Beneficial Soil Microbiome for Sustainable Agriculture Production

Carmine Crecchio, Tanja Mimmo, Davide Bulgarelli, Ilaria Pertot, Youry Pii, Michele Perazzolli, Marina Scagliola and Stefano Cesco

Abstract The projected increase in world population and the need to reduce the reliance on non-renewable inputs, such as synthetic agrochemicals, are challenging the current vision of agriculture. In particular, to achieve a fair and sustainable global food security, disruptive changes in crop production are unavoidable. A promising strategy proposes to exploit the metabolic capabilities of soil microbial communities, i.e., the microbiome, to conjugate stable yield with reduced impact on the agroecosystem. In this chapter, we introduce the microbiome populating the

C. Crecchio (✉) · M. Scagliola
Department of Soil, Plant and Food Sciences, University of Bari Aldo Moro,
Via Amendola 165/a, 70125 Bari, Italy
e-mail: carmine.crecchio@uniba.it

M. Scagliola
e-mail: marina.scagliola@uniba.it

T. Mimmo · Y. Pii · S. Cesco
Faculty of Science and Technology, Free University of Bozen-Bolzano,
Piazza Università 5, 39100 Bolzano, Italy
e-mail: TMimmo@unibz.it

Y. Pii
e-mail: youry.pii@unibz.it

S. Cesco
e-mail: stefano.cesco@unibz.it

D. Bulgarelli
Plant Sciences, School of Life Sciences, The James Hutton Institute,
University of Dundee, Invergowrie, Dundee DD2 5DA, Scotland, UK
e-mail: d.bulgarelli@dundee.ac.uk

I. Pertot · M. Perazzolli
Department of Sustainable Agro-Ecosystems and Bioresources,
Research and Innovation Centre, Fondazione Edmund Mach,
Via E. Mach 1-38010S, Michele all'adige, TN, Italy
e-mail: ilaria.pertot@unitn.it

M. Perazzolli
e-mail: michele.perazzolli@fmach.it

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root-soil interface from an evolutionary perspective. Next, we discuss the molecular bases of plant-microbe interactions in soil and how these interactions impact plant growth, development and health. We illustrate how plant-probiotic members of the microbiome can be isolated from soil and further characterized for their biological activities, a key pre-requisite for translational applications. In addition, we focus on paradigmatic examples of soil microbes turned into inoculants for agriculture, their fate on soil, their impact on the native microbiome and the beneficial effects exerted on crop production.

Keywords Beneficial soil microbiome · PGPR · Plant-microbiome interactions Bio-fertilizers · Bio-fungicides · Bio-herbicides · Dynamic soil microbiota

1 Introduction

The soil microbiome defines the community of microorganisms, and their encoded functions, inhabiting the soil environment. The soil microbiome acts as a main source of inoculum for the rhizosphere, the thin layer of soil adhering to and influenced by plant roots. Bacteria, fungi and, in specific environments, protozoa and algae too, coexist in the rhizosphere, bacteria being the most abundant (Tate 1995). Such a microbial diversity within the rhizosphere mirrors a functional diversification in the interactions with plants. For instance, some rhizosphere microorganisms, pathogens, can have detrimental effects on their host whereas others may exert beneficial effects, ranging from stimulating plant growth to reducing the damage from soil-borne pathogens.

Therefore, understanding the structure and function of the rhizosphere microbiome has a great potential for discoveries both in basic science and translational agriculture. Studies conducted with both model and cultivated plants revealed that the recruitment and maintenance of the microbiome is affected by different factors. Plant morphology creates different habitats that give rise to distinct microbial composition in the vicinity of and associated with plant roots (Bulgarelli et al. 2013; Edwards et al. 2015). Likewise, plant rhizodeposition, i.e. the release of organic compounds serving in general as nutrients for soil microorganisms, is a key factor that discriminates among microbial groups and species, thus directly influencing microbiome proliferation and diversity (Rosier et al. 2016). Considering the relatively high concentration of nutrients, particularly carbon sources, the rhizosphere microbiome is mainly composed of copiotroph microorganisms, respect to oligotrophs, which survive in much lower carbon concentrations, such as non-amended mineral bulk soils. The main soil characteristics, such as pH, micro and macronutrients content and availability, redox and water potential, as well as environmental factors do contribute to shape microbiome composition and activity, too.

Due also to human activities, environmental health is worldwide declining causing a decrease in nutrient availability, a loss of productivity and desertification. This situation is further exacerbated by the continuously increasing amount of pollutants released into the environment. Besides traditional solutions, the use of beneficial microorganisms as bio-fertilizers and biocontrol agents is promising to achieve sustainable agricultural production. Nevertheless, how these microbes beneficially affect plants and how they interact as a community and between each other is still not clearly understood (Fig. 1).

The aim of this review is to comprehensively describe (i) the structure and the functioning of the microbiome thriving at root-soil interface and (ii) the mechanisms by which plant and microbes interact; (iii) the methods to estimate active bacteria in soil and how they can be screened and isolated; (iv) agricultural uses of beneficial soil bacteria, focusing on current as well as potential applications; (v) other bio-simulants alternative or cooperating with beneficial bacteria.

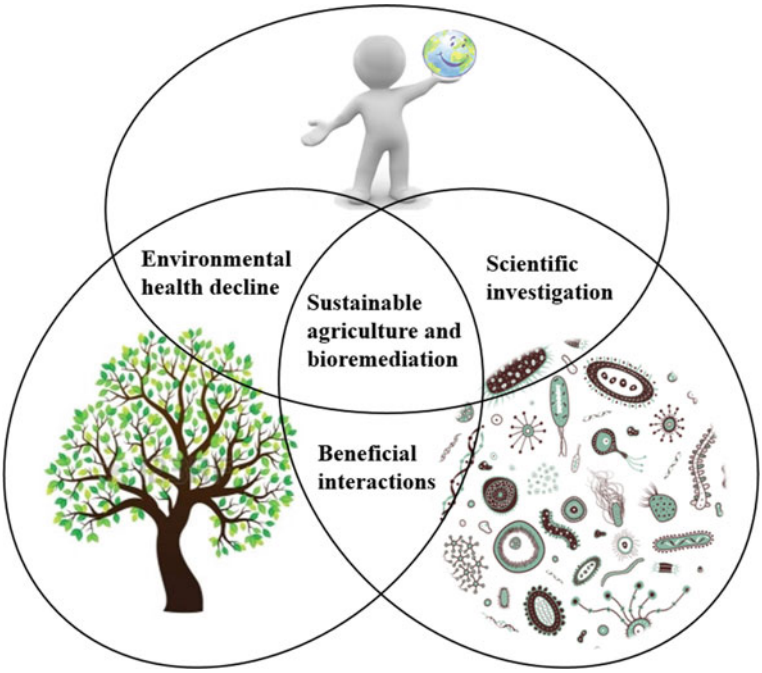

Fig. 1 Relevance of scientific investigations to study beneficial soil microbiome and their applications for a sustainable agriculture and to contrast negative effects of human activities on environmental health. Unsustainable agricultural practices have negatively affected environmental health, causing the degradation of soil properties. Soil microbiome plays a key role in preserving soil chemical and biological balance, through beneficial interaction with plants. On these basis, scientists aim is to investigate the mechanisms underlying the plant-microbiome interactions to achieve sustainable agriculture and bioremediation

2 Structure and Functioning of Plant-Associated Soil Bacteria

Since the inception of the transition from the aquatic to the terrestrial environment, early land plants became exposed to a wide variety of microorganisms, including bacteria, fungi and protists (Kenrick and Crane 1997). The establishment of interactions with this 'pristine' soil biota represented a hallmark for plants' adaptation to the new ecosystem: fossil evidence indicates that plants engaged in symbiosis with arbuscular mycorrhizal fungi, microorganisms capable of enhancing plant's mineral uptake form soil, as early as ~400 million years before present (Lambers et al. 2009). Likewise, phylogenetic investigations support the hypothesis that a single evolutionary innovation, likely occurred ~100 million years before present, has granted to certain angiosperms the capacity to develop specialized symbiotic relationships with nitrogen-fixing bacteria (Werner et al. 2014). In the last decade, advances in experimental and computational approaches have allowed scientists to unlock and catalogue the diversity of microbes interacting with plant roots at an unprecedented depth (Lebeis et al. 2012). These investigations revealed that plants host complex microbial communities, *the plant microbiota*, of which arbuscular mycorrhizal fungi and nitrogen-fixing bacteria can be considered 'extreme forms' of a continuum of symbiotic associations that comprises mutualism, commensalism and parasitism (Schlaeppi and Bulgarelli 2015). Indeed, the plant microbiota and their encoded genes, *the plant microbiome*, represent a still untapped resource of plant probiotic functions, such as enhanced mineral acquisition from soil and indirect pathogen protection (Bulgarelli et al. 2013).

