Chapter 16
Fungal Hydrolytic Enzymes Produced by Plant Growth-Promoting Rhizobacteria (PGPR)

Lucky Duhan, Deepika Kumari, Rohit Verma, and Ritu Pasrija

Abstract Roots are the lifeline of plants and besides anchorage, they are a source of nutrients incorporation from soil. Although health-giving roots safeguard plants' fitness, but adjacent soil is also a dwelling place for various microbial pathogens, which might attack the roots. To neutralize this, soil has plant growth-promoting rhizobacteria (PGPR), which are generally free-living and populate around plants' roots. They defend plants from various biotic and abiotic stresses, as well as enhance soil texture for superior plant growth. The PGPR involves various species, like *Pseudomonas*, *Bacillus*, *Azospirillum*, *Rhizobium*, *Enterobacter, Agrobacterium, Serratia,* etc., but *Bacillus* and *Pseudomonas* are most predominant. They encourage robust plant growth, in both direct and indirect manner. The direct mechanism refers to nutrient uptake, release of siderophores, seed germination, etc. While indirect mechanisms include release of enzymes like chitinase, protease/elastase, cellulase, catalase, β-(1,3)-glucanase, etc., and hydrogen cyanide, and antibiotics. The hydrolytic enzymes synthesis/secretion is under stringent regulation and shields the roots from pathogens attack, including fungi. The enzymes targeting fungal microbes, either generate disturbance in the cell wall structure, interfere with membrane composition, impede hyphal formation, cause myco-parasitism, etc., leading to fungal cell death. Indirect mechanisms also involve induced systemic resistance (ISR) and reinforce the roots by evolving physical and chemical barriers to withstand adverse conditions.

The PGPR-mediated fungal biocontrol suggests their imperative role in sustainable pathogen management and ultimately supporting plants' well-being besides yield. This chapter summarizes the PGPR role in fungal control, especially through their hydrolytic enzymes.

Keyword Hydrolytic enzymes · Induced systemic resistance (ISR) · MOA · Myco-parasitism · PGPR · Phytopathogens

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1 Introduction

In the modern era, one of the biggest challenges before mankind is feeding the expanding population; this has placed a substantial liability on farmers and governments to increase the yield and crops' quality. To achieve this goal, various modern agricultural tools and hybrid seed varieties are developed and employed to significantly escalate the agricultural production. However, despite the deployment of these practices, different plant diseases cause significant reduction (~30%) in yields, which puts a huge economic burden on the producers and country (Sayyed et al. 2012). To combat these plant diseases, cultivators often turn towards chemical pesticides that are overpriced and have detrimental after-effects on the ecosystem as well. So, to circumvent these drawbacks of chemicals-based pesticides, renewed attempts involve inculcation of a safer and inexpensive practice involving the Plant Growth-Promoting Rhizobacteria (PGPR) that boost seed germination, root development, water utilization, resistance development against plant pathogens, etc., which finally promote plant growth and yields.

In 1904, a German scientist named Hiltner, coined the term "rhizosphere" referring to the soil around the plants' roots, which is rich in varied bacterial population density (100–1000 folds) than bulk soil. These bacteria form micro-colonies and constitute ~15% of the root surfaces (Gray and Smith 2005). "PGPR" refers to a heterogeneous group involving several bacterial species that populate the rhizosphere and promotes plant growth through separate mechanisms. Thus, it is predictable that the rhizosphere is a region of immeasurable microbes' interactions with plant roots, as root secretions act as a major nutrient source for these microbes and support efficient geo-cycling of nutrients. In general, PGPR can perform functions as biofertilizers, biostimulator, rhizo-mediator, and biopesticides (Table 16.1).

PGPR classification: Different criteria can be used for their classification and these are discussed here.

Based on Location Depending upon the interrelation with plant roots, PGPR can be categorized into two types: the first is **extracellular PGPR** (ePGPR), which are

Table 16.1 Classification of PGPR according to their use and mechanism of action (MOA)

Class	Description	Mechanism of Action (MOA)
Biopesticide	PGPR improve plant growth and yield by inhibiting the phytopathogens.	By production and release of hydrolytic enzymes, antibiotics, siderophores, hydrogen cyanide (HCN), induced systemic resistance (ISR), etc.
Biofertilizer	PGPR improve plant growth and yield by supplying growth nutrients.	By nitrogen fixation and utilization of insoluble nutrients from the soil
Phyto-stimulator	PGPR improve plant growth and yield by supplying of different phytohormones for various functions in plants.	By production of phytohormones i.e., indole acetic acid (IAA), gibberellic acid (GA), cytokinesis, ethylene, jasmonic acid (JA), etc.

Source: Adapted from Shah et al. (2018)

found predominantly inside the rhizosphere or in between root cortex cells. These include species of *Agrobacterium*, *Arthobacter*, *Bacillus*, *Caulobacter*, *Erwinia*, *Micrococcus*, *Pseudomonas*, *Serratia,* etc. The second group is **intracellular PGPR** (iPGPR), restricted to specific sections in root nodules (Gray and Smith 2005). In particular, Rhizobacteriaceae family bacteria reside in these zones, which contain *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium*, *Frankia,* etc. Experiments have validated the contribution of both these categories in improving the yields, by generating resistance in plants that too without any side effects (Vessey 2003; Gray and Smith 2005).

