Importance of Biofilm Formation in Plant Growth Promoting Rhizobacterial Action

Chapter · September 2010

DOI: 10.1007/978-3-642-13612-2_4

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Abstract Among the diverse soil microflora, plant growth promoting rhizobacteria (PGPR) mark an important role in enhancing plant growth through a range of beneficial effects. This is often achieved by forming biofilms in the rhizosphere, which has advantages over planktonic mode of bacterial existence. However, the biofilm formation of PGPR has been overlooked in past research. This chapter

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D.K. Maheshwari (ed.), *Plant Growth and Health Promoting Bacteria*,
Microbiology Monographs 18, DOI 10.1007/978-3-642-13612-2_4,

focuses on new insights and concepts with reference to improved PGPR effects caused by the biofilm formation by PGPR and its impact on overall plant growth promotion, compared with the planktonic lifestyle of PGPR. Beneficial PGPR play a key role in agricultural approaches through quorum sensing in their biofilm mode. The in vitro production of biofilmed PGPR can be used to give increased crop yields through a range of plant growth mechanisms. They can be used as biofertilizers through improved N_2 fixation and micro- and macronutrient uptake. Further, higher levels of plant growth with PGPR have been observed due to their production of plant growth regulators and their abilities to act as biocontrol agents, which are carried out by the production of antibiotics and other antimicrobial compounds. The microbial inoculant industry would also benefit greatly by developing biofilmed PGPR with N_2 fixing microbes. Biofilmed PGPR can be manipulated to achieve results in novel agricultural endeavors and hence is as an area which needs a deeper probing into its potential.

1 Introduction

The soil represents a favorable habitat for diverse populations of microbes which have made inquisitive minds probe into their function and activities since time immemorial. The intrinsic roles they play in terrestrial ecosystems have a direct effect on plant growth and soil quality. This feature has led to considerable attention being paid to improve plant growth promotion using effective microorganisms in sustainable agriculture. By and large, this is attributed to the ability of microbes to "turnover" nutrients and to bind particles in soil which is essential for plant growth.

Among the plant associated soil microbial communities, root colonizing beneficial bacteria (rhizobacteria), known as plant growth promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova 2009), are recognized as one of the predominant groups that wield a range of beneficial effects in enhancing plant growth. This is achieved by an array of activities including N_2 fixation, increasing the availability of phosphate and other nutrients in the soil, phytostimulation, suppression of plant diseases, synthesis of antibiotics and the production of phytohormones (Sivan and Chet 1992; Zehnder et al. 2001). Excellent reviews on the PGPR action on roots and mycorrhizosphere are found in Bending et al. (2006) and Spaepen et al. (2009). The success of PGPR in agriculture is attributed to their effective colonization of plant roots (Raaijmakers et al. 1995; Bolwerk et al. 2003) and subsequent growth to form microcolonies or biofilms, which is their common occurrence in a successful plant–microbe interaction (Saleh-Lakha and Glick 2006).

Biofilms are mass colonies of single or multispecies of microbial cells adherent to biotic or abiotic surfaces and/or in intimate contact with each other, encased in a self produced matrix of extracellular polymeric substances (EPS). Less complex biofilms with lower numbers of cells are variably described as microcolonies, aggregates, or cell clusters (Morris and Monier 2003; Ramey et al. 2004). The microcolony is the basic growth unit of a biofilm, and this mode of biofilms is predominant in almost all natural environments (Lappin-Scott and Costerton 1995). The colonization of plant surfaces by plant-associated microbial populations shows similarities to the formation of biofilms on abiotic surfaces with certain genetic determinants common to both processes (Molina et al. 2003).

