salinity in cassava (Table 18.1). Transcriptomic analysis reveals that SAHA upregulated the expression of allene oxide cyclase which is a catalyzing agent and catalyzes important step in biosynthesis of JA. This study demonstrated that the HDAC inhibitor is an effective small molecule for alleviating salinity stress in crops and could improve the understanding of the mechanisms by which histone acetylation regulates responses to abiotic stress in cassava. SAHA treatment can reduce $Na^+$ concentration in both leaves and stems. Plants are able to survive high salinity stress conditions through the maintenance of $K^+$ and $Na^+$ homeostasis using several transporters (Patanum et al. 2017).

The amount of hydroxamic acid (Hx) concentration in plant varies from species to species. There is no evidence available about level of hydroxamic acid in cereal seeds (Epstein et al. 1986), but concentration of Hx continuously increased as discussed above in wheat and maize. It reaches maximum after germination in maize

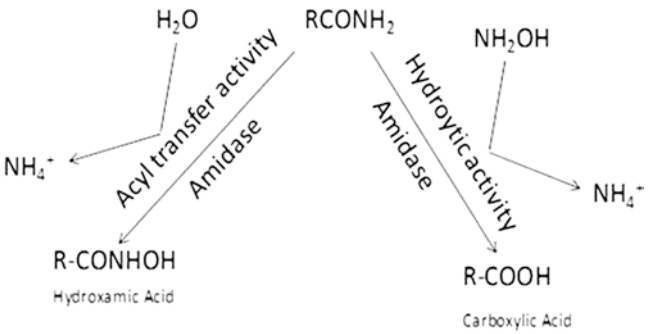


**Fig. 18.1** Types of reactions catalyzed by amidase. (Modified from Bhatia et al. 2013)

**Table 18.1** List of hydroxamic acid derivatives and their applications

| Hydroxamic acids derivatives | Applications | References |
|---|---|---|
| Benzohydroxamic acid | Antitumor, antineoplastic | Bhatia et al. (2012) |
| Acetohydroxamic acid (Lithostat) | To treat ureaplasma, anemia, anti-HIV agent | Pandey et al. (2011) |
| Fatty hydroxamic acids | Anti-inflammatory to treat chronic asthma | Haron et al. (2012) |
| Deferoxamine B (Desferal) | Antimalarial | Giannini et al. 2015) |
| α-Aminohydroxamic acid | Anti-HIV agent, psoriasis inhibitor | Munster et al. (2001) |
| Marimastat | To treat small cell lung cancers | Muri et al. (2002) |
| Inhibitor of LTA4 | Anti-inflammatory | |
| Idrapril | Render cardioprotective effects | |
| N-formyl hydroxylamine BB-3497 | Antibacterial agent | |
| Cyclic hydroxamic acids | Provide resistance against pathogen and insects | Copaj et al. (2006) |
| Unsaturated and middle-chain hydroxamic acid | Wastewater treatment,nuclear technology | Haron et al. (2012) |
| Nicotinyl hydroxamic acid | Tyrosinase and melanin inhibitor | Chen et al. (2011) and Bhatia et al. (2014) |
| Spiropiperidine hydroxamic acid (SAHA) | Anticancerous | Bosiack et al. (2011) |
| Long-chain hydroxamic acids | As surfactants in detergent industry | Jahangirian et al. (2011a, b) |
| Poly hydroxamic acid | Used for gravimetric analysis and scavenging of heavy metal ions | Hassan et al. (2011) |

and wheat (Argandona et al. 1980). Thus, the level of Hx depends upon the cultivation of crops (Klun and Robinson 1969). Hydroxamic acid is synthesized in all the plant species, but relative levels of Hx in roots and aerial part of plants are altered within species and cultivar (Argandona et al. 1981). The amount of Hx is predominantly more in stems as compared to leaf tissue. However, no significant concentration of Hx was reported in xylem exudates or guttation drops in maize and wheat (Argandona and Corcuera 1985; Guthrie et al. 1986). Subsequently, Hx level also varies within leaves. Younger leaves contain more Hx as compared to older leaves. Hx levels are more in the vascular bundles as compared to the leaves of maize (Argandona and Corcuera 1985) and wheat (Agandona et al. 1987). Furthermore, lateral veins contain higher amount of Hx as compared to the central vein of maize leaves (Argandona and Corcuera 1985). But Hx could not be detected in lower epidermal tissues of wheat leaves. Steler region contains more Hx level as compared to cortex in maize seedlings.