Based on Mechanisms Apart from location, direct or indirect impact is also a valid criterion to categorize PGPR, as shown in Fig. 16.1. Direct impacts involve nitrogen fixation, phosphorous and potassium solubilization, release of siderophores, seedling enhancement, etc., promoting nutrient uptake and growth of the plants. On the contrary, indirect influences comprise antagonistic compounds production like antibiotics, hydrolytic enzymes, etc., that provide resistance, especially against fungal phytopathogens, as compiled in Table 16.1. Additionally, fungal pathogens are rendered ineffective due to the mycoparasitism (parasite to fungi) activity of PGPR, and ultimately protecting the plant roots (Woo and Lorito 2007). Besides, rhizobacteria also augments the plant defense called "Induced Systemic Response (ISR)" by activation of a latent resistance system containing physical and chemical barriers (Loon et al. 1998). Intriguingly, the enhancement is not restricted to the nodular area but also protects the distal parts of plants. This response involves signaling pathways and employs compounds such as jasmonic acid (JA), ethylene and other components like antibiotics, siderophores, and hydrolytic enzymes, which exhibit synergism in inducing ISR against the phytopathogens. The next section explains

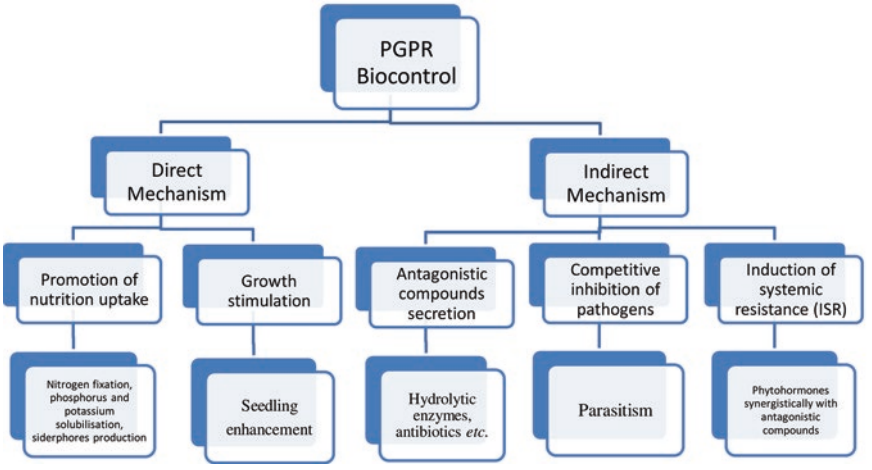

Fig. 16.1 Various mechanisms involving PGPR-mediated biocontrol of phytopathogens. Biocontrol may be done by one or more than one mechanism acting in synergism

the various components of PGPR secretions, with major emphasis on hydrolytic enzymes.

2 PGPR Secretions

PGPR emancipate various metabolites and hydrolytic enzymes, which doesn't allow the fungal phytopathogens to carry out a successful attack.

2.1 Hydrolytic Enzymes

The PGPR are efficient in production of several different hydrolytic enzymes i.e., chitinase, glucanase, protease/elastase, cellulase, catalase, etc. These enzymes have activities against several phytopathogens including fungi, thereby restricting several plant diseases. Although the hydrolytic enzymes perform their function via various mechanisms, but the major one remains degrading the glycosidic bonds in fungal wall chief component chitin. This inhibits hyphal formation in fungi, a crucial step in deeper fungal penetration in plant tissues.

Besides enzymes, various antibiotics, toxins, or volatile compounds are also synthesized by PGPR, which are extremely target specific and thus prevent varied pathogens from attacking plant root nodules. It is reported that physical factors such as pH, temperature, and moisture content influence antibiotic production (Shanahan et al. 1992). *Pseudomonas* secretes lipopeptides, hydrogen cyanide (HCN), phenazines, pyoluteorins, etc. (Haas and Keel 2003). Alongside, different antibiotics, antibacterial, antivirals, and cytotoxic agents effective against insects like antifeedant and anti-helminthic molecules are also produced.

2.2 Antibiotics

PGPR-mediated antifungal activity is also due to the release of antibiotics (Haas and Keel 2003). These antibiotics are a heterogeneous group of organic, low-molecular-weight compounds (Duffy et al. 2003). The *Bacillus* strains are associated with production of more than 20 different antibiotics, most important being Kanosamine, Zwittermycin A, Iturin A (Cyclopeptide), Bacillomycin, Plipastatins, etc. (Volpon et al. 2000). Haas and Defago categorized the antibiotics into two subclasses: (1) diffusible antibiotics, which involve five classes – phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, and cyclic lipopeptides, and (2) volatile antibiotics, like hydrogen cyanide (HCN) (Haas and Defago 2005).

2.3 Siderophores

PGPR produce siderophores, which are low-molecular weight and high-affinity iron chelating compounds that help in ion uptake through channels across cell-membrane. Their importance can be estimated from the fact that iron in soil is actually present in bound state, which is inaccessible for plants' usage and can lead to iron deficiency and reduced yield. The siderophores facilitate iron solubilization and absorption by its chelation from various organic and inorganic sources (Wandersman and Delepelaire 2004). These compounds bind easily to insoluble ferric ions (Fe^{3+}) and facilitate their absorption as soluble form. Siderophores are broadly divided into four classes – hydroxamates, carboxylate, catecholates, and mixed type.