As outlined by Saleh-Lakha and Glick (2006), these bacterial assemblages have the capability to communicate chemically with one another through quorum sensing, functioning as a single unit. Thus, PGPR when they are in biofilm mode should perform well in inhibiting competing organisms, nutrient uptake, quick responses, and adaptation to changing environmental conditions. However, the natural existence of PGPR in the soil has not been adequately investigated, and the knowledge of biofilmed mode of PGPR and their actions is vastly unexplored. Some reports have highlighted that the plant-associated biofilms have a higher ability to protect themselves from external stress and microbial competition that are characteristic of the rhizosphere, and also to produce beneficial effects in plant growth promotion (Ramey et al. 2004; Seneviratne et al. 2008a, b, 2009). Additionally, it has been shown that naturally occurring or in vitro produced effective PGPR inocula have many potential uses evidently in agricultural and biotechnological settings (Seneviratne et al. 2008b).

Most bacteria appear to form biofilms and this multicellular mode of growth likely predominates in nature as a protective mechanism against hostile environmental conditions (e.g., *Pseudomonas aeruginosa*, Costerton et al. 1995; Costerton and Stewart 2000; Walker et al. 2004). Biofilms, in general, have unique developmental characteristics that are different to freely swimming planktonic cells or nonbiofilm-forming cells. Molecular and genetic studies have identified that biofilms differ considerably from individual microbes in planktonic mode of growth in vital characteristics such as gene expression (Davies et al. 1993; Vilain and Brözel 2006) and physiological functions (Dow et al. 2007). Further, Stoodley et al. (2002) reported that as a result of biofilm structure, physiological adaptation, and the adherent nature of microbial cells in biofilms, they show an elevated antimicrobial tolerance.

Thus, the role of biofilm architecture in plant–microbe interactions cannot be negligible and identification of plant growth improvements through developed biofilmed inocula would have a great scope in plant growth promotion. The impact of microbial biofilms in plant growth promotion has not received adequate attention and studies of beneficial biofilm communities are thus of special interest. This chapter focuses on new insights and concepts with reference to improved PGPR effects caused by the biofilm formation by PGPR and its impact on overall plant growth promotion, compared with the planktonic lifestyle of PGPR. In addition, their potentials in agricultural innovations are also discussed.

2 Occurrence of PGPR Biofilms in Plant–Microbe Interaction

It is well known that most microorganisms in the rhizosphere exist as biofilms rather than their planktonic mode (Watnick and Kolter 1999; Davey and O'Toole 2000). Biofilms associated with the plant roots have been found to be beneficial for plant growth, yield, and crop quality. PGPR biofilm formation and plant growth promotion are governed by effective root colonization of the host plant (Saleh-Lakha and Glick 2006). However, to date biofilm-mediated PGPR actions have not been described adequately. Therefore, evidences found in literature for occurrence of PGPR biofilms in plant–microbe interactions and their possible mechanisms are discussed in this section.

Common plant-associated bacteria found on leaves, roots, and the soil such as *P. putida*, *P. fluorescens*, and related pseudomonads, together with a majority of other natural isolates, have been reported to form effective biofilms (Ude et al. 2006). Bloemberg et al. (2000) noted that the plant growth promoting *P. fluorescens* discontinuously colonized the root surface, developing as small biofilms along epidermal tissues. In contrast, dense and confluent biofilms on root surfaces were observed in studies analyzing pathogenic *Pseudomonas* spp. (Bais et al. 2004; Walker et al. 2004). Although the fundamental cause of these different observations is uncertain, it is evident that the root biofilms of *Pseudomonas* spp. can range from relatively small multicellular clusters to extensive biofilm networks.

Microbes in root-associated biofilms depend basically on root exudates for food and nutrition (Bais et al. 2006). Although the quantities of organic compounds exuding from plant roots are not large, seldom exceeding 0.4% of the C photosynthesized, they exert a very strong influence on the soil microorganisms and may be significant in affecting plant nutrient availability (Rovira 1969). By providing organic compounds as a nutrient source, these root exudates take a central role in being a major plant-derived factor and in triggering of root colonization (Lugtenberg et al. 1999) and biofilm associations (Walker et al. 2004). Some studies have also suggested that the biofilm formation at root sites is triggered by a plant-derived component similar to that seen in *Rhizobium*-legume and other bacterial interactions (de Ruijter et al. 1999), which has happened to be organic compounds of root exudates in this case. The role played by root exudates is further confirmed by Espinosa-Urgel et al. (2002) by observing that *P. putida* can respond rapidly to the presence of root exudates in soils, converging at root colonization sites and establishing stable biofilms.