Studies conducted with both model plants (Bulgarelli et al. 2012; Lundberg et al. 2012) and crops (Peiffer et al. 2013; Edwards et al. 2015) indicated that the soil type is a major determinant of the structural and functional composition of the plant microbiota. A prediction of this observation is that a major source of inoculum for the plant microbiota is the soil and, in turn, its characteristics modulate microbiota's recruitment. Comparative studies indicate that a taxonomically congruent group of bacteria, namely members of the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, largely dominate the microbial assemblages associated with both monocotyledonous and dicotyledonous plant species (Hacquard et al. 2015). Intriguingly, this observation is in striking contrast with the broad taxonomic diversity of the soil biota (Fierer et al. 2012). Therefore, the plant microbiota emerges as a distinct community not randomly assembled from the soil biota but rather, as the outcome of specific recruitment cues modulating the proliferation of bacteria thriving in association with plant roots. A survey of the available literature indicates that this selection is operated, at least in part, by the host plant itself. In particular, a multilayered process triggered by a substrate-driven selection, i.e. the plethora of organic compounds released through rhizodeposition, represents an initial recruitment step for the plant microbiota, whose composition is ultimately fine-tuned by other or additional host-molecular mechanisms (Bulgarelli et al. 2013; Hacquard et al. 2015).

Recent breakthrough discoveries brought the host immune system (Jones and Dangl 2006) at center stage in the recruitment and maintenance of the plant microbiome. For instance, studies conducted with the model plant *Arabidopsis thaliana* indicate that a fully-functional immune system is required for the assembly of the endogenous, non-pathogenic, root microbiota (Lebeis et al. 2015; Hiruma et al. 2016). These observations are further strengthened by the fact that microbial components capable of 'dialing' into the plant immune system, such as the Type-III secretion system (Guttman et al. 2014), represent a distinctive feature enriched in and discriminating between plant-associated and unplanted soil microbiome (Ofek-Lalzar et al. 2014; Bulgarelli et al. 2015). From an evolutionary perspective, crop plants are the result of man-driven on-going selection processes, designated domestication and diversification, which progressively differentiated modern cultivated varieties from their wild ancestors (Abbo et al. 2014). In a relatively narrow time scale, i.e., since the dawn of agriculture ~10,000 years before the present, these selection processes markedly impacted on the genetic and phenotypic characteristics of crop plants (Doebley et al. 2006). Perhaps not surprisingly, a critical appraisal of the current literature suggests that domestication and diversification impacted also on the root-associated microbial communities (Perez-Jaramillo et al. 2016). Therefore, humans should be considered as a determinant of the plant

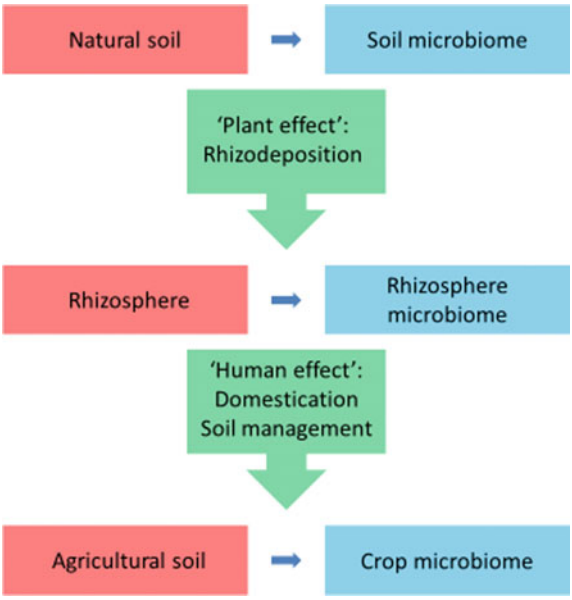

Fig. 2 Progressive differentiation of the agricultural microbiome from the native soil biota. Soil characteristics drive the composition of the soil microbiome. Plant colonization, through multiple processes including, but not limited to, rhizodeposition sculpts the microbiome thriving at the root-soil interface. Human intervention can contribute to the process of microbiome differentiation directly, through agricultural practices modifying soil properties, and indirectly, through the cultivation of defined plant genotypes derived from domestication and breeding selection

microbiome. For this reason, a detailed understanding of plant-microbe interactions taking place at the root-soil interface, both in model and domesticated plants will be paramount to unlock the potential of the microbiome for agriculture (Fig. 2). In the next section, we will critically discuss the recent insights into this research field and we will highlight promising avenues for future investigations.

3 Mechanisms of Plant-Bacteria Interactions

Within the microbiome associated with plant roots, plant growth-promoting rhizobacteria (PGPR) is the group of rhizosphere bacteria, which can exert positive effects on the growth of plants, basically through two kinds of mechanisms. The direct mechanisms include those modes of action by which PGPR can promote the growth of plants regardless of the presence of pathogens (Lugtenberg and Kamilova 2009), as in the case of the improvement of mineral nutrients acquisition (Pii et al. 2015a; Alegria Terrazas et al. 2016) and by the modulation of hormones levels (i.e. auxins and ethylene) in the root tissue (Glick 2012). The indirect mechanisms encompass all those actions that are aimed at protecting plants from the attack of pathogenic microorganisms (Lugtenberg and Kamilova 2009), by activating the induced systemic resistance (ISR) and by inducing the synthesis of stress related compounds (Parray et al. 2016) (Fig. 3).

3.1 Direct Mechanisms

Among the direct mechanisms, the enhancement of plant mineral nutrition is one of the best studied, since plant growth and productivity strongly depend on the bioavailability of mineral nutrients in the rhizosphere (Pii et al. 2015a). PGPR can facilitate the acquisition of mineral resources in different ways, e.g. biological nitrogen fixation, phosphorus solubilization, production of siderophores and manipulating the biochemical and molecular pathways devoted to nutrient acquisition (Pii et al. 2015a, 2016; Alegria Terrazas et al. 2016).

(a) *Biological nitrogen fixation*

The biological nitrogen fixation (BNF) is the conversion of molecular nitrogen (N_2) to ammonia (NH_3) catalyzed by nitrogenase enzyme (Kim and Rees 1994), and it can be carried out by both symbiotic N_2-fixing bacteria (i.e. rhizobiaceae and *Frankia*) and free-living diazotrophs that are capable of establishing non-obligate relationships with host plants, such as *Cyanobacteria*, *Azospirillum*, *Azotobacter*, and *Azoarcus* (Bhattacharyya and Jha 2012; Bhat et al. 2015). Nevertheless, it has been estimated that the free-living diazotrophs can contribute only with a small amount of bioavailable N to the plants, whilst the most efficient N_2 fixation process

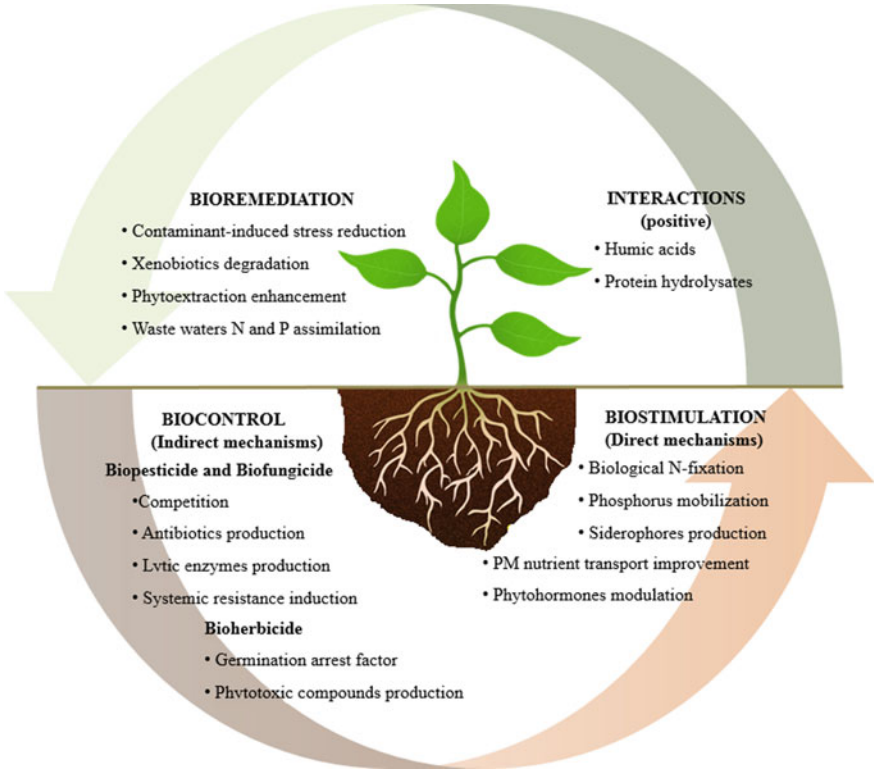

Fig. 3 Schematic overview of the plant beneficial microbial functions on plants and environment. PGPR can positively affect plant growth and development by several activities, directly as well as indirectly. The direct mechanisms enhance plant nutrient acquisition, while indirect mechanisms protect plants against pests. Likewise, these beneficial microorganisms are considered an interesting resource in bioremediation, enhancing plants in extraction, degradation and elimination of several soil contaminants. PGPR would seem to have a positive interaction with other bio-stimulants, such as humic substances and protein hydrolysates, increasing their beneficial effects on plants

occurs within specialized organs, the root nodules, that are developed following the establishment of a symbiotic relationship, i.e. between legume plants and rhizobia (Jones et al. 2007).