3 PGPR-Mediated Biocontrol

In general, biocontrol refers to the use of living organisms to suppress the growth of pathogen by either direct (parasitism, hyper-parasitism, commensalism), indirect (competition, systemic acquired or ISR), or hybrid antagonistic modes (like production of antibiotics, lytic enzyme, siderophores, volatile organic substances, etc.) (Heimpel and Mills 2017). Free-living PGPR restrain bacterial, nematode, viral, and fungal pathogens by controlling microbial balance near plant roots (Kenawy et al. 2019).

Fungal pathogens cause a lot of diseases in plants and their effective control is required for improved harvest and quality. The common fungal pathogens attacking plants are summarized in Table 16.2. The biocontrol of fungal phytopathogens through the PGPR involves metabolites secretion including hydrolytic enzymes, which play the central role in suppressing the fungal infections (Gangwar et al. 2016).

The fungal pathogen exists in diverse morphological forms, such as spores, hyphae, or fruiting bodies. Thus, an effective response requires rhizobacteria to recognize all these structures. The fungal phytopathogen exists in close proximity to the rhizobacteria, enabling direct target recognition, penetration, and lysis of pathogenic cells (Shaikh and Sayyed 2015). Although, the attachment can either be direct connection of bacteria and target cell, or entrapment of phytopathogen into the rhizobacterial biofilms.

Here, although lysis seems the only method in biocontrol, but various other mechanisms exist in combating these plant fungal pathogens. Some of major mechanisms in effective biocontrol are following:

(a) *Niche Competition* – The PGPR contest with fungal pathogens for the niche and outcompetes the fungal phytopathogens from plant tissue and soil (Loper and Henkels 1997).
(b) *Antibiotics, Siderophores Production* – These compounds manipulate the metabolism of fungal phytopathogen and restrict pathogen growth (Beneduzi et al. 2012).

Table 16.2 Various fungal pathogens causing plant diseases

Fungal pathogen	Target plant species	Disease	References
Syncephalastrum racemosum	Potatoes, onions, carrots, fleshy organs, etc.	Soft rots	Misra et al. (2016)
Phytophthora sp.	Jarrah	Root rots	Rea et al. (2010)
Puccinia	Wheat, oats, rye, barley	Rusts	Uchida et al. (2006)
Alternaria solani	Potato	Early blight	Abuley and Nielsen (2019)
Phytophthora infestans	Potato	Late blight	Small et al. (2015)
Gibberella circinata	Woody plants	Cankers	Wingfield et al. (2002)
Claviceps purpurea	Wheat, rye, barley, and other grasses	Ergots	Giesbert et al. (2008)
Rhizoctonia solani	On the whole lawn irregularly	Brown and yellow patches	Giesler and Yuen (1998)
Fusarium sp.	Potatoes, onions, carrots, fleshy organs	Dry rots	Heltoft et al. (2016)
Fusarium sp.	Mango	Leaf spot	Sultan et al. (2019)
Phragmidium satoanum	Rose	Leaf rust	Ono and Wahyuno (2019)
Puccinia arachidis	Groundnut	Leaf rust	Sathiyabama and Balasubramanian (2018)
Uncinula necator	Onions,cucumbers, Grains, alfalfa	Powdery mildew	Doster and Schnathorst (1985)
Erwinia amylovora	Pea and apple	Fire blight	Braun-Kiewnick et al. (2011)
Fusarium graminearum	Wheat, rye, barley, potatoes	Scab	O'Donnell et al. (2000)
Uromycladium tepperianum	Sengon	Gall rust	Lestari et al. (2013)
Xanthomonas oryzae	Rice	Leaf blight	Wongkhamchan et al. (2018)
Bipolaris maydis	Maize	Leaf blight	Kumar et al. (2016)
Fusarium oxysporum	Potatoes, alfalfa	Wilts	Pietro et al. (2003)
Erwinia amylovora	Apple	Fire blight	Gaucher et al. (2013)
Phomopsis sp.	Various plants	Seed decay	Li et al. (2015)
Pythium and Fusarium	Various plants	Damping off	Mao et al. (1997)
Glomerella cingulata	Apple	Leaf spot	Liu et al. (2016)

(c) *Hydrolytic Enzymes Production* – Hydrolytic enzymes refer to various proteases and lipases, which together work and degrade the cell wall of fungal phytopathogens (Sayyed et al. 2013).

(d) *Mycoparasitism* – Refers to parasitism on fungal pathogen (Woo and Lorito 2007).

(e) *Induction of ISR* – ISR improve antifungal activity of plants by strengthening the physical and chemical barriers (Beneduzi et al. 2012).

3.1 The PGPR Released Hydrolytic Enzymes

PGPR secretes various extracellular hydrolytic enzymes i.e., chitinase, protease, cellulose, β-(1,3)-glucanase, etc., which play a key task in the inhibition of fungal phytopathogens growth (Wang et al. 2019). These hydrolytic enzymes cleave the cell wall units of fungi, including, chitin, proteins, cellulose, hemicelluloses, glucans, etc., thus inhibit the hyphal formation and penetration deep into plant tissues.