Most species of bacteria use the quorum sensing to coordinate their gene expression according to the local density of their population. This signaling mechanism modulates and coordinates bacterial interactions with plants, including the control of tissue maceration, antibiotic production, toxin release, and horizontal gene transfer (HGT) (von Bodman et al. 2003). It is one of the main regulatory mechanisms in the formation of biofilms and it is seen that most beneficial phenotypes of PGPR are under its control (Loh et al. 2002). Quorum sensing of PGPR is mediated by an array of signal molecules which include (a) acylated homoserine lactones (AHLs) among proteobacteria; (b) gamma-butyrolactones in Streptomyces species; (c) *cis*-11-methyl-2-dodecanoic acid (also called DSF) in species of *Xanthomonas*, *Xylella*, and their relatives; and (d) oligopeptides among gram-positive microbes (Danhorn and Fuqua 2007). The AHLs-mediated cell-to-cell communication is mostly common among rhizospheric bacteria. The AHLs act as population density sensors and facilitate the communication between different cells (Pierson et al. 1998). Although the AHLs-based quorum sensing is characterized by the proteins LuxI-type protein, AHL synthase, and LuxR-type protein, exceptions have been reported for *Vibrio harveyi* and *P. fluorescens* F113, as they replace the LuxI-type with LuxM AHL and HdtS AHL synthase, respectively (Case et al. 2008). The AHLs-mediated quorum sensing is widely detected in *Pseudomonas* spp. than any other root colonizing bacteria (Juhas et al. 2005). The root-associated biocontrol agent *P. fluorescens* 2P24 requires AHLs for biofilm formation and therefore control of take-all disease on wheat (Wei and Zhang 2006).

It is evident from above information that biofilm formation by PGPR is common in the rhizosphere and that quorum-sensing-based cell-to-cell communication could play a key role in the action of PGPR in green agricultural approaches. The importance of discovering effective forms of PGPR biofilms leads us to the next section, where we focus on their potential applications in futuristic agricultural systems.

3 PGPR Biofilms in Futuristic Agriculture

The current public concerns on the detrimental side effects in the use of agrochemicals have lead to search other avenues of gaining better crop productivity. Of these, an increasing interest has been shown in the use of biofertilizers comprising of beneficial microorganisms, which improves plant growth through the supply of plant nutrients in a manner sustaining environmental health and soil productivity (O'Connell 1992). However, an inconsistency in the field application of such microbial inocula has limited its widespread commercial application, most probably due to the incapability of such inocula to successfully compete with indigenous microflora in establishing themselves in the rhizosphere (Van Elsas et al. 1986; Bent and Chanway 1998).

This failure can be overcome by the introduction of bacterial inoculants in the form of biofilms, thus protecting the inoculants against adverse environmental conditions such as high salinity, tannin concentrations, low pH, heavy metals, predation by earthworms, the competition by native soil populations (Seneviratne et al. 2008b), and the resistance to protozoan grazing (Matz et al. 2004). In this respect, the use of well-characterized PGPR biofilms is remarkable than solitary PGPR since the biofilm formation is an added advantage for PGPR to colonize effectively on or in the plant root, where they can compete well with indigenous microflora along with improved plant growth promotion. This has been made evident by Timmusk et al. (2005) who reported that *Paenibacillus polymyxa* forms biofilms around the root tip and behaves as a root-invading bacterium attributing a possible mechanism in biocontrol and drought tolerance-enhancing activities. Apart from the root colonization, recent observations have been made that the bacterial colonization of biotic fungal surfaces leading to the formation of fungal–bacterial biofilms (FBB) renders the biofilms enhanced metabolic activities in comparison to monocultures, leading to improved biofertilizing and biocontroling effects (Seneviratne et al. 2008a, b). Further, as speculated by Seneviratne and Jayasinghearachchi (2003), the establishment of such biofilms of rhizobia with common soil fungi provides a plausible strategy for the rhizobial survival.