(b) *Phosphorus solubilization*

Soil can contain large amount of phosphorus (P), both organic and inorganic, however it is estimated that less than 1% of P is readily available for plants (Bhattacharyya and Jha 2012; Alegria Terrazas et al. 2016). The available P form for plants occurs as monobasic phosphate ($H_2PO_4^-$), which displays an approximate 10 μM concentration in the soil solution (Bieleski 1973; Marschner 2011); it is therefore clear that immobilized P forms have to be made available for plants,

through solubilization processes in the case of inorganic forms or by enzymatic mineralization in the case of organic P (Giles and Cade-Menun 2014; Gerke 2015).

Concerning the inorganic P, mainly bound to iron (Fe), aluminum (Al) and calcium (Ca) in soil (Igual et al. 2001; Gyaneshwar et al. 2002), the release of organic acids (formic, gluconic, 2-ketogluconic and shikimic acid) represents the major process through which phosphate-solubilizing microorganisms (e.g. *Pseudomonas, Burkholderia, Enterobacter, Bacillus, Penicillium and Aspergillus*) are able to enhance P bioavailability, through direct chelation of the counter ions (Fe, Al, Ca) (Hinsinger 2001; Hunter et al. 2014); in addition, the exudation process is often coupled with the cotransport of protons across the plasma membrane causing an acidification of the external media that further induce the acid dissolution of the immobilized P sources (Alegria Terrazas et al. 2016). With regard to the organic sources, it has been demonstrated that phosphatase activity exerted by microorganisms can mineralize the different P-containing molecules, present in the soli, and release orthophosphate groups (Rodríguez et al. 2006). According to a recent survey, the microbial genera featuring the ability of utilizing P from organic sources encompass for instance *Bacillus, Enterobacter, Klebsiella, Lactobacillus, Penicillium and Pseudomonas* (Azeem et al. 2015).

(c) *Siderophores production*

Fe is an essential micronutrient for all the living organisms and it can occur in two oxidation states, i.e. Fe(II) and Fe(III); however, in aerobic environments Fe is mainly present as Fe(III), which forms insoluble (oxy)hydroxides that are sources of Fe not available for both plants and microorganisms (Colombo et al. 2014; Mimmo et al. 2014). Commonly, microorganisms acquire Fe from the external environment by synthesizing and releasing low molecular weight organic compounds, generally termed microbial siderophores (MSs) (Lemanceau et al. 2009), which display a very high affinity for Fe(III), with an affinity constant ranging from 10^{23} to 10^{52}. MSs form very stable complexes with Fe(III), which are then transported into microbial cells through specific transporters (Neilands 1981; Guerinot 1994; Hider and Kong 2010). It has been suggested that plants can exploit Fe mobilized by MSs. Such hypothesis has been tested by using different experimental approaches; for instance, the use of radiolabelled Fe-MS complexes and natural Fe-pyoverdine complexes have allowed to demonstrate that plants are able to acquire Fe from MSs-chelated form (Crowley et al. 1988; Duijff et al. 1994a, b; Walter et al. 1994; Yehuda et al. 1996; Siebner-Freibach et al. 2003; Jin et al. 2006; Vansuyt et al. 2007; Robin et al. 2008).

However, the relative contribution of this mechanism to the whole amount of Fe acquired by plants is not quantified yet. In addition, with respect to grasses (monocots non graminaceous) it should be also considered that due to the high stability of the Fe(III)-MSs complexes, the exchange reactions between Fe(III)-MSs and phytosiderophores (PSs) could be very limited (Colombo et al. 2014). Nonetheless, it is suggested that monocots are able to use Fe(III)-MSs as Fe sources through an indirect mechanisms based on MSs degradation by microorganisms

(Duijff et al. 1994b; Robin et al. 2008). More recently, several pieces of evidence have showed that the inoculation of Fe-deficient plants, grown in either artificial or calcareous soils, with siderophores-producing PGPR can alleviate the symptoms of Fe deficiency, demonstrating a role for these microorganisms in favoring the root acquisition of the nutrient via also, at least in part, an effect in its soil availability (Sharma et al. 2003; de Santiago et al. 2009, 2013; Pii et al. 2015b).

(d) *Effects on biochemical/molecular mechanisms*

The improvement in mineral nutrition, achieved through the inoculation of plants with PGPR, can be indeed due to the increase bioavailability of key nutrients at the rhizosphere (as described above), but also to an enhanced ability of the plants themselves to take up nutrients. Evidence concerning these aspects has emerged following the observations that the PGPR *A. brasilense* could affect the efflux of protons from wheat roots, which was restored also in plants pretreated with orthovanadate. These results corroborate the idea that the PGPR could act directly on plasma membrane (PM) H^+-ATPases (Bashan et al. 1989; Bashan 1990). Several experiences, carried out with different model plants and different PGPRs, have further strengthened these observations (Bertrand et al. 2000; Canellas et al. 2002, 2013). In the case of mineral nutrition, PM H^+-ATPases generate and maintain a transmembrane H^+ electrochemical gradients, which is required for the transport of several nutrients (e.g. $H_2PO_4^-$, sulfate SO_4^{2-} and nitrate, NO_3^-) across the plasma membrane (White 2003). Therefore, the enhanced H^+ extrusion induced by PGPR inoculation might be suggested as a further mechanism through which bacteria could improve the uptake of mineral nutrients.

Similarly, the inoculation of seed rape with the soil-isolate *Achromobacter* induced an increased accumulation of NO_3^- in plant tissues, which was ascribed to a putative activity of the PGPR on the constitutive high-affinity transport system (cHATS) for NO_3^- (Bertrand et al. 2000; Nacry et al. 2013). Still concerning the N nutrition, *Phyllobacterium* STM196 was shown to induce in *A. thaliana* plants the upregulation of *NRT2.5* and *NRT2.6* genes, which was hypothesized to function as transceptors for a systemic signal generated by the inoculation with the PGPR (Mantelin et al. 2006; Kechid et al. 2013).

Also in the case of Fe, it was observed that the fungus *Trichoderma asperellum* could induce an increased Fe uptake in Fe sufficient cucumber and *Lupinus albus* by enhancing the activity of the root Fe-chelate reductase, the enzyme devoted to the reduction of Fe(III) to Fe(II) prior the uptake via IRT1 transporter (de Santiago et al. 2013; Zhao et al. 2014). Furthermore, it has been demonstrated that *Bacillus subtilis* GB03 could stimulate Fe sufficient *A. thaliana* plants to induce the molecular mechanisms (e.g. Fe reduction, H^+ extrusion and genes expression) correlated with the response to the lack of Fe (Zhang et al. 2009). More recently, Pii et al. (2016) showed that the influence of *A. brasilense* on the Fe acquisition mechanisms in cucumber plants was independent on the Fe nutritional status, suggesting that the PGPR might differently affect the different actors (Fe-chelate

reductase, FeII transporter, PM H^+-pump) of the mechanism underlying Fe acquisition by roots.

(e) *Modulation of phytohormones*

Plant hormones play an important role in plants growth, development and in the response to environmental stimuli (Taiz and Zeiger 2006; Glick et al. 2007). An important aspect of phytohormones activity in plants is related to the plasticity of the root system, which shows a surprisingly high adaptability depending on the availability of the nutrient sources (Kloepper et al. 2007). Furthermore, it has been demonstrated that also PGPR-derived phytohormones (i.e. auxin, gibberellin, cytokinin) can have a role in altering the root architecture (Vacheron et al. 2013). In spite of the fact that many PGPR can produce gibberellins, cytokinins or both and that they can affect plants growth, a detailed understanding of the role played by these bacterially-synthesized hormones in plants growth promotion is still lacking (Glick 2012). Several PGPR, as well as pathogenic bacteria, symbiotic and free-living rhizobacteria, have been reported to synthesize auxin (indole-3-acetic acid, IAA) as secondary metabolite and to release it in the external medium (Scagliola et al. 2016).