3.1.1 The Fungal Cell Wall: Weaker Link

The cell wall is a protective barrier in fungi and guards against external environmental stresses, but also controls morphogenesis, as well as helps in plant-fungal interaction (Latge and Beauvais 2014). The criticalness of cell wall in maintaining the integrity of the fungal cell and is regarded as an outstanding target for antifungal compounds (Geoghegan et al. 2017). The fungal cell wall is made up of approximately 80% of the fibrillar cross-linked polysaccharides. The major components are chitin, glucans, mannans, polyphosphate, and glycoproteins. These are cross-linked together and build the skeleton of the cell wall (Bowman and Free 2006), (Geoghegan et al. 2017). The fibrillar polymers are surrounded with the complex gel-like matrix including polyglucuronic acid, xylomannoprotiens, polyphosphate, etc. (Table 16.3).

About 20–30% of the proteins exist as glycoproteins and either form the structural framework of the cell wall (Srinorakutara 1998) or perform various functions like aiding in water movement, preventing desiccation, or signaling proteins (receptors) involved in regulation, etc. (Cox and Hooley 2009). Therefore, disturbing the homeostasis or degrading the integrity is one of the most employed mechanisms of hydrolytic enzymes mediated fungal combating.

Hydrolytic enzymes have the capacity of destroying the fungal cell wall structure and integrity (Budi et al. 2001). They function by breaking or disturbing the glycosidic bonds forming the chitin polymers and results in lysis of cell walls, inhibition of germ tubes, and hyphae formation (Shaikh and Sayyed 2015; Kim et al. 2003). Wu et al. studied the control of paper seedling wilt disease, caused by a thread-like fungus *R. solani,* which is confronted by various hydrolytic enzymes, namely, chitinase, β-1,3-glucanase, peroxidase, catalase, superoxide dismutase (SOD), polyphenol oxidase, phenylalanine ammonia lyase, etc., from *Bacillus subtilis* SL-44 (PGPR). These enzymes fracture the mycelia and thus result in leaking of cell material, ultimately leading to fungal cell death in the pepper plant (Wu et al. 2019). Hydrolytic enzymes also act synergistically with other anti-fungal by-products of PGPR. Someya et al. demonstrated the synergistic effects of hydrolytic enzymes of *Serratia marcescens* B2, with efficacy of *Pseudomonas fluorescens* LRB3W1 antifungal compounds, against cabbage *Fusarium* yellows, caused by *F. oxysporum*. Reportedly, the fungal cell wall and hyphae degradation were more effective than PGPR hydrolytic enzymes alone (Someya et al. 2007). In the next section, some major hydrolytic enzymes and their mode of action are discussed in detail.

Table 16.3 List of major components of fungal cell walls

Fungal classification	Fibrous polymers	Gel-like polymers
Basidiomycota	Chitin β-(1–3), β-(1–6) glucan	Xylomannoproteins α (1–3) glucan
Zygomycota	Chitin chitosan	Polyglucuronic acid, glucuronomannoproteins, polyphosphate
Ascomycota	Chitin β-(1–3), β-(1–6) glucan	Galactomannoproteins α (1–3) glucan
Chytridiomycota	Chitin glucan	Glucan
Oomycetes	β-(1–3), β-(1–6) glucan cellulose	Glucan

Adapted from Gow and Gadd (1995)

Chitinase

Chitinase [EC 3.2.1.14] is the chief hydrolytic enzyme released by PGPR. Its antifungal activity is well-known and as the name suggests, it acts on polymer chitin, present in fungal cell wall. Chitin polymer is formed by β-1,4 linkages between N-acetyl-D-glucosamine (NAG or GlcNAc) subunits, as shown in Fig. 16.2 (Pillai et al. 2009). The purified enzyme works as efficiently as chitinase coding genes in bacteria (Kim et al. 2003). In general, chitinases are found in a number of chitin-containing microbes like insects, crustaceans, yeasts, and fungi, and also in many non-chitin synthesizing cells of bacteria, higher plants, viruses, animals, etc. (Sharp 2013). Table 16.4 is a compilation of chitinase released by various PGPR, which suppress fungal phytopathogens effectively.

Besides bacteria, the cloning and purification of *CHIA* (Chitinase Acidic) gene, encoding chitinase has also been tried for controlling fungal phytopathogens. Oppenheim and Chet effectively controlled the *S. rolfsii* and *R. solani* fungal pathogens, by cloning, expressing, and purifying *CHIA* gene (*S. marcescens)* product in *E. coli* (Oppenheim and Chet 1992). Similar results were obtained in producing chitinase, chitosanase (chitosan), and protease enzymes from *B. cereus* QQ308, which suppressed spore germination and tube formation in *F. oxysporum, F. solani,* and *P. ultimum* on Chinese cabbage plant (Chang et al. 2007). Jones et al. rather followed the forward genetics approach and inactivated the *ChiA* gene in *S. marcescens* to make chitinase mutants and studied its effect on growth of *F. oxysporum* in pea plants (Jones et al. 1986). These various studies prove that indeed chitinase enzyme can be used as controlling means against fungal phytopathogen.

Types of Chitinase enzymes – The chitinase can be divided into two main groups.

