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Engineering root microbiomes for healthier crops and soils using beneficial, environmentally safe bacteria.

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Abstract

The Green Revolution developed new crop varieties, which greatly improved food security worldwide. However, the growth of these plants relied heavily on chemical fertilizers and pesticides, which have led to an overuse of synthetic fertilizers, insecticides, and herbicides with serious environmental consequences and negative effects on human health. Environmentally friendly plant-growth promoting methods to replace our current reliance on synthetic chemicals and to develop more sustainable agricultural practices to offset the damage caused by many agrochemicals are proposed herein. The increased use of bioinoculants, which consist of microorganisms that establish synergies with target crops and influence production and yield by enhancing plant growth, controlling disease, and providing critical mineral nutrients, is a potential solution. The microorganisms found in bioinoculants are often bacteria or fungi that reside either within external or internal plant microbiomes. However, before they can be used routinely in agriculture, these microbes must be confirmed as non-pathogenic strains that promote plant growth and survival. In this article, besides describing approaches for discovering plant-growth promoting bacteria in various environments, including phytomicrobiomes and soils, we also discuss methods to evaluate their safety for the environment and for human health. (189 words)

Keywords: Biofertilizer, biosafety, PGPR/B, biopesticide, phytomicrobiome

Introduction

Microbes function as biofertilizers, biopesticides, and plant growth promoters and have been utilized to enhance crop growth in numerous countries around the world, but especially in developing and emerging nations (Bashan et al. 2014). Companies worldwide have supplied nitrogen-fixing inoculants to farmers for decades as well as formulations of plant growth-promoting (PGP) microbes, both fungi and bacteria, to enhance crop production (Wood, 2015). Many microbial products are also used by home gardeners and for organic agriculture, and large-scale, commercial farms in China, the U.S., and Europe are beginning to adopt biological materials as substitutes for chemical fertilizers and pesticides (Parnell et al. 2016). Replacing these compounds is critical for agricultural sustainability (Kecskés et al. 2016, Menendez and Garcia-Fraile 2017), but there is a huge gap in information about the effectiveness of PGP microbes based on laboratory studies versus their performance in the field. It is not always clear how useful many of the bioinoculants discovered in the laboratory are once they are tried in the field or whether or not they or their products might have untoward effects on non-target organisms, including humans.

This review focuses on certain Plant Growth-Promoting Bacteria (PGPB or PGPR for rhizobacteria), which are becoming better known for their potential to promote sustainable agriculture. Currently, bioinoculants are available mostly as single entities (Bashan et al. 2014) but are also being formulated as consortia of multiple bacteria and fungi, which have synergistic PGP traits to: 1) enhance the growth of different crops (Yanni et al. 2001, Laabas et al. 2017); 2) exhibit biocontrol activity (Bach et al. 2016, N. Khan et al. 2017); 3) prime the plant for more efficient pathogen defense (Aziz et al. 2016); and/or 4) increase crop nutritional value (Egamberdiyeva 2007). In some cases, PGPB help the plant grow under extreme conditions, such

as nutrient deficiency, aridity, salinity, and drought (Shinde et al. 2017, A. Khan et al. 2017, Wang et al. 2012, Vílchez and Manzanera 2011). To find the bacteria that are the most effective, they first must be isolated from their original sources, their identity determined, and the traits they possess to support plant growth rigorously evaluated. Moreover, their success under both laboratory and natural conditions needs to be determined, and their potential risks to other plants, animals, and humans must be evaluated. Finally, the question of whether natural soil microbiomes are negatively affected by adding foreign microbes must also be addressed.

The Basics of Soil Microbe Discovery Research

Isolating soil microbes (and/or their DNA) and evaluating their potential as bioinoculants. Both cultivation-dependent and -independent methods are used for constructing inventories of PGPB from their natural habitats, typically soil, roots (rhizoplane or rhizosphere), or internal tissues of plants. For cultivation-dependent analyses, non-selective and selective culture media are traditionally used to find bacteria that readily grow under artificial conditions. The development of inoculants requires that the PGPB multiply in culture, are easily propagated, positively affect plant growth, and are safe for humans and the environment. Many microbes have been cultivated using an enrichment media method whereby a soil sample is mixed with water and the suspension serially diluted onto a non-selective medium such as nutrient agar or any generalized medium that contains a carbon source, amino acids, and salts (Sanders and Miller 2010). A large number of different species of organisms with varied morphologies are likely to grow on non-selective agar plates, so plate washes or individual colonies are subjected to another round of selective media to isolate microbes that grow under more stringent conditions. Subsequent steps often require the use of a culture medium that reveals a particular

PGP trait (Menendez and Garcia-Fraile 2017). Once a single species is isolated, it is usually identified by 16S ribosomal (rRNA) gene sequence analysis. Many of these steps are illustrated in Fig. 1.