Broad spectrum of hydroxamic acid application has been studied in Chilean cultivars where the amount of hydroxamic acid levels was reported maximum at fourth or fifth days after seed germination. Interestingly, the level of DIBOA continuously decreased, and it became unmeasurable in some cultivars after tenth day of developmental stage, while conversion of benzoxazinoid hydroxamic acids derived from

2-hydroxy-2H-1,4-benzoxazin-3(4H) fluctuated in cereals and wheat callus culture (Zuiiiga et al. 1990). Prospective controls of hydroxamic acids in breeding programs for developing aphid-resistant cereal cultivation have been studied. Hydroxamic acid level in wheat (*Triticum aestivum* L.) reduced aphid correlation, but performance of aphid effect had considerably decreased in primitive diploid and tetraploid wheat (Thackray et al. 1990; Copaja et al. 1991).

## 18.4 Applications of Hydroxamic Acid

### 18.4.1 Histone Deacetylation by Hydroxamic Acid

Histone deacetylase (HDAC) is a class of enzymes that remove the acetyl groups from the histone proteins having an ε-N-acetyl lysine amino acid. This elimination of acetyl group allows DNA strand to wrap histone more tightly and regulates acetylation and deacetylation, thereby affecting the expression of DNA. Any change in the expression and mutations in HDACs gene leads to the development of tumor due to uncontrolled cell proliferation, cell cycle, and apoptosis (Giannini et al. 2015).

### 18.4.2 Effect of Hydroxamic Acid Against Antibiotic-Resistant Bacteria

Since pathogenic strains are becoming resistant to existing antibiotics, new approaches have to be explored. One such approach is the use of peptide deformylase (PDF). These are important enzymes which play a crucial role in bacteria for the synthesis of cell wall and plasma membrane. They belong to metallohydrolases family which is the most studied enzyme and an attractive target for drug design (Wei et al. 2000). These enzymes require $Fe^{2+}$ ion for their catalytic activity. In PDF ferrous ions bond loosely and hence can easily oxidized into ferric ion, resulting in the inactivation of enzyme. Therefore, in order to develop new PDF inhibitor moieties to counteract the pathogenic bacteria, new strategies and chemical compounds must be developed. PDF can be used as antibacterial drug design because (1) it is present in all bacteria (2), the gene present with this activity is important for bacterial growth in vitro, and (3) it closely resembles with various metallohydrolases. Since PDF is a metallohydrolase, hydroxamic acid can potentially inhibit this enzyme. Actinonin is a known hydroxamate-containing inhibitor of various metallohydrolases and acts as a chelating group that binds metal ion of the enzyme and inhibits its activity (Jayasekera et al. 2000; Wei et al. 2000).

### 18.4.3 Antibacterial Activity of Hydroxamic Acids

Hydroxamic acids play an important role in defense mechanism of several plants and thus function as natural pesticides. The cyclic hydroxamic acids 2,

4-dihydroxy-1, 4-benzoxazin-3-one (DIBOA) and 2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA) act as a defense molecule in cereals against insects and pathogenic microorganisms. *Erwinia* spp. cause soft rot disease in maize, but maize protects itself by secreting DIMBOA. DIMBOA is also secreted for the management of *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Piriformospora aeruginosa*, *Pseudomonas indica*, *and Yersinia enterocolitica* (Varma et al. 2001; Pepeljnjak et al. 2005).