This leads us to confirm that the in vitro production of such biofilmed inocula with PGPR can be utilized to satisfy the future demand of augmented crop production attributed to increased N_2 fixation, nutrient uptake, plant growth promoting agents, and biocontrol of diseases, through a range of mechanisms described below.

3.1 PGPR Biofilms as Biofertilizers

The plant growth promoting rhizobacterial species which flourish in the rhizosphere of plants have been seen to stimulate plant growth, yield, and crop quality by a plethora of mechanisms. This has led to a considerable number of PGPR being tested as biofertilizers, mainly because they provide inorganic nutrients to plants by mineralizing organic and insoluble inorganic forms of nitrogen, phosphorous, and sulfur that plants cannot utilize directly (Mendez-Castro and Alexander 1983) as well as providing essential micro and macro nutrients. This has been made evident by the possession of N_2-fixing properties in many PGPR species including *Bacillus* spp., *Azotobacter* spp., *Azospirillum* spp., *Beijerinckia* spp., *Pseudomonas* spp. (Dobereiner 1997; Reis et al. 1994; Vance 1997), and *Rhizobium* and *Bradyrhizobium* spp. (Dobereiner 1997; Vance 1997).

Such PGPR have been seen to valuably carry out their N_2-fixing ability in the biofilm mode as well, as shown by many studies. Jayasinghearachchi and Seneviratne (2004a) demonstrated that a fungal-rhizobial biofilm (FRB) (*Bradyrhizobium elkanii* SEMIA 5019 and *Penicillium* spp.) biologically fixed N_2 more effectively, as revealed by nitrogenase activity and N accumulation, in comparison to the rhizobial strain grown as a monoculture. A developed biofilmed inoculant of this FRB was also seen to significantly increase N_2 fixation (by ca. 30%) compared with a rhizobium-only (conventional) inoculant when applied to soybean (Jayasinghearachchi and Seneviratne 2004b). The ability to increase N availability by ca. twofold and a high nitrogenase activity, even under a very high soil NO_3^- concentration, were observed in the direct application of FRBs to soil, compared with the monocultures (Seneviratne and Jayasinghearachchi 2005). Yet another PGPR *Azospirillum brasilense*, a free-living N_2 fixer, was found to interact with roots of wheat and maize, forming dense biofilms and thereby promoting their host plant's growth (Assmus et al. 1995; Burdman et al. 2000).

Of the PGPR used to date, two genus most widely known are *Rhizobium* and *Bradyrhizobium* and their symbiotic N_2 fixation through inoculation to legume crops is well known (Dobereiner 1997; Vance 1997). Recent reports have indicated that these symbiotic bacteria may have the potential to be used as PGPR with nonlegumes. Seneviratne et al. (2009) have recently observed the heavy colonization of FBBs/FRBs on root hairs of rice (*Oryza sativa*), tea (*Camellia sinensis*),