1. *Endo-chitinases* – Cause random cleavage of chitin polymer at internal positions of linear chitin polymer which produce the diacetylchitobiose dimer, as well as GlcNAc soluble multimers like chitotriose and chitotetraose (Sahai and Manocha 1993).
2. *Exo-chitinases* – These are further sub-divided into two.

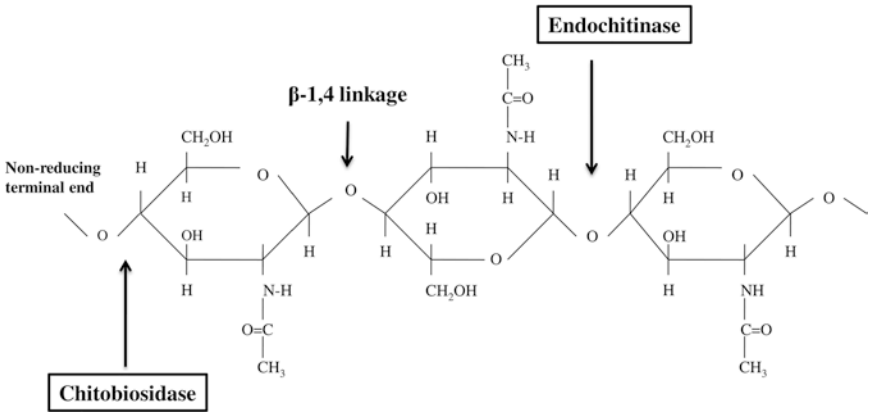

Fig. 16.2 Sites of chitinase enzyme on chitin polymer in cell wall of fungal phytopathogens. Endochitinase catalyses random splitting of chitin polymer at internal positions. Chitobiosidase catalyses release of di-acetylchitobiose in chitin microfibril, starting from non-reducing end. 1–4-β-glucosaminidases splits the endochitinases and chitobiosidases oligomeric products, generating monomers of GlcNAc

(a) Chitobiosidases (E.C.3.2.1.29) – Catalyse the release of diacetylchitobiose of chitin microfibril from the non-reducing end.
(b) 1–4-β-glucosaminidases (E.C.3.2.1.30) – Split endochitinases and chitobiosidases, generating monomers of GlcNAc (Sahai and Manocha 1993).

Glucanase

Glucanases refer to a category of hydrolases that breaks the glucosidic bond in glucans, a polysaccharide made of glucose monomers. Among these, β-1,3-glucanases [EC 3.1.1.6] are found in various microbes like bacteria, fungi, and higher plants (Simmons 1994). β-1,3(1,6)-glucans polysaccharides are a major structural framework component, having β-1,3-linked backbone and β-1,6-linkages in the fungal cell wall. The β-1,3-glucanase hydrolytic enzymes are released from various PGPR and cause lysis of β-1,3(1,6)-glucans polysaccharides thus, inhibiting the hyphal cell growth, ultimately leading to their death (Goswami et al. 2016; Fridlender et al. 1993) (Fig. 16.3). These are further sub-divided into two divisions.

1. *Exo-1,3-glucanases (EC 3.2.1.58)* – Catalyse hydrolysis of the fungal cell wall via sequential breakdown of glucose residues from the non-reducing end of glucan polysaccharides (Mouyna et al. 2013).
2. *Endo-1,3-glucanases (EC3.2.1.39)* – Catalyse hydrolysis via random breakdown of the glucan polysaccharide into oligosaccharides units (Mouyna et al. 2013).

Various groups took efforts to study glucanases in detail and made successful attempts at their purification. β-1,3 glucanase from *Pseudomonas cepacian* was

Table 16.4 Various microbes showing hydrolytic antifungal cell wall lysis activities in different host plants

Microbes releasing hydrolytic enzymes	Hydrolytic enzyme	Host plant	Target fungus species	References
S. plymuthica C48	Chitinase	Mustard crop	*Botrytis cinerea*	Frankowski et al. (2001)
S. marcescens QMB1466	Chitinase	Pea	*F. oxysporum*	Jones et al. (1986)
S. marcescens	Chitinase	Cotton	*Sclerotium. rolfsii* and *R. solani*	Oppenheim and Chet (1992)
Bacillus cereus QQ308	Chitinase, chitosonase, protease	Chinese cabbage	*F. oxysporum, F. solani, Pythium. Ultimum*	Chang et al. (2007)
Bacillus strain EBS8	Chitinase	Maize	*F. verticillioides*	Abiala et al. (2015)
S. marcescens	Chitinase (into the *Rhizobium meliloti*)	Alfalfa	*R. solani*	Sitrit et al. (1993)
B. subtilis 30VD-1	Chitinase, Protease	*Pea*	*Fusarium* sp.	Khan et al. (2018)
Streptomyces griseus	Chitinase	Cotton	*F. oxysporum, A. alternata, R. solani, F. solani*	Anitha and Rabeeth (2010)
Paenibacillus sp. strain 300 and *Streptomyces sp.* strain 385	β −1,3-glucanase, Chitinase	Cucumber	*F. oxysporum* f. sp. *cucumerinum*	Singh et al. (1999)
P. cepacia	β-1,3-glucanase	Soil borne	*R. solani*, *S. rolfsii*, *P. ultimum*	Fridlender et al. (1993)
B. subtilis NSRS 89–24	β-1,3-glucanase	Rice	*P. grisea* and *R. solani*	Leelasuphakul et al. (2006)
P. aeruginosa PGPR2	Protease	Mung-bean	*Macrophomina sp., Rhizoctonia sp.* and *Fusarium sp.*	Illakkiam et al. (2013)
B. subtilis SL-44	Lytic enzymes include chitinase and β-1,3-glucanase	Pepper	*R. solani*	Wu et al. (2019)
P. fluorescens LRB3W1	Chitinase	Cabbage	*F. oxysporum*	Someya et al. (2007)
Paenibacillus jamilae HS-26	Cellulase, Chitinas, protease, glucanase	Cucumber	*Fusarium* sp., *Alternaria* sp., *R. solani,* etc.	Wang et al. (2019)