In plants IAA, also in conjunction with other hormones, controls cell division, extension and differentiation, vascular bundles development, the tropic responses to light and gravity, and initiate lateral and adventitious roots formation (Sachdev et al. 2009; Overvoorde et al. 2010). The PGPR-secreted IAA can affect the aforementioned plant developmental processes, since the endogenous pool of auxin can be integrated by the external amount absorbed (Spaepen et al. 2007; Glick 2012). In addition, bacterial IAA can contribute in increasing root surface and length, thus providing plants with a better access to the nutrient sources in soli (Xie et al. 1996; Mayak et al. 1999). Furthermore, IAA plays also a fundamental role as reciprocal signaling molecule, inducing the expression in both plants and microorganisms of key genes that are required for a proficient establishment of the interaction between plants and PGPR (Spaepen and Vanderleyden 2011).

With a completely opposite mechanism, PGPR expressing the 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which cleaves the ethylene precursor ACC into 2-oxobutanoate and ammonia (Arshad et al. 2007), can facilitate and improve plant growth by modulating the levels of ethylene (Glick 2014). The hormone ethylene is found in all higher plants and it is involved in key developmental processes as well as in the responses to a wide variety of environmental stresses (Abeles et al. 1992). The so-called “stress ethylene” occurs in two subsequent peaks that differ in magnitude, being the first one much smaller than the second one; whilst the first peak of ethylene is believed to induce the activation of defense and/or protection mechanisms, the second peak is generally originated from the de novo synthesis of ACC and has detrimental effects on plant growth, inducing senescence, chlorosis and leaves abscission, thus further exacerbating the environmental stress (Glick 2014).

PGPR exhibiting ACC deaminase activity, belonging to the genera *Acinetobacter*, *Achromobacter*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* (Parray et al. 2016), are thought to function as sink for ACC, thus mitigating the effect of the massive production of ethylene, and, as a consequence, its negative effects on plants growth (Glick 2014). Therefore, plants inoculated with ACC deaminase rhizobacteria feature an enhanced root and shoot growth, an improved ability to establish symbiotic relationships with both rhizobia and mycorrhiza and a better N, P and potassium (K) uptake (Nadeem et al. 2007, 2009; Shaharoona et al. 2008).

3.2 Indirect Mechanisms

The indirect mechanisms of interaction are mainly involved in biocontrol and they may be distinguished in three main groups, namely *Competition, Production of antibiotics and lytic enzymes, Induced Systemic Resistance,* based on the features of the mechanism itself. It is worth noting that for long time they have been raising the interest of the scientific community for the idea of using bacteria expressing these phenotypic traits in place of chemical pesticides in the pest defense programs.

(a) *Competition*

PGPR can out-compete pathogenic microorganisms on two different levels, (i) competition for niches and (ii) competition for essential nutrients. Despite being it not clearly demonstrated, some pieces of evidence indicate that the direct competition between PGPR and pathogens can reduce the severity and the incidence of the diseases. For example, Innerebner et al. (2011) showed that the treatment with *Sphingomonas* sp. induced a strong reduction of the pathogens load in *A. thaliana* plants infected by *Pseudomonas syringae* pv. tomato, also preventing the development of the disease symptoms. As mentioned above, in Fe limiting conditions, PGPR can synthesize microbial siderophores (MSs) to solubilize the micronutrient from sparingly available sources. In this case, the production of MSs can represent also an effective biocontrol mechanism, since they can prevent phytopathogens from acquiring sufficient amount of Fe, thus limiting their ability to proliferate (Lugtenberg and Kamilova 2009); this is mainly due to MSs having generally higher affinity for Fe than the siderophores produced by pathogenic fungi (Schippers et al. 1987).

(b) *Production of antibiotics and lytic enzymes*

The production of a wide range of antibiotic compounds (e.g. 2,4-diacetylphloroglucinol, phenazine, pyrrolnitrin, tensin, zwittermicin A, xanthobaccin) is another phonotypical trait of PGPR associated with biocontrol, especially against the proliferation of pathogenic fungi (Whipps 2001; Haas and Keel 2003; Compant et al. 2005; Mazurier et al. 2009). The biosynthesis of

antibiotics is dependent from the general metabolic state of the bacterial cells that is determined, for example, by nutrient availability and external stimuli (Duffy and Défago 2000). Indeed, it is worth highlighting that each PGPR strain might produce more than one antibiotic compounds, whose synthesis might be induced by different environmental conditions (Duffy and Défago 1999).

For these reasons, some of the isolated biocontrol PGPR strains have been commercialized to replace the common chemical pesticides; nevertheless, some phytopathogenes can still develop resistance against specific antibiotics. Besides antibiotics, several biocontrol PGPR strains have been shown to produce lytic enzymes, including chitinases, cellulases, glucanases, proteases and lipases, that can compromise the integrity of the cell walls of many phytopathogenic fungi. Several pieces of research have demonstrated that PGPR producing these enzymes are effective in preventing the infection of a wide range of fungi, among which *Botrytis cinerea*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Phytophthora* spp., *Rhizoctonia solani* and *Pythium ultimum* (Singh et al. 1999; Frankowski et al. 2001; Kim et al. 2008).

(c) *Induced Systemic Resistance*

The triggering of the so-called plant induced systemic resistance (ISR) is defined as a physiological state of enhanced defensive capacity that can be elicited by either a non-pathogenic organism or specific environmental stimuli; in this status, the plant's innate defenses are "primed", therefore they result potentiated and react faster against subsequent pathogens attack (van Loon et al. 1998). In addition, this induced resistance is systemic because the defensive capacity is enhanced not only in the site of the primary infection, but it is spread all over the plant organism; this is mainly due to the involvement of a jasmonate- and/or an ethylene-based signaling, which stimulates the plant defense mechanisms toward a wide range of pathogens (Verhagen et al. 2004). The signaling inside plants is in turn stimulated by bacterial determinants, as for instance the flagellar proteins, chitin, β-glucans and cyclic lipopeptide surfactants (Annapurna et al. 2013).

4 Characterization of Beneficial Soil Bacteria

A key step to use beneficial microbiome for sustainable agricultural practices is the screening and the isolation of beneficial species from different soil environments. Independently of the plant growth promotion traits researched, the first approach in this quest is the collection of rhizosphere soil that, after serial dilutions, is plated on agarized medium. If the nutrient agar is non-selective for any plant growth promoting activity (enriched of nutrients), the higher is the number of the colony forming units, the higher is the probability to isolate bacteria with potential plant growth promoting traits. Alternatively, the growth medium can be selective for one of the main plant growth promoting traits, such as P-solubilizing activity,

siderophore and indole-3-acetic acid production, therefore reducing the number of isolates but, on the other hand, pre-selecting bacterial strains on the basis of the desired phenotypical trait. Besides a microbiological, morphological and biochemical characterization of potential beneficial bacteria, as well as 16S rDNA sequencing and phylogenetic analysis, quantitative assays for the main activities should be performed for an in vitro evaluation of the potentiality of isolated strains. Two research papers (Ahmad et al. 2008; Dastager et al. 2010), among many others, described in detail the analytical procedures to determine production and release of indoleacetic acid, NH_3, HCN and siderophores, phosphate and Zn oxides solubilization, ACC-deaminase activity, all potential beneficial activities. Another key feature that should be evaluated before considering the possibility of using beneficial soil bacteria in agriculture is the persistence of microbe in the soil, where competition for nutrients with autochthonous microbial populations very likely occurs. For an easy detection in soil, bacteria can be transformed by a plasmid carrying a green fluorescent protein (Miller and Lindow 1997). Many other molecular tools are also available to follow the fate and the persistence of a bacterial inoculum in a fresh soil. PCR-based approaches, consisting of DNA extraction from soil, amplification by PCR/qRT-PCR of a selected region targeting specifically the inoculating bacterium have been routinely performed in the last two decades. Since many factors affect the persistence, including genetic and metabolic characteristics of the inoculum, density and frequency of the inoculum, physical-chemical soil properties, autochthonous microbial community structure and functioning, genetic and biochemical properties of the plant to be grown and the related agricultural practices, environmental aspects, each case should be accurately evaluated at the laboratory scale before being used at the field one.