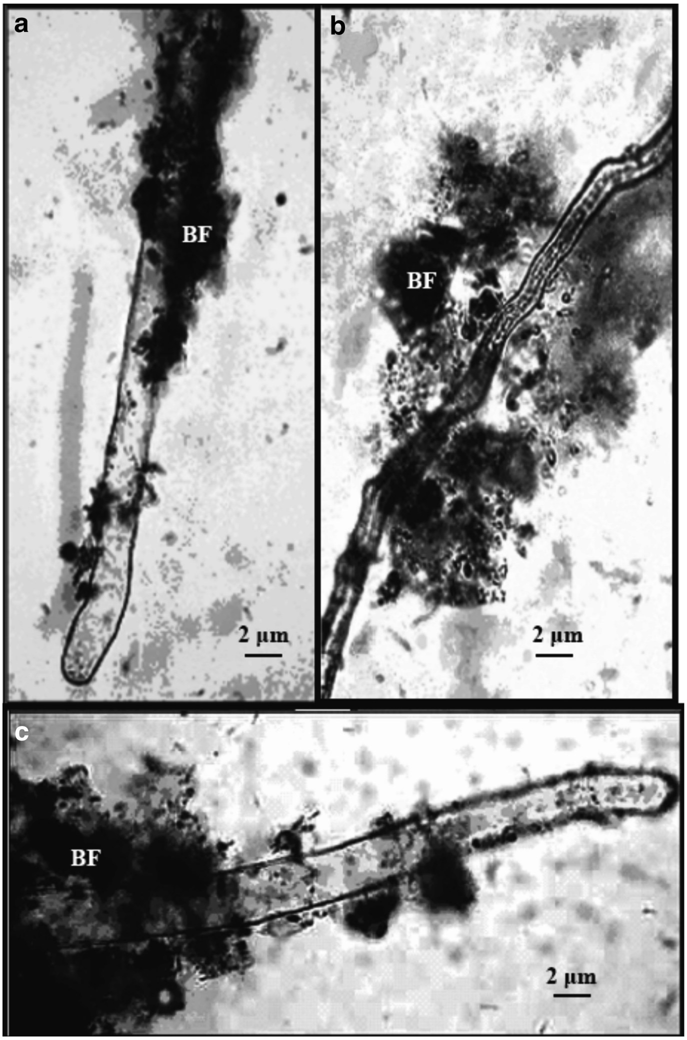

Fig. 1 Root hairs of rice (**a**), tea (**b**), and anthurium (**c**) colonized by microbial biofilms (BF), when fungal–bacterial biofilms (FBB) or fungal–rhizobial biofilms (FRB) were inoculated under axenic conditions. Darkness is due to cotton blue stain absorbed by the extra cellular polymeric substances (EPS) produced by the BF. Reprinted from Seneviratne et al. (2009)

Anthurium (*Anthurium andraeanum*), and wheat (*Triticum aestivum*) (Fig. 1). It has been suggested that such FRBs may act as "pseudonodules," fixing N_2 biologically on the roots of nonlegumes. Further, it was found that recommended chemical fertilizers may be reduced by about 50% by applying such in vitro produced biofilmed biofertilizers (BBs). When BBs were applied with chemical fertilizers to micropropagated Anthurium plantlets, leaf number and chlorophyll content increased by ca. 60% and 100%, respectively, compared with chemical fertilizers alone application (KACN Seneviratne, unpublished). The BBs alone application increased root length of Anthurium by ca. 65%, compared with chemical fertilizers alone application.

Phosphorus (P) is a highly limited nutrient in some soils and hence phosphate-solubilizing bacteria play an important role in the P nutrition in plant growth. Seneviratne and Jayasinghearachchi (2005) have shown that the application of FRBs directly into soil increased P availabilities by 15-fold and that the biofilmed inocula can be effectively used in biosolubilisation of rock phosphate. This was amply demonstrated by an increased P solubilisation (up to ca. 230%) when biofilms developed from *Penicillium* spp., *Pleurotus ostreatus*, and *Xanthoparmelia mexicana*, a lichen fungus, were used compared with the fungus-only cultures (Jayasinghearachchi and Seneviratne 2006a; Seneviratne and Indrasena 2006).

Apart from the major nutrients required for plant growth, some studies have also shown that coinoculation of PGPR inocula enhanced the uptake of micronutrient such as Zn, Cu, and Fe (Bashan 1998). Coinoculation of *Pseudomonas* BA-8 + *Bacillus* OSU-142 increased Fe and Zn contents of leaves up to 50.5 and 35.5%, respectively, compared with the control (Esitken et al. 2005). Investigations of the modes of action by PGPR are increasing at a rapid pace to exploit them commercially as biofertilizers. The benefits of such combinations of mixed cultures or biofilms can be manipulated to overcome the challenges facing for more widespread utilization of PGPR as biofertilizers.

3.2 PGPR Biofilms as Plant Growth Promoting Agents

Numerous studies have demonstrated an improvement in plant growth and development in response to seed or root inoculation with various microbial inoculants capable of producing plant growth regulators (Zahir et al. 2004). Important plant growth promoting substances commonly produced by rhizosphere bacteria include auxins (indolyl-3-acetic acid), gibberellins, and cytokinins (Brown 1974).