### 18.4.4 Insecticidal Property of Plant-Derived Hydroxamic Acid

Hydroxamic acids reduced the survival and reproduction of aphids. Different varieties of cereals like wheat, maize, and rye produce different types of hydroxamic acids that hamper the growth of aphids (*Metopolophium dirhodum*). It has been reported that aphids fed with DIMBOA have poor survival rate as compared to aphids fed with diets lacking DIMBOA. Copaj et al. (2006) reported that a high hydroxaminic acid level in maize has similar relation with the resistance to the European corn borer *Ostrinia nubilalis*. Indeed, secondary metabolites can act as shielding agents in plants against insects either causing direct toxicity or as repellent (Janzen et al. 1977). A different concentration of hydroxamic acids can have diverse effect of aphid interaction in several gramineae. Some of the derivatives of hydroxamic acids, in particular DIMBOA-l, have been demonstrated to be inhibitory against insects (Klun et al. 1967; Long et al. 1977), fungi, and bacteria (Corcuera et al. 1978; Lacy et al. 1979).

### 18.4.5 Hydroxamic Acid in Wastewater Treatment and Nuclear Technology

Hydroxamic acids have also been reported to have potential use in wastewater treatment and nuclear technology to evolve new methods to reduce contaminating metal ions. This serves as a promising approach to clean wastewater contaminated with heavy metal ions (Haron et al. 2012).

### 18.4.6 Hydroxamic Acid in Analytical Chemistry and Detergent Industry

Hydroxamic acids have important role in analytical chemistry as reagents for gravimetric and spectrophotometric analysis of metal ions (Hassan et al. 2011). Owing to their ability to form complex with metal ions, long-chain hydroxamic acids are also used as surfactants in the detergent industry (Jahangirian et al. 2011a, b).

### 18.4.7 Regulation of Hydroxamic Acid Derivatives in Plant Signaling

Allene oxide cyclase (AOC) plays a key rate determining step in JA biosynthesis and JA derivatives such as methyl jasmonate (MeJA) which have reduced salinity stress in soybean (Yoon et al. 2009). Similarly, accumulation constitutive transcripts of AOCs elevated plant tolerance capability against salinity stress in tobacco cell lines (Yamada et al. 2002) and wheat (Zhao et al. 2014). Interestingly, SAHA treatment sturdily induced the mRNA expression level of MeAOC4. These findings suggested that SAHA application in plant can help JA signaling pathways which improves the plants tolerance ability against salinity stress. Another plant hormone which involves abscisic acid (ABA) inhibits seed germination, and the regulation of ABA biosynthesis has a role in the maintenance of seed dormancy. 9-Cis-epoxycarotenoid dioxygenase (NCED) catalyzes the reaction and is considered as a rate-limiting enzyme during ABA biosynthesis. Previously, in vitro study has argued that two hydroxamic acids, i.e., D4 and D7, used as inhibitors of carotenoid cleavage dioxygenase (CCD) and NCED of decrease germination time of tomato (*Solanum lycopersicum* L.) seeds constitutively by greater expression of *NCED1* (Awan et al. 2017). Further, no effect on seedling growth of tomato was observed in terms of height, dry weight, and fresh weight post-seed germination. Moreover, effect of chemical on seed germination in a tetracycline-inducible LeNCE D1 transgene of tobacco was highlighted where seed germination was controlled through chemical induction of NCED gene expression and the chemical inhibition of the NCED protein. Application of tetracycline increased germination timing and delayed hypocotyl emergence as similar to exogenous application of ABA and opposite to the D4 treatment (Awan et al. 2017). Similar effect was also monitored where D4 application improved germination percentage in lettuce seeds under thermo-inhibitory temperatures.