Since only active microorganisms interact with plant roots and, more in general, drive biogeochemical processes in soil, it is fundamental to understand which part of the total microbiome may have, actually or potentially, a role for sustainable agricultural production. Cells are considered active when actually involved in biochemical transformations and ready to respond to substrate input; differently, potentially active microorganisms can switch in few minutes to hours and contribute to ongoing processes, responding to changes in substrates availability or environmental conditions (De Nobili et al. 2001). Since it is reasonable that beneficial soil bacteria may enhance plant growth and health once they are added to soil, instead of or in cooperation with chemical fertilizers, it is important to understand the transitions (active/potentially active/dormant) they can undergo after their inoculum, as well as the transitions eventually occurring in the already existing microbial community.

Many approaches, recently reviewed by Blagodatskaya and Kuzyakov (2013), allow the estimation of different physiological states in soil, including plate counts, fluorescent microscopy combined to complementary staining, biochemical determinations (ATP and PFLA content, enzymes activities) and molecular methods such as microarray and real time-PCR, by using RNA instead of DNA.

5 Agricultural Use of Beneficial Soil Bacteria

5.1 Fate in Soil of Microbial Inoculants

Beneficial microbial inoculants can be used to treat seeds in drum priming (Bennett and Whipps 2008) or before sowing (Walker et al. 2002), plant roots before transplanting or directly the soil by drenching or incorporating the biocontrol agents into it (Weller 1988). In spite of the increasing interest on the use of beneficial soil bacteria in agricultural soil, scarce information is available on their survival after application. Most of the available data refer to few specific strains, mainly studied as model microorganisms or developed as commercial products. Although some general conclusions on the fate of the introduced inoculant in soil can be drawn, the precise values of survival rates should refer to the specific strain and conditions tested.

The persistence of the microbial inoculum in the soil or in the rhizosphere is a key factor for efficient biocontrol activity, therefore understanding the conditions that optimize the survival rate could be crucial to guarantee a successful practical application. On the other hand, the presence of high concentrations of an introduced strain may imply a disturbance of the native microbial communities; therefore, the survival may have an indirect relevance also on the risk assessment of the application of inoculants to soil (Weller 1988).

When the inoculants are introduced in soil, they are exposed to soil environmental conditions and the competition with native microorganisms, which in general results in a decrease of their populations (Raaijmakers et al. 2009). In addition, the oligotrophic soil environment quite often does not suit the nutritional requirement of the introduced inoculum. Therefore, a basic distinction between survival in the rhizosphere and in the bulk soil should be made when studying the fate of an introduced microorganism in the soil. When considering the bulk soil, besides the intrinsic characteristics of the strain, the specific conditions of the soil play a relevant role on its survival (i.e. physical, chemical and biological characteristics, temperature, relative humidity, pH). In fact, the survival under different soil condition can result in very different patterns. On the other hand, besides the soil composition, the rhizosphere is deeply influenced by the root exudates, which in turn depend on the plant genotype, its physiological/nutritional status, but also on the cross talk with the microbial inhabitants (Dutta and Podile 2010). The residing microflora and microfauna in the bulk soil or rhizosphere may also deeply directly influence the survival of the introduced microorganism. In fact, a microbial population reacts to the introduction of an external strain with a complex pool of responses (Perazzolli et al. 2016). Nevertheless, it should be noted that the role of the natural microbial antagonisms on the survival in soil of the added microorganisms is more an assumption than a proven evidence. In fact, although it is commonly accepted that introduced inoculants are greatly influenced by the native soil microbial communities, not much is known regarding these interactions and the specific effect on the survival of the inoculant (Raaijmakers et al. 2009).

The lack of data on the survival of microbial inoculants in soil is mainly due to the difficulties of monitoring their fate. Before the advent of the non-culturable techniques based on the detection of the DNA of the specific introduced microorganism, most of the studies of the fate of an introduced microorganism relied on the retrieval of the cells (colony forming units, CFUs) of the introduced inoculants on selective media. In most cases, this implied the selection of antibiotic-resistant mutants in the colonies of the target microorganism (Troxler et al. 1997). Although most of the studies demonstrated the absence of biological and physiological difference compared to the wild types, the bias introduced by the resistance to antibiotics might not theoretically excluded. In addition the counting of CFUs tend to underestimate the real numbers of viable bacterial cells when they tend to stick together (Gamalero et al. 2004) or when they lose their colony-forming ability under stress soil conditions, for example in the case of some gram-negative bacteria as *Pseudomonas fluorescens*. The Kogure's direct viable count can partially solve the latter problem (Troxler et al. 1997). The introduction of the green fluorescent protein (GFP) gene to mark strains is a powerful tool that allowed the in situ visualization of the patterns of colonization of the selected strain especially in the rhizosphere (Gamalero et al. 2004; Chen et al. 2005; Poonguzhali et al. 2008). The specific detection and/or quantification by PCR approaches improved dramatically out possibility to monitor the fate of the introduced inoculants, making it a routine analysis especially for the registration requirements (Segarra et al. 2016). PCR-based detections methods however are not exempt from biases: for example underestimation due to a low efficiency of the DNA extraction from soil matrix, overestimation of viability, in fact the detected DNA can originate from dead cells, etc. (Savazzini et al. 2008) (Table 1).

Table 1 Approaches to investigate the survival of microbial inoculants

Techniques		Advantages	Disadvantages
Culturable	Plate counting	Simple and cheap	• Antibiotic resistant mutants utilization • Underestimation of real number
	Green fluorescent protein labelling	In situ localization	Mutants utilization
Un-culturable	DNA approaches (PCR/qRT-PCR, microarray, etc.)	Rapid and simple	• Underestimation of real number (low efficiency of DNA extraction) • Overestimation of viability (dead cells, etc.)
	RNA approaches (PCR/qRT-PCR, microarray, etc.)	Targeting viable cells	Low and un-reproducible RNA extraction yields (RNA degradation)

In general, the population of introduced inocula declines gradually after field application, because of exposure to abiotic stress and antagonism with indigenous resident microbiota. However, some specific patters can be recognized according to the type or strain of the microorganism applied.

(a) *Pseudomonas* spp.

Once introduced in soils, *Pseudomonas* spp. tend to face an initial sharp decline in the number of culturable cells and then establish themselves at a basal stable number (Troxler et al. 1997; Fischer et al. 2010; Gao et al. 2012). In general, *Pseudomonas* spp. survives better in the rhizosphere, than in the bulk soil (Hase et al. 2000; Fischer et al. 2010), indicating a general good rhizosphere competence of strains belonging to this genus. However, once the viable-but-non-culturable cells are compared to the culturable ones of *P. protegens* CHA0 (previously *P. fluorescens*), it appears that the survival of the microorganism is indeed declining over time, but less sharply than expected from the mere CFUs counting (Troxler et al. 1997). Differences between total cells and CFUs counts are reported also for other strains, for example *P. fluorescens* A6RI (Gamalero et al. 2004). In the bulk soil, the soil type has a relevant influence on the survival rate over time, and especially on the occurrence of the viable-but-non-culturable *P. protegens* CHA0 cells (Hase et al. 2000). Although the organic amendments and substrate composition and origin are known to strongly influence the survival of biocontrol agents in soil (Hoitink and Boehm 1999), the cause of this non-culturable status of the cells is not the deprivation of a single nutrient or multiple nutrients (Hase et al. 1999). The non-culturable status is not a physiological strategy to improve survival under adverse conditions, but it is more a reaction to stress conditions, in particular oxygen limitation combined with reducing conditions (Mascher et al. 2000; Troxler et al. 2012) and soil pH (Mascher et al. 2014).

Root colonization is related to the concentration of root exudates, which vary along the root. In fact *P. fluorescens* A6RI densities during time were found to vary according the zoot zone, with a fast decrease in the parts, which include apex, elongation and young hairy zone and a more stable concentration pattern in the older part of the root, supporting the hypothesis of a relation between root exudates concentration and proportion of culturable *Pseudomonas* cells (Gamalero et al. 2004). Interestingly the biocontrol agent *P. fluorescens* 32 was reported to show a wave-like oscillations along wheat roots, most probably linked to cell growth and death cycles, with a more pronounced oscillation in conventionally managed than in organically managed soils (van Bruggen et al. 2008).

When *P. protegens* CHA0 cells are applied on the soil surface, they spread in the entire soil profile, with some differences between covered (ley) and uncovered soil. The presence of ley guarantees a better survival in the top layer in comparison to uncovered soil and relatively high concentration up to a depth of 150 cm, below which it markedly decreases. In the uncovered soil, the highest numbers of cultivable cells concentrate in the soil immediately above the plow pan, followed by a sharp decrease and a gradual increase. Just after the release on the soil, the

P. protegens CHA0 cells spread along the walls of the macropores, but then, during time, they also colonize the micropores. The water movement into soil is most probably the main factor causing the spread of cells in the vertical profile. However, the cells concentration and/or transportation may depend also on other factors related to the microhabitat. For example the presence of roots, relative humidity, physical barriers (e.g. the plow pan), the activity of earthworms can positively influence the survival pattern in the vertical soil profile (Troxler et al. 2012).