Studies by Bandara et al. (2006) revealed that higher acidity and PGP hormone levels were produced by FBBs of beneficial rice endophytes than their mono- or mixed cultured forms with no biofilm formation. Their studies on a large collection of microbes also revealed the existence of a significant negative relationship between the production of indoleacetic-acid-like substances (IAAS) and pH in liquid culture media of FBBs, but not in mixed cultures with no biofilm formation. This high acidity reflects high IAAS production when biofilms are formed. Thus, the use of biofilmed inocula, rather than the conventional practice of plant inoculation with monocultures or mixed cultures of effective microbes, may help achieve the highest microbial effect. Another recent study on early growth of rice showed that the contribution of developed biofilmed inocula in enhanced release of organic acids and PGP substances led to ca. 25% increase in plant dry weight compared with conventional monocultured inocula (Seneviratne et al. 2009). In further studies, biofilmed inocula showed lower pH, higher IAAS, and rice plant dry weights than the monocultured inocula (MLMAW Weerasekara, unpublished). The biofilmed inocula showed a fourfold increase in H^+ secretion to the culture medium, compared with the monocultured inocula. Negative relationships were observed between pH of both types of the inocula and plant dry weight (Fig. 2a) or soil NH_4^+ (Fig. 2b). This implies that the inoculated biofilmed inocula colonize the rhizosphere, producing high acidity and IAAS (Seneviratne et al. 2008a), and the high acidity in microsites causes to an increase of plant available NH_4^+ (Xu et al. 1997) in the soil solution near root hairs, which helps increase the plant growth. Therefore, in vitro production and application of more effective combinations of such beneficial biofilmed inocula would play an important role in the inoculant industry. However, this needs further research to fully understand the effects and potentials of the biofilmed inocula in the plant growth promotion. It is clear from the above studies that one of the most plausible mechanisms of plant growth promotion by PGPR is the production of plant growth regulators. Further, the effectiveness of using such PGPR in their biofilmed mode in the production of higher levels of plant growth promoting substances is also noticeable.

3.3 PGPR Biofilms as Biocontrolling Agents

Biocontrolling has been seen as a well-suited alternative or supplement in contrast to conventional methods of disease control of which microbial biocontrolling

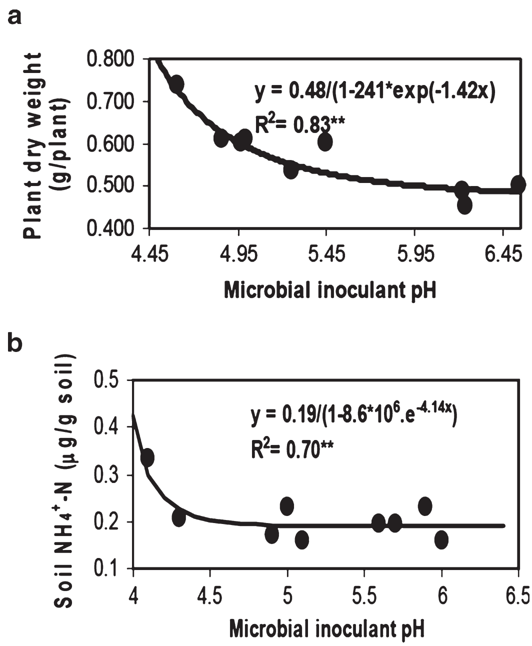

Fig. 2 Relationships between (**a**) microbial inoculant pH of both biofilmed and conventional inocula and rice plant dry weight, and (**b**) the microbial inoculant pH and soil NH_4^+, when inoculated in a soil pot experiment. The biofilmed inocula represent relatively low pHs

agents have emerged as favored options due to their complex mode of action and success in bringing out a reduced risk of resistance. For example, the extensive studies of root-associated pseudomonads have revealed that many of these promote the growth of host plants or are used as biocontrol agents (Lugtenberg et al. 2001). *P. fluorescens* has been reported to coat plant roots by forming a biofilm, which may protect roots against soil bacterial and fungal pathogens (O'Toole and Kolter 1998; Walker et al. 2004). The promising nature of PGPR strains as means of plant protection via disease suppression was amply demonstrated by Raupach and Kloepper (1998) in finding the occurrence of a consistent protection against pathogens when mixtures of PGPR were present, possibly in the biofilm mode.