purified, by growing it on a laminarin (in brown algae) as a carbon source and found to be active (pH 5.0) (Fridlender et al. 1993). The β-1,3-glucanase of *Bacillus subtilis* NSRS 89–24 was even cloned and purified, having a molecular weight of 95.5 kDa. The optimal activity at pH 6.5–9.5 and 50 °C (Leelasuphakul et al. 2006). However, β-1,3-glucanase from *Trichoderma harzianum* is reported to be around

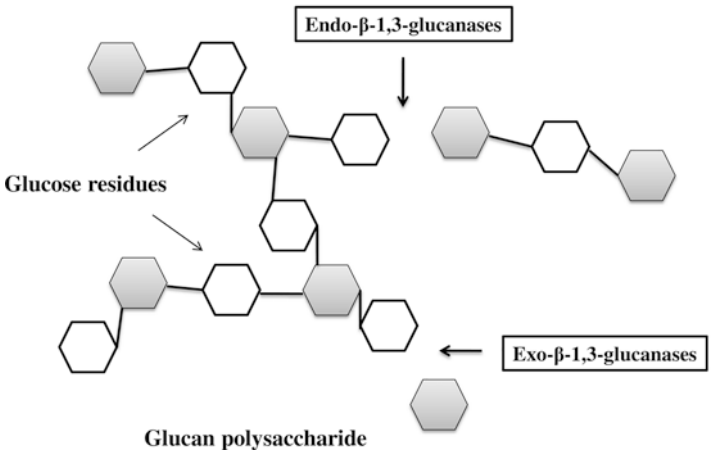

Fig. 16.3 The mechanism of action of different glucanases on β-1,3-glucans. Exo-1,3-glucanase cause sequential breakdown of glucose residues of glucan polymers. Endo-1,3-glucanase cause random breakdown of the glucan polymers

29 kDa and active at pH 4.4 and 50 °C. Its K_M and V_{max} are 1.72 mg/ml and 3.10 U/ml, respectively, with laminarian as substrate (Noronha and Ulhoa 2000).

The inhibitory effects of β-1,3-glucanases from different PGPR are reviewed in Table 16.4. β-1,3-glucanase and chitinases from *Paenibacillus* sp. 300 and *Streptomyces* sp. 385, against *F. oxysporum* f. sp. *cucumerinum*, instigated cucumber' vascular wilt (Singh et al. 1999). β-1,3-glucanase from *Pseudomonas cepacia* cause fungal cell wall lysis in phytopathogens-: *R. solani*, *S. rolfsii*, and *P. ultimum;* and thus reduce diseases progression by 85%, 48%, and 71%, respectively (Fridlender et al. 1993). The inhibitory effects of β-1,3 glucanase from *Bacillus subtilis* NSRS 89–24 contained *Pyricularia grisea* and *R. solani* with MIC values of 12.5 mU/ml and 3.13 μg/ml, respectively. Further, β-1,3 glucanase act synergistically with antibiotics and show better results together than alone (Leelasuphakul et al. 2006).

Protease

Fungal cell wall possesses various proteins and peptide units to provide essential structural framework. The PGPR proteases are extracellular and its intervened hydrolysis is not a mere theoretical possibility to disturb the cell wall integrity, but indeed substantiated with experiment based studies (Jadhav et al. 2017). Proteases [E.C. 3.4.24] play an important role in the phytopathogenic fungi biocontrol, as either alone or in synergism with other PGPR secretions. Although several microbes produce proteases, but it is the PGPR secreted proteases only which are primary in biocontrol activities against *Aspergillus flavus, A. niger, A. wentii, A. alternata, Byssochlamys fulva,* etc. (Sayyed et al. 2019; Tewari et al. 2019). It is reported that

extracellular proteases are chiefly released by *Bacillus sp.* (Sookkheo et al. 2000). Scientists claim that protease also display slight specificity against specific protein structures in cell wall of fungi.

The function of the proteases is shown in Fig. 16.4. Different studies of proteolytic enzymes active against fungal phytopathogens are compiled in Table 16.4.

The protease enzyme from *P. aeruginosa* mungbean rhizosphere was purified and molecular weight of purified protease is around 33 kDa, with activity of 235 U/mL at pH 6.0 and 40–70 °C temp (Illakkiam et al. 2013). However, Sookkheo et al. (2000) could purify three extracellular proteases from *B. stearothermophilus* strain TLS33, naming them as S, N, and B, with weights of 36, 53, and 71 kDa respectively. The optimal activities is rather reported at varied pH of 8.5 (72 °C), 7.5(78 °C), and 7.0 (90 °C) respectively (Sookkheo et al. 2000). It again reaffirms protease to be broad category.