## 18.5 *P. indica* Symbiosis Association with Plant Roots Modulated Phytohormone Signaling

Promotion of plant growth is most evident in *P. indica* infected plants. It is reported that phytohormones released by plants under colonization with endophytes leads to plant growth promotion (Khatabi et al. 2012). *P. indica* is reported to promote initial stage of plant vegetative growth, thus leading to an early switch to the generative stages of host development (Vahabi et al. 2013). Plant root system is a direct target of colonizing endophytes. Auxin is a key chemical signal for root development during plant-microbe interactions (Hilbert et al. 2012; Franken 2012). Promotion of root growth by beneficial microbes is widely studied (Das et al. 2012). Associated microbes change the root architecture by interfering with the plant-auxin pathways (Rajasekaran et al. 2007). The culture filtrate of *P. indica* produces substances like IAA. This helps in regulation of plant growth and lateral root development (Swanson

et al. 1992). A higher level of IAA was found in colonized roots of 3-day-old barley seedlings when compared to control. *P. indica* strains with silenced *piTam1* gene were reported to have compromised IAA production and decreased colonization of barley roots in biotrophic phase (Modi et al. 2014).

Ethylene has an important role in plant development, germination, flower and fruit ripening, leaf senescence, and programmed cell death (Vahabi et al. 2015). In *Arabidopsis*, colonization with *P. indica* interferes with ethylene signaling components resulting in increased root colonization and inhibition of growth promotion (Pal et al. 2015). It is reported that repression of ethylene-responsive genes is involved in barley when colonized by *P. indica*. Regulation of host-microbe association and root physiology is induced by phytohormones such as cytokinin, gibberellins, jasmonate, salicylic acid, and strigolactone. These are how the associated signaling networks and phytohormones work together to generate compatible fungus-host interaction. This processes lead to root growth promotion and greater biomass accumulation (Kilam et al. 2017).

The investigation of *P. indica* mycelium extracts showed that mycelium extracts (1% v/v) reduced the hairy root growth, while treatment by podophyllotoxin (PTOX) and 6-methoxy podophyllotoxin (6MPTOX) after 2 h of production significantly stimulated root dry weight (Tashachori et al. 2016). It also has the ability to synthesize hydroxamic acid a secondary metabolite, which functions like a natural pesticide (Varma et al. 2001). It has been strongly advocated that *P. indica* has significance as a biofertilizer and biocontrol agent (Waller et al. 2005; Varma et al. 2012). *P. indica* reveals several positive consequences on diverse crop plants and has become an important candidate in biotechnological and microbiological research (Barazani and Baldwin 2013). It was reported that *P. indica* induce methionine synthase activity which facilitates methionine cycle of ethylene biosynthetic pathway (Peškan Berghöfer et al. 2004) during its colonization with plant roots via immune suppression, surprisingly explains the broad host range of the fungus (Schäfer et al. 2007; Jacobs et al. 2011).

Ethylene was reported to be involved in *P. indica*-plant interaction which modulates the interaction between them via signal molecules of fungi as well as plant receptors at the root cell surface after the fungal spore reside to attain the desired compatibility. Interestingly, ethylene signal magnitude contributes to the colonization of plant roots by *P. indica* where ethylene signaling either inhibits or enhances the growth of hyphae depending on the magnitude of signaling (Camehl et al. 2013). It is now confirmed that to establish symbiotic relationship, ethylene signaling network requires definite biochemical or genetic role to establish a communication across the symbionts as well as host plants to promote physiological benefits to each partner (Ansari et al. 2013).

## 18.6 Symbiosis Association Elevated Nutrient Uptake

The mutual interaction with *P. indica* and host plant provides enhanced nitrate/nitrogen uptake (Sherameti et al. 2005; Yadav et al. 2010). Increase in endogenous content of N, P, and K was observed in chickpea and black lentil plants colonized with *P. indica* (Nautiyal et al. 2010). In contrast, deficiency of Fe and Cu was surpassed when inoculated with *P. indica* (Gosal et al. 2011). Kumar et al. (2011) reported that *P. indica*-treated plants were able to uptake and transport P which may be related to increased plant growth and development via their various regulatory, structural, and energy transfer processes (Fig. 18.2). Further, *Z. mays* inoculated with *P. indica* mutant where, a phosphate transporter was knocked out; there was a reduction in endogenous content of phosphate (Yadav et al. 2010; Ngwene et al. 2013).