(b) *Bacillus* spp.

Similarly to *Pseudomonas* spp., *Bacillus* cells persist better on the rhizoplane and rhizosphere than bulk soil (Cao et al. 2011). Regarding root colonization patterns after application, *B. subtilis* SQR initially colonizes the root tip and elongation zone of the primary roots and then spread on the elongation and differentiation zones of the plant primary roots and on the lateral root junctions (Cao et al. 2011). *Bacillus megaterium* C4 also colonizes firstly the primary and lateral roots and then in the lateral root junctions, however without being found on the root tip (Liu et al. 2006). Many *Bacillus* strain can penetrate the root, most probably from the crack formed at the lateral root junction, and establish within the root tissues (Cao et al. 2011; Liu et al. 2006).

Similarly to *Pseudomonas* strains or other bacterial inoculants (Larkin 2016; Segarra et al. 2016), the long-term survival of vegetative cells *Bacillus* strains in bulk soil is also very limited. For example *B. subtilis* GB03 cells cannot be detected in bulk soil at the end of the growing season (Larkin 2016). However, the origin of the strain seems to be relevant in the successful colonization of bulk soil. For instance the survival in the rhizosphere of *B. cereus* B11, a strain isolated originally from non-rhizosphere soil, was not improved by the presence of the roots (Young et al. 1995). The initial rapid decline of the *B. subtilis* vegetative cells in bulk soil is mainly due to nutritional starvation, but also to the antagonistic effect of indigenous microorganisms (Podile 1994; Tokuda et al. 1995). *Bacillus* strains may need repeated applications and some time to functionally adapt to the soil abiotic and biotic conditions (Jeong et al. 2013). Although vegetative cells decline, *Bacillus* strains can stabilize and survive for long time thanks to the formation of spores (Tokuda et al. 1995). Spores are known to be more resistant to the adverse effect of soil biotic and abiotic stress: for example, when *B. cereus* B11 are introduced as spores its survival was much higher compared to the introduction as vegetative cells (Young et al. 1995).

As in the case of *Pseudomonas*, soil abiotic conditions can affect the survival of *Bacillus* strains. For example montmorillonite and high soil matric potentials enhance the survival (Lee and Stotzky 1999), but, differently from *Pseudomonas*, soil temperature is irrelevant (Schmidt et al. 2004). Specific inoculum carriers, as for example biochar, may support a better survival (Sun et al. 2015).

5.2 Impact of Inoculants on Native Microbial Communities

While the survival of bacterial inoculants in the rhizosphere and bulk soil have received a lot of attention because of its relation to the desired effect of the inoculum, the impact on the native soil microbial populations attracted the interest of researchers only in recent times thanks to the recent advances in metagenomics technologies (Maron et al. 2011). Biocontrol inoculants had negligible impacts on species diversity and abundance of cultivable (Girlanda et al. 2001; Moenne-Loccoz et al. 2001; Thomas and Sekhar 2016) and uncultivable soil microorganisms (Felici et al. 2008; Kim et al. 2010; Piromyou et al. 2011; Gao et al. 2012; Guo et al. 2012; Chowdhury et al. 2013; Jeong et al. 2013; Yin et al. 2013; Krober et al. 2014; Kong et al. 2016). Effects of biocontrol inoculants (*e.g.* *Bacillus* spp., *Brevibacillus* spp., *Klebsiella* spp., *Pseudomonas* spp. and *Serratia* spp.) on native soil microbial communities are related to the inoculant establishment and survival in soil (Ambrosini et al. 2016), but are commonly limited in terms of intensity (Björklöf et al. 2003; Correa et al. 2009; Piromyou et al. 2011; Chowdhury et al. 2013; Jeong et al. 2013; Krober et al. 2014; Kong et al. 2016; Larkin 2016), time (Scherwinski et al. 2008; Gao et al. 2012; Guo et al. 2012; Yin et al. 2013; Krober et al. 2014; Thomas and Sekhar 2016) or space (Moenne-Loccoz et al. 2001). Biocontrol inoculants decreased the abundance of soil plant pathogens, such as *Ralstonia solanacearum* in *B. amyloliquefaciens* ZM9-inoculated soil (Wu et al. 2016), and attenuated the impact of phytopathogenic fungus *R. solani* on the rhizosphere and phyllosphere lettuce microbiome (Erlacher et al. 2014), Particularly, it has been shown that *Firmicutes* abundance increased in *Bacillus* spp.-inoculated soils, while *Proteobacteria* and *Gammaproteobacteria* abundances slightly decreased (Jeong et al. 2013; Krober et al. 2014). Changes in the abundance of *Cyanobacterium* spp., *Beta-proteobacteria* spp., *Staphylococcus* spp., and *Bacillus* spp. were detected at early stages after *P. fluorescens* 2P24 soil inoculation (Yin et al. 2013), while *Arthrobacter* spp. and *Nocardioides* spp. were specifically identified in soils inoculated with the biocontrol strain *B. subtilis* B579 (Chen et al. 2013). Interestingly, putative PGPRs belonging to *Acinetobacter* spp., *Azospirillum* spp., *Bacillus* spp., *Bradyrhizobium* spp., *Burkholderia* spp., *Dyella* spp., *Mesorhizobium* spp., *Pseudomonas* spp., *Psychrobacter* spp., *Rhizobium* spp. and *Stenotrophomonas* spp. increased in soils inoculated with *B. amyloliquefaciens* ZM9 (Wu et al. 2016) or *B. subtilis* Tpb55 (You et al. 2016), suggesting that biocontrol inoculants might increase the proportion of beneficial microorganisms in the plant microbiota. However, microbiome changes elicited by biocontrol bacteria are generally smaller than those caused by plant growth (Girlanda et al. 2001; Piromyou et al. 2011; Krober et al. 2014) and environmental conditions (Scherwinski et al. 2008; Chen et al. 2013). Impacts of biocontrol bacteria were also related to the soil chemical and biological properties (Schreiter et al. 2014; Li et al. 2015) and, according to the ecological theory, soil microbial communities with a greater diversity are less susceptible to disequilibria caused by the invading microorganisms (Fliessbach et al. 2009). Thus, the soil

microbiome is resilient to the perturbation that may be caused by biocontrol inoculants (Moenne-Loccoz et al. 2001), indicating that biocontrol agents should possibly compete with indigenous communities to occupy niches and display antagonistic properties against the phytopathogens (Perazzolli et al. 2016).

5.3 Plant Bio-stimulation by Inoculants

The term bio-stimulants indicates those substances that are promoting the plant growth without being nutrients, soil amendments or pesticides, and being applied in little amounts. However, the lack of legal and regulatory definition at world level prevents an accurate classification and listing of all the substances and microorganisms covering the concept of bio-stimulants (du Jardin 2015). Nevertheless, some class of compounds and microorganisms (e.g. humic and fulvic acids, protein hydrolysates and N-containing compounds, seaweeds extracts, chitosan and biopolymers, and PGPR) are widely recognized as bio-stimulants by the scientific community, the regulators and the stakeholders (Calvo et al. 2014; Halpern et al. 2015). For the proposes of the present chapter we will review experimental evidence about the biostimulants properties only in the case of PGPR applied to horticultural and fruit crops. In the case of horticultural crops, the majority of the experiments have been carried out under controlled conditions (e.g. growth cabinet, greenhouse) and only few in open field. In this latter case, both broccoli and lettuce, inoculated with *Brevibacillus reuszeri/Rhizobium rubi* and *R. leguminosarum* bv. *phaseoli* strain P31, respectively, showed a promotion in the growth of the root system, an increased yield and an enhanced macro- and micronutrients uptake, highlighting the efficacy of the candidate PGPR strains (Chabot et al. 1996; Yildirim et al. 2011). In contrast, the growth promotion experiments carried out by inoculating PGPR in fruit crops (e.g. apple, apricot, banana, cherry) have been predominantly carried out in open filed conditions, also adopting different inoculation strategies as compared to horticultural crops (foliar spray vs. root dipping) (Esitken et al. 2002, 2003, 2006; Kavino et al. 2010; Ryu et al. 2011). The PGPR used for fruit crops inoculation belonged to the genus *Pseudomonas* and *Bacillus* and, in particular, *Bacillus* sp. OSU-142 was able to increase the production, the weight and the quality parameters in the aforementioned fruits (Esitken et al. 2002, 2003, 2006; Kavino et al. 2010; Ryu et al. 2011). In spite of these encouraging data, the main concerns in using PGPR inoculants in open field conditions for horticultural and fruits production are i) the persistence of bacterial strains in soil after the application and their interaction with the autochthonous microflora, as well as ii) the survival of PGPR in the bio-stimulants commercial formulation during the storage. Apple trees inoculated with *Pseudomonas* spp. showed approximately 2-fold increase in the fruit yield after two years of cultivation, suggesting that the PGPR strains were effectively persistent and active in the orchard (Aslantas et al. 2007). However, this specific evidence was obtained in freshly planted pre-inoculated material, so that PGPR could efficiently colonize roots without being