An array of studies has confirmed that bacteria when they are in the biofilm mode perform well as biocontrol agents, mainly because the plant is made less sensitive to infection by the formation of biofilms by bacteria on the plant root (Bais et al. 2004; Rudrappa et al. 2008). Owing to the heterogeneous nature of biofilms, it is likely that the biofilm formation on the plant roots protects the plants against soil borne diseases through resistance mechanisms such as cell–cell communication via quorum-sensing (Danhorn and Fuqua 2007) and production of antibiotics against pathogens (Russo et al. 2006).

Biofilms bring about disease suppression through a variety of roles played by antibiotics. Such microbial communities have a significant resistance to antibiotics compared with planktonic bacteria of the same species (Stewart and Costerton 2001), while some biofilms have the ability to produce different antibiotics (Leifert et al. 1995; Raaijmakers et al. 2002; Yu et al. 2002; Risøen et al. 2004; Roberts and Stewart 2005).

In addition, biocontrolling agents of PGPR have been shown to successfully establish in plants, when they were applied as biofilmed inocula. Jayasinghearachchi and Seneviratne (2006b) confirmed this in vitro by using a *Pleurotus ostreatus* – *Pseudomonas fluorescens* biofilm which was seen to increase endophytic colonization of tomato by *P. fluorescens*, a biocontrolling agent, by over tenfold compared with inoculation of *P. fluorescens* alone. The PGPR *Paenibacillus polymyxa* provides protection from pathogens through the synthesis of several antibiotics, when it forms biofilms by predominantly colonizing the root tips of *Arabidopsis thaliana*, as revealed by fluorescence microscopy and electron scanning microscopy (Timmusk et al. 2005). *Bacillus subtilis*, another biocontrolling PGPR, protects plant roots from pathogenic bacteria by mechanisms which include biofilm formation and antibiotic and surfactin production (Bais et al. 2004; Cavaglieri et al. 2005). Surfactin possesses antimicrobial activity, and pathogens those reach inside the biofilms are killed by high surfactin concentrations (Bais et al. 2004).

Bacteria used to accomplish biocontrolling exert their action also through producing antimicrobial secondary metabolites, which target the competing microorganisms (Mazzola et al. 1992; Raaijmakers et al. 2002; Haas and Keel 2003). Some *Pseudomonas* strains secrete antimicrobial compounds such as exoproteases, antibiotics, HCN, or metabolites with antifungal activity known as phenazines (Molina et al. 2003). These compounds have the capacity to eliminate competititors from the rhizosphere with a plethora of studies demonstrating their prospect as biocontrol agents (Thomashow 1996; Chin-A-Woeng et al. 2000; Kremer and Souissi 2001). Studies outlined above highlight the potential of using biofilmed PGPR with increased microbial action to carry out biocontrol feats in conventional agriculture and organic farming systems.

4 Conclusions

Although developing biofilms has been the axis around which many recent studies have evolved in diverse areas of biotechnology, the investigation of the involvement of PGPR in such biofilms is yet in its infancy. The capability of PGPR to colonize plant roots proficiently and carry out a range of benefits to the plant has made it one of the predominant soil microbial groups. Regulatory mechanisms, such as quorum sensing, exhibited by PGPR have made them stable partners in biofilms, placing them on a higher pedestal compared with their existence alone. PGPR biofilms have been shown to play a fundamental role in futuristic agricultural approaches such as biofertilizers, plant growth promoters, and biocontrolling agents. A heightened interest in recent times in inoculant technology has thrown much importance on the designing and developing of PGPR biofilmed inocula. The beneficial results they yield encourage the deeper delving into its applications and the innovative future perspectives. The importance of biofilm formation in PGPR action is thus an area which needs much more in depth exploration to bring out its true potential.

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