Further, it has been highlighted that iron deficiency in the growth medium could induce Hx level in maize (Manuwoto and Scriber 1985a, b), while lower temperature reduces Hx levels in maize roots (Thompson et al. 1970). Nitrogen application has more impact on Hx level. In gramineae cultivars, nitrogen application increased Hx level, while no significant effect of nitrogen was reported in some maize cultivars (Manuwoto and Scriber 1985a, b).

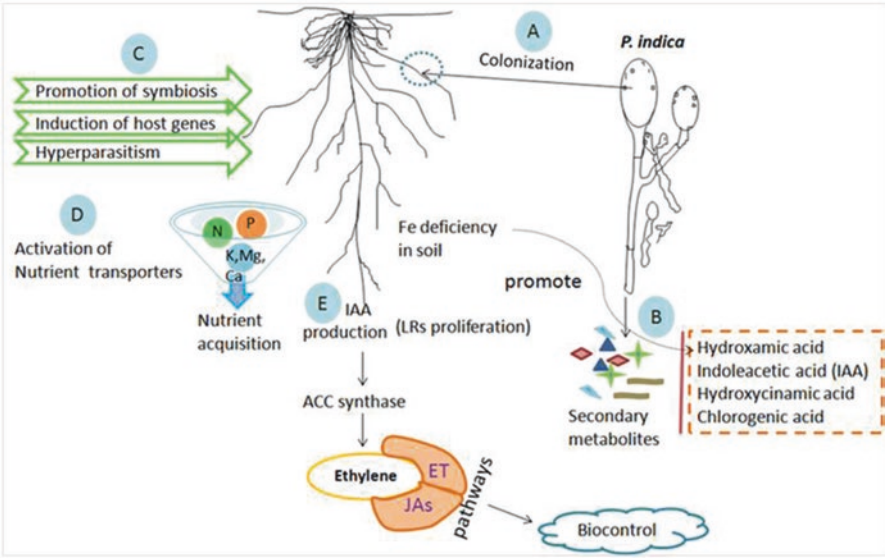


**Fig. 18.2** *P. indica* association with host roots and its role in host development. The first step shows colonization of hyphae with roots (**a**). After successful colonization, several secondary metabolites are secreted by *P. indica* hypha (**b**). Secondary metabolites promote symbiosis, induction of host genes, and hyperparasitism (**c**). Subsequently, *P. indica* balances nutrients level in plants through elevated efficacy of different nutrient transporters (**d**), and activation of JAs/ET signaling pathways leads to regulation of defense response (**e**)

## 18.7 Conclusion and Future Prospects

*Piriformospora indica* synthesizes secondary metabolites including hydroxamic acid having multifunctional roles in growth, protection, stress tolerance, and plant disease management of agricultural crops. It has the potential to manipulate physiochemical properties of the roots and might genetically reprogram root proliferation through mutualistic association. Hydroxamic acids can be synthesized naturally as well as enzymatically. Enzymatic approach can be used directly for medicinal purposes, plant growth and protection, and nutrient acquisition through hormonal regulation. Several hydroxamic acid derivatives have been chemically synthesized which can be applied in the agricultural field for improved disease control and management leading to improved crop protection. Hydroxamic acid derivatives are used as antibacterial agent, biocontrol agent, gene regulator in plant metabolism, and mineral uptake. Hydroxamic acid being an effective metal chelator, its role in iron chelation in agricultural soil needs to be investigated at molecular and cellular level in greater details, especially in those scenarios where severe iron deficiency exists in soil. Moreover, symbiotic relationship with phytohormone and *P. indica* colonized roots needs further investigation. Owing to these advantages of hydroxamic acids, research in mass production of *P. indica* in bioreactors using plant tissue culture technique can be a step closer toward commercialization of this agriculturally important compound.

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