out-competed by soil microflora; on the other hand, such practice is not applicable, for instance, in already established orchard. Furthermore, the persistence of the introduced bacterial strains in the rhizosphere is monitored within very few studies and this is due to the difficulties in specifically recovering the inoculated bacteria (Von Felten et al. 2010). Moreover, the inoculation with fresh bacterial cultures is hardly feasible from an agronomical point of view, therefore the research is orienting towards the development of dry inoculation methods (Bashan et al. 2014; Ruzzi and Aroca 2015). At present, the most popular techniques for the delivery of PGPR into the field is the encapsulated formulation, wherein bacteria are immobilized within a polymeric matrix, as for instance calcium alginate that might occasionally contain also other substances like humic acids, skimmed milk, starch or bentonite (Young et al. 2006; Minaxi and Saxena 2011; Wu et al. 2012).

5.4 *Beneficial Plant-Associated Microorganisms as Bio-fungicides*

The discovery of the antagonistic properties against phytopathogens of several plant-associated microorganism stimulated, already long time ago, the idea of their possible implementation as biological fungicides (Henis and Chet 1975; Stutz et al. 1986) (Fig. 3). Most of the strains used as bio-fungicides, were isolated from suppressive-soils (Chet and Baker 1981) or identified as effective antagonists of phytopathogens in dual-culture plates (Tjamos et al. 2004). These screening approaches may explain why most of the existing active ingredients of bio-fungicides are acting mainly by the production of lytic enzymes, siderophores and antibiotics or as hyperparasites (Markovich and Kononova 2003; Bennett et al. 2006; Vinale et al. 2008; Santoyo et al. 2012; Atanasova et al. 2013). Only recently the induction of resistance in plants by beneficial plant-associated microorganisms received increasing attention, due to the promising results obtained under controlled conditions (Perazzolli et al. 2012). However its tangible efficacy under field condition is still debated (Dagostin et al. 2011). In spite a wide number of species and strains have been shown to possess antagonistic properties against numerous phytopathogens, the active ingredients of the most part of the existing bio-fungicides are mainly few bacterial strains, belonging to the genera *Bacillus* and *Pseudomonas* (Jacobsen et al. 2004; Weller 2007) and fungal strains, belonging to the genera *Trichoderma* and *Coniothyrium minitans* (Whipps and Gerlagh 1992; Vinale et al. 2008).

Once the suitable strain is identified, the beneficial microorganism is commonly produced in industrial fermenters (submerged or solid state fermentation), formulated and applied in large quantities to soil (inundated biocontrol) (Montesinos 2003). One of the most important roles of formulation is guaranteeing a sufficient shelf-life of the plant protection product, in order to be compatible with the business model of the distributing company and the practical needs of the growers

(Segarra et al. 2015). Fungicides based on microbial stains are subject to the same authorization protocol as chemical ones, meaning that they must be proved to be not toxic for humans, animals and the environment. In several countries (e.g. European Union), the bio-fungicide must also be proved to be sufficiently effective against the target disease. In addition, to maximize it efficacy under field conditions, the mechanism of action, the best timing and conditions of application must be identified. Therefore, before placing a bio-fungicide on the market a large body of evidences must be collected and it is not surprising that the most studied strains in literature are indeed the active ingredients of the main commercial products (Velivelli et al. 2014). The depiction of the existing biofungicides is out of the scope of this review and we refer to existing literature (Whipps and Gerlagh 1992; Druzhinina et al. 2011; Santoyo et al. 2012) and we concentrate the discussion mainly on the most important traits for the success and the weaknesses of the existing strains.

Easiness of fermentation at industrial scale, production of spores, which guarantees a good shelf life of the biofungicide, wide spectrum of activity against several bacterial and fungal pathogens and the multiple modes of action, makes *Bacillus* strains the most commercially successful bacterial active ingredients of fungicides. Currently available biofungicides are based on strains belonging to *B. subtilis*, *B. amyloliquefaciens* and *B. pumilus.* These strains antagonize pathogens by direct antibiosis (Stein 2005), but can also induce resistance on plants (Santoyo et al. 2012). Efficacy not only depends on the successful root colonization, but also on the method of application, the plant species and its physiological status (Szczech and Shoda 2006; Pertot et al. 2013).

Similarly to *Bacillus*, *Pseudomonas* strains can colonize rhizosphere and directly (antibiosis) and indirectly control (ISR) a wide range of plant pathogens (Weller 2007). Existing commercial biofungicides are based on *P. fluorescens* species complex (Garrido-Sanz et al. 2016). They can also be efficiently produced in fermenters, however, differently from the *Bacillus* biocontrol strains, they do not form spores (Weller 2007). Therefore, the formulation of Gram negative bacteria is relatively complex compared to spore-forming Gram positive bacteria (Segarra et al. 2015). Difficulties in the formulation, the short shelf-life and the precise conditions of storage may explain why, in spite of relative good efficacy under field conditions, *Pseudomonas* strains have been less attractive for industrial development of biofungicides, compared to *Bacillus*.

5.5 *Beneficial Plant-Associated Microorganisms as Bio-herbicides*

Weeds constitute a serious issue in agricultural production, since they compete with crops and they are thus associated with a limitation in crops productivity and yield (Harding and Raizada 2015). In this context, the agricultural management has been

changed by the introduction of selective herbicides, which enable controlling weeds without affecting non target crops, or at least reducing the effect on them, due to differences in plants at biochemical levels (Mithila et al. 2011). However the use of a limited variety of herbicides, impacting on the same biochemical mechanisms, has led to the development of resistant weeds population in response to the selective pressure, thus exacerbating the need of finding new weeds control methods to preserve the agricultural productivity (Green and Owen 2011; Mithila et al. 2011; Darmency 2013). In this context, the biological control, i.e. the introduction of organisms in the ecosystem to prevent the development of not desired species, has received a great attention in the last decades, mainly focusing on the possible use of bacteria and fungi as controlling organisms (Charudattan 2001; Li et al. 2003; Bailey et al. 2011). Within the biological control of weeds, two main approaches can be distinguished: (i) the classical methods, which is based on the release of natural predators and/or pathogens of the undesired species with the awareness that they will persist in the environment, hindering pests growth throughout the whole ecosystem, and (ii) the bioherbicide strategy (also known as inundative biological control), wherein fungal spores or bacteria are propagated and then released only within the managed area at a concentration that would not naturally occurs in the ecosystem (Dane and Shaw 1996; TeBeest 1996; Auld et al. 2003; Caldwell et al. 2011) (Fig. 3).

A wide number of bacteria have been investigated for the possibility to be used as bioherbicies. *Pseudomonas fluorescence* is generally characterized for its ability to promote the growth of plants (Gamalero et al. 2005), however several strains showed inhibitory effects on germination and growth of plants. The strain D7 was isolated from winter wheat (*Triticum aestivum*) and was shown to feature an inhibitory activity on the germination and growth of downy brome (*Bromus tectorum*) (Gealy et al. 1996), most likely exerted by a combination of extracellular peptides and a lipopolysaccharide (Gurusiddaiah et al. 1994). To date, the most thoroughly studied is the strain WH6, which was shown to impair the germination of 21 monocot and 8 dicots species, with the exception of the modern maize hybrids (Banowetz et al. 2008). The germination inhibition is mediated by the Germination Arrest Factor (GAF), which was characterized as 4-formilaminooxy-L-vinylglycine, synthesized from the amino acid homoserine (Banowetz et al. 2008; McPhail et al. 2010; Halgren et al. 2013). This class of compounds are known to interfere with pyridoxal phosphate-dependent enzymes, including those involved in the ethylene biosynthetic pathway (Halgren et al. 2013). *Xanthomonas campestris* is another bacterial species that has been studied as potential candidate as bioherbicide; several strains (e.g. JT-P482, LVA-987) have been shown to control grasses as annual bluegrass (*Poa annua*) and horseweed (*Conyza canadensis*), however, to date, the effector phytotoxic compounds have not been identified yet (Imaizumi et al. 1997; Boyette and Hoagland 2015).