The *P. aeruginosa* PGPR2 protease has antifungal activities against *Macrophomina sp., Rhizoctonia sp.,* and *Fusarium sp.*, and maximum activity is reported against *M. phaseolina* in agar disc diffusion assay with a distinct inhibition zone at pH 6.0 (Illakkiam et al. 2013). The protease, chitinase, and chitosanase from *B. cereus* QQ308 are active against *F. oxysporum, F. solani,* and *P. ultimum* infections in Chinese cabbage (Chang et al. 2007). Proteases released by PGPR are mainly extracellular; the quantity of proteases is very high and shows hydrolytic activity under harsh environmental conditions as well.

Cellulase

Celluloses are microfibrils, rigid, insoluble, and crystalline structures. Cellulase enzyme system mainly involves the combination of the three major hydrolytic enzymes, involving endo-1,4-β-glucanase enzymes [EC 3.2.1.4], exo-1,4-β-glucanase enzymes [EC 3.2.1.91], and β-glucosidases [EC 3.2.1.21], which can break the glycosidic linkages (Lynd et al. 2002). They hydrolyse the 1,4-β-D-glucosidic linkages in cellulose, and thus recycle this polysaccharide (Jayasekara

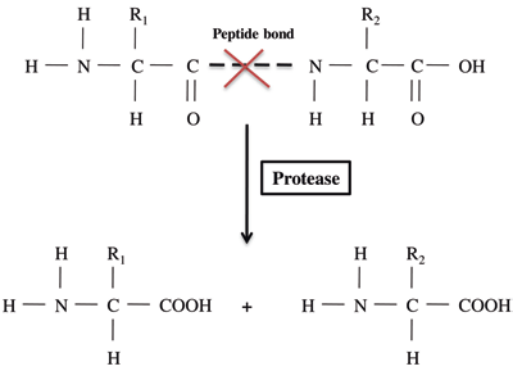

Fig. 16.4 Actions of protease on peptide bond of protein molecule causing proteolysis

and Ratnayake 2019). Although there are not enough conclusive evidences to prove fungal cell wall degradation by cellulase enzymes alone, but rather studies support the synergistic participation of glucanase other hydrolytic enzymes with cellulase. Many PGPR release cellulolytic enzymes and thus help in the breakdown of cellulose in microbial cell wall (Tang et al. 2020). Some studies involving role of cellulase enzyme in antifungal activities are discussed here as well (Table 16.4). Wang et al. reported various lytic enzymes, break the fungal cell wall (*Fusarium* spp., *Alternaria* sp., *R. solani,* etc.), from the *P. jamilae* HS-26 (rhizobacteroid) strain, both qualitatively and quantitatively. The mixture of enzymes, after 3 days, show cellulase, glucanase, and protease enzymes level reaching up to 62.76 ± 1.35 U/mL, 4.13 ± 0.53 U/mL, and 15.56 U/mL, respectively (Wang et al. 2019). This study endorses synergistic roles of cellulase in the degradation of fungal cell wall. Another mechanism of fungal inhibition with cellulolytic enzymes is its synergism with mycoparasitism in *Phytophthora* and *Pythium spp.* (Picard et al. 2000). These reports conclude that either the exact mechanism is still unknown and needs to be explored or separate ways could be employed for effective biocontrol by cellulases.

Limited literature is available on cellulases and one study involves purification from *B. licheniformis* (Isolate 380) with 20 kDa size, and maximum activity of this carboxymethyl cellulase is 0.14 UEA mL-1 min-1 (Marco et al. 2017). Cellulase enzyme from *B. subtilis* YJ1 have a molecular mass of 32.5 kDa and appear to be an endo-1,4-glucanase enzyme at 6.0 pH and 50–60 °C temp, (Yin et al. 2010).

4 Mycoparasitism in Antifungal Response

Mycoparasitism is an indirect mode of inhibition of fungal cells and refers to obtaining nutrients from living fungal cells. It involves different phases, starting with: attachment, detection, contact, and penetration, followed by nutrient acquisition as shown in Fig. 16.5 (Woo and Lorito 2007). Mycoparasitism activity can be shown in two ways: necrotrophic and biotrophic. Necrotrophic mycoparasites destroy the host mycelium in the early stages of parasitism and use the nutrients that are released from dead host cells. Necrotrophic mycoparasites are more hostile and violent in comparison to biotrophic parasites. These mycoparasites show a broad range of host choices and infinite mode of parasitism. The parasitic activity of necrotrophic parasites is due to the secretion of hydrolytic enzymes, antibiotics, and other antagonistic compounds (Sahai and Manocha 1993). On the other hand, in biotrophic parasitism, the biotrophs fulfil their need of nutrient from living host instead of dead cell (Scott 1976). Biotrophic mycoparasites show a narrow host range, and implicate haustorial structures development for nutrients uptake from the host fungal cells (Sahai and Manocha 1993).