Although the above steps seem easy to accomplish, cultivation-dependent methods are often problematic because not all bacterial soil isolates can be grown *in vitro*. Indeed, it has been estimated, based on the discrepancy between the numbers of cells directly counted in an environmental sample versus the number growing in culture medium, that only approximately 1% of environmental microbes are cultivatable (Katz et al. 2016). Nevertheless, the current state-of-the-art is that microbes must be cultured if they are to be used as commercial inoculants, but many possibilities exist as to why certain strains cannot be grown in artificial media. Some bacteria may depend on other microbial species to catabolize a substrate that neither species can break down alone or because two or more bacteria may synthesize a particular metabolite or antibiotic only in the presence of a partner or in a consortium (D'Onofrio et al. 2010, Adnani et al. 2017). Such relationships make it highly unlikely that certain microbes will be cultivated on standard media. However, cultivation techniques continue to be improved, and bacteria missed in previous attempts are being identified. Thus, it is highly likely that new methods and media may help in the search for cultivatable PGPB. In the meantime, elucidating soil and plant-associated bacterial biodiversity without having to go through a culturing step is where advances in molecular biology, genomics, and bioinformatics have not only helped identify new natural products but have also provided insight into the diversity of microbial populations in a variety of environments. These techniques may also help to distinguish pathogenic from non-pathogenic species through a deep analysis of microbial genomes.

For cultivation-independent methods, successful DNA extraction from soil microbes is required, and often the heterogeneous material that makes up soil, namely, clay particles, organic matter, humic acids, etc. interfere with extracting high-quality material for PCR analysis. Fortunately, the methods and efficacy of pursuing cultivation-independent approaches have improved significantly in recent years and have been embraced by both environmental microbiologists and natural product chemists, albeit with different aims.

Once high-quality environmental DNA (eDNA) is obtained, several strategies may be employed for species identification, with the ultimate goal of examining the collective genomes of all the microorganisms within a community to get a better idea of which microbes can be used as future inoculants. Commonly, the 16S ribosomal RNA gene is chosen for community analysis (Winsley et al. 2012) although other highly conserved genes may be used as well (*gyrB*, *rpoB*). However, the use of certain primers in metagenomic sequencing may lead to problems because the primers are often not as universal as they are expected to be. In addition, some bacterial groups can be overrepresented in an environment whereas others may be under- or even not represented at all (Schloss et al. 2011, Wang and Qian 2009).

Another approach is the use of whole-genome shotgun sequencing, whereby total eDNA, composed of random short fragments representative of the microbial community are pooled and assembled with sequence assembly algorithms (Sharpton 2014). This method is said to produce a more comprehensive sample of a complex environment and also sheds additional light on the abundance of various species and the overall diversity within the sample. Furthermore, for plant microbiome analysis, e.g., nodule microbiomes, the plant DNA may need to be subtracted from the total shotgun array of sequences. To bypass this difficulty, alternative isolation methods, using single cell extractions, can be used and then followed by multiple displacement DNA

132 modification and traditional 16S rDNA screenings (Levy et al. 2018). With the increased
133 availability of sequenced plant genomes, plant vs. microbe DNA sorting will become less of a
134 problem (Busby et al. 2017) and allow a more efficient and reliable analysis. The cultivation-
135 independent method reveals the larger scale phylogenetic relationships of bacterial species in a
136 particular environment because it includes those microbes that cannot be cultured (Ellis et al.
137 2003, Ranchou-Peyruse et al. 2006, Štursa et al. 2009).

138

139 **Assays for Finding Potential PGPB.**

140 **Traits for promoting plant growth**. A number of assays, which might predict the
141 isolates' potential performance as PGPB *in planta*, are used to select strains for inoculation.
142 Although phenotypic analyses are important for a first screening, *in planta* studies are absolutely
143 required to ensure that the microbial isolates generate a positive growth effect.

144 Nitrogen fixation is one of the most important of all PGPB traits, and several
145 methodologies have been employed to measure the nitrogen-fixing capabilities of a strain (e.g.,
146 Wertz et al. 2012). However, the ability to grow in an N-limited environment is not a test for
147 nitrogen fixation ability because the media used are rarely completely depleted of nitrogen,
148 which leads to false positives (Martínez-Hidalgo et al. 2014a). For example, bacteria that either
149 effectively scavenge N or have 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase
150 activity may survive for a time on an N-free medium (Schwartz et al. 2013). The apparent Nif+
151 phenotype on –N media and ethylene synthesis inhibition are linked because ACC, the precursor
152 to ethylene, a plant growth inhibitor, is converted by AcdS to ammonia and α-ketobutyrate.
153 Ammonia serves as a nitrogen source for the bacteria and the reduction in ethylene synthesis
154 further promotes growth. *In planta* or *in vitro* analyses with N^{15} provide more accurate

155 measurements for nitrogenase