Besides bacteria, fungi have been also investigated as potential bioherbicides and a survey of the scientific literature about this topic highlighted that mainly three fungal genera have been studied for this aspect, i.e. *Colletotrichum*, *Phoma* and *Sclerotinia*.

Several species belonging to the genus *Colletotrichum* are able to control weeds like hemp sesbania (*Sesbania exaltata*) and spiny cocklebur (*Xanthium spinosum*) (Auld et al. 1988, 1990; Schisler et al. 1991); the mechanisms used by these fungi to inhibit plants growth has not been clarified yet, however the analysis of the genome revealed the presence of sequences putatively associated with pathogenesis, as for instance cell wall degrading enzymes (Gan et al. 2013). In addition, *Colletotrichum* spp. encode also for the biochemical pathway for the biosynthesis of indole-3-acetic acid, whose analogues and derivatives are well established herbicides (Grossmann 2010; Gan et al. 2013).

Among the genus *Phoma*, the species *P. macrostoma* has been thoroughly investigated since it was observed to specifically inhibit the growth of dicot plants (Bailey et al. 2011, 2013; Smith et al. 2015). The strategy adopted by *P. macrostoma* aiming at contrasting the growth of plants consists in the production and release of macrocidins, a member of the tetramic acid family, that impair the photosynthesis especially in the new leaves, suggesting that this effector molecule could be transported within plants via phloematic stream (Graupner et al. 2003; Bailey et al. 2011). Nevertheless, the exact mechanism of action of macrocidin remains to be elucidated. In addition, *P. macrostoma* has been shown to produce also anthraquinone pigment (Quereshi et al. 2011); similar pigments have been isolated from other fungi and can provoke necrosis on the leaf of both wheat and legumes in a light dependent manner (Bouras and Strelkov 2008; Andolfi et al. 2013). However, also in these cases the exact mode of action of the active principle still remains elusive.

Two species of the genus *Sclerotinia*, *S. minor* and *S. sclerotinium*, have been investigated for they proved herbicidal actions against dandelion and creepy thistle (*Cirsium arvense*) (Abu-Dieyeh and Watson 2007; Skipp et al. 2013) and it was observed that the production of oxalate is required so that fungi can express their virulence towards target plants (Magro et al. 1984; Briere et al. 2000). The production of oxalic acid can be stimulated by the addition of succinate to the growth media, thus inducing a higher virulence of the fungi; oxalate is thought to acidify the cell wall, which will enable the degradation through acid hydrolase, and to interfere with the activity of polyphenol oxidase, which play a role in plant defense (Cessna et al. 2000).

In conclusion, considering the need of continuously producing new herbicides to meet the challenge of controlling the development of resistant weeds, the use of bioherbicides could offer higher advantages as compared to traditional herbicides. The most claimed benefits of bioherbicides is the environmental compatibility, which is basically due to (i) the target specificity (Auld and Morin 1995), (ii) the rapid degradation of the effector molecules (Li et al. 2003) and (iii) the inability of the bioherbicide species to freely propagate in the environment (Johnson et al. 1996; Hoagland et al. 2007). In addition, the costs associated with the development and production of a bioherbicide are reported to be generally lower as compared to those required for synthetic herbicides (Auld and Morin 1995; Li et al. 2003). At present, several microbial species have been considered for this role in the

agriculture; nevertheless, to further implement this strategy of weeds control, it will be necessary to develop techniques that allow obtaining the same efficacy observed in small-scale conditions also at the field scale.

5.6 Other Environmental Applications of PGPR Inoculants

Besides the above-described uses of beneficial bacteria in crop production, they have been applied in distinct areas of bioremediation. A comprehensive evaluation of these aspects has been reviewed by de-Bashan et al. (2012). There are clear pieces of evidence that the association of PGPR, arbuscular mycorrhizal (AM) fungi and rhizobia helps restoring vegetation in semiarid areas, undergoing desertification, of southern Europe (Requena et al. 1997, 2001) and tropical regions (Founone et al. 2002) although, so far, no long-term field trial has been reported (Fig. 3).

Inoculation with PGPR enhances the capacity of plants to contain, degrade or eliminate different contaminants from soils, such as pesticides, heavy metals, crude oil (Glick 2003; Mendez and Maier 2008). In general, beneficial bacteria assist plants to overcome contaminant-induced stress, i.e. by ACC-deaminase activity (Huang et al. 2004) or enhance their growth by IAA hormone production (Khan et al. 2009); sometimes rhizosphere microorganisms living in association with plant roots contribute to the decontamination through xenobiotic degradation.

The removal of metals from the environment by concentrating them within the biomass of the plant (phytoextraction) is improved by PGPR and AM fungi by enhancing general growth of plant (the larger the plant biomass, the higher the removal) or by increasing metal mobilization by microbial metabolites (Reichman 2007; Lebeau et al. 2008). Mine tailings, lacking plant cover and soil structure, are a relevant source of metal pollution and pose a long-term health hazard to nearby urban areas. Phytostabilization, that is the use of plants as ground cover, is limited by the weak capacity of most plant species to grow under high metal concentration, low pH, lack of water-retaining clays and essential minerals. Several studies demonstrated the feasibility for phytostabilization of the use of PGPR such as *Azobacterium chroococcus, B. megaterium* and *Arthrobacter* spp. (previously isolated from tailings), in combination (often) with reduced doses of compost or chemical fertilizers (Petrisor et al. 2004; Grandlic et al. 2009).

The bacteria inoculation effects in marginal ecosystems (desertified lands, tailings, highly contaminated soils) is usually more evident than in agricultural for the (generally) low content of competing heterotrophic bacteria in the first (Mendez and Maier 2008). Therefore, the replacement of autochthonous community occurs more easily and lasts longer. Finally, *A. brasilense* has been used to enhance the capacity of microalgae (a kind of "unicellular plant" that may respond to beneficial bacteria as do "eukaryotic plant") to reduce nitrogen and phosphorus in municipal wastewaters (de-Bashan and Bashan 2008).

6 Other Bio-stimulants and Their Interaction with Beneficial Bacteria

Humic substances (humic and fulvic acids, humins) have been shown beneficial to plant growth, yield and nutrition. Calvo et al. (2014) summarized structural characteristics of humic substances, how their activity is related to the source of organic matter and the time of its transformation (Berbera and Garcia 2014) and present a comprehensive summary of publications, reporting effects of humic and fulvic acids on morphology, growth and physiology of various crops (Pinton et al. 1999).

Although the aims of this review do not deal with these aspects, it is worth noting that most of the effects induced by humic substances on plants are similar to those induced by PGPR, (for instance increased uptake of nutrients, enhanced tolerance to abiotic stresses, changing in root structure). Accordingly, a study by Befrozfar et al. (2013) demonstrated that a combination of PGPR and humic acids increased yield in basil plants.

Protein hydrolysates, obtained from both animals and plants, have been reported to enhance nutrient uptake and yields in plants, to stimulate carbon and nitrogen metabolism, as well as defenses to biotic and abiotic stresses. Despite the initial concerns about the safety of protein-based products, Corte et al. (2014) clearly demonstrated that hydrolysates showed no toxic effects on soil microbiota and yeasts, opening a new frontier in the field of plant bio-stimulants (Fig. 3).

7 Conclusions and Future Challenges

The comprehension of structure and functioning of whole soil microbiome and the isolation of new beneficial soil microorganisms could surely contribute to the urgent need to improve crop production (to meet the challenge of feeding the growing population) and to protect plants from biotic and abiotic stresses with always more environmentally-friendly approaches (to meet the challenge of saving non-renewable resources). At the same time, these pieces of information are undoubtedly future goals necessary to sustain the growing market of bio-stimulants. Therefore, efforts should be spent at the scientific level in order to make more efficient PGPR available for long-term field trials. A promising research avenue aims at moving beyond the one-microbe-at-a-time approach, i.e., where only individual strains are deployed for a given crop/conditions. Combining the metabolic potential of two or more microbes, for instance plant growth promotion with pathogen protection, will be likely a key towards more consistent results. Therefore, the study and the application of microbial consortia and synthetic microbiotas is gaining momentum in this research field. Concurrently fundamental research should focus on the biochemical and molecular processes affected in plants by these microorganisms. Akin to the role envisaged for the human microbiota in personalized medicine, an increased knowledge of plant-microbiota interactions will allow

scientists to rationally design crop- and soil-specific solutions. Together, projected outcomes of these investigations, combined with advancements in other research fields, such as plant breeding and agronomy, hold the key to ensure a fair and sustainable future for the planet.

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