Mycoparasitism property of PGPR, especially by actinomycetes, could act as a game changer in the field of biocontrol of fungal phytopathogens (Barnett and Binder 1973). For direct physical attachment, PGPR can recognize various forms of fungi, involving spores, fruiting bodies, hyphae, etc. The mycoparasitism normally

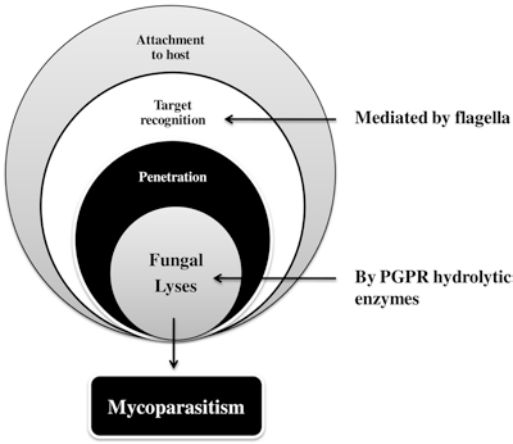

Fig. 16.5 PGPR parasitism on fungal pathogen. PGPR hydrolytic enzymes show lytic activities against cell wall of fungal phytopathogens

involves help of various compounds involving hydrolytic enzymes such as chitinases, proteases, glucanases, cellulose, etc., along with other PGPR products (Fig. 16.5) (Chet et al. 1990). Chet et al. reported the release of lytic enzymes from *S. marcescens* which caused inhibitory activity against *S. rolfsii* in beans and *S. solani* in cotton, for effective biocontrol. The cloned and purified chitinase enzyme extracted from *S. marcescens* caused effective outburst of hyphal tips of *S. rolfsii* (Chet et al. 1990). Bolwerk et al. reported that *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 showed parasitism on *F. oxysporum* hyphae with the help of phenazine-1-carboxamide (PCN) and other lytic secretions, thus helped in the biocontrol of foot and root rot in case of tomato plants (Bolwerk et al. 2003).

5 Induced Systemic Resistance (ISR) in Combating Fungi

Induced Systemic Resistance (ISR) is an acquired process to expand plants' defensive competency manifolds against various biotic infections and other environmental challenges (Loon et al. 1998). This defensive ability is called systemic because it increases plants' endurance at not infection site, but also at rest other sites, and protect from any future attack from fungi or other phytopathogens.

It's warranted that various PGPR products should act synergistically and induce ISR in plants. These include siderophores, pyoverdin, antibiotics, and hydrolytic enzymes. The role of enzymes in the induction of ISR has not been studied in detail, and limited reports exist. The ISR associated enzymes are chitinase, β-1,3-glucanase, peroxidase (PO), polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), lipoxygenase (LOX), ascorbate peroxidase (APX), phenylalanine ammonia-lyase (PAL), protease, etc. (Annapurna et al. 2013). Lawrence et al. report three moderately early blight resistant tomato varieties that have higher chitinase and

β-1,3-glucanase (antifungal isozymes) levels; compared to the non-resistant varieties for *A. solani* (Lawrence et al. 2000). Dumas-Gaudot et al. recorded the short-term increase of the chitinase and sometime β-1,3-glucanase activities as an induced defense response in plants towards fungal phytopathogens (Dumas-Gaudot et al. 1996). Bargabus et al. reported an increase in systemic resistance of sugar beet plant by chitinase and β-1,3-glucanase released from *Bacillus pumilus* (Bargabus et al. 2004) Therefore, it is conclusive that there is some involvement of PGPR hydrolytic enzymes in promoting ISR, but yet, there is a lot more scope in exploring the well-defined mechanism behind ISR induction activities of PGPR hydrolytic enzymes.

6 Conclusions and Future Prospects

The possibility of PGPR in protecting plants from attack of fungal pathogens, and thereby enhancing yield and quality of crops is feasible. It is promising due to release of several antifungal components, like hydrolytic enzymes, antibiotics, siderophores, defensive hormones, etc. All these factors play an important role in sustainable plant disease control, including fungal phytopathogens. Different hydrolytic enzymes target the multiple cell wall components i.e., chitinase, pectinase, glucanases, cellulases, and effectively guard from fungi attack. The PGPR maintain microbial balance in rhizosphere, enhance the seed, and ensure absorption of nutrients. Thus, it improves harvest and strength of plants cultivated for economic reasons. These hydrolytic enzymes effectively bring mycoparasitism of PGPR, along with increased ISR. The synergism between various PGPR released components further augments the affectivity. The natural biocontrol of fungal phytopathogens is promising as it can efficiently decrease the reliability on chemical fertilizers and promote organic farming, which is fast catching attention. Sikkim, in India, has already committed to 100% organic farming. The commercial production of PGPR secretions in combination with nanoparticles would be a splendid biofertilizer, as these would not result in acquired resistance in fungal species, like various chemical fertilizers.

However, like the two sides of the coin even PGPR have their own share of complications as well. PGPR exhibit some shortcoming as well, like cyanide can inhibit the growth of some plants. The auxins accumulation in rhizosphere can impede roots development. Some compounds of PGPR secretion negatively affect nodulation in plants or induces foliar chlorosis in soybeans. Therefore, we can conclude that responsible manipulation of PGPR has promising potential to act as an alternative to current agriculture practices, in controlling pathogens and ensuring plant health and productivity with sustainability. However, despite a lot of research on the production of hydrolytic enzymes in the last 40 years, the functioning of PGPR is still not fully understood and requires more efforts and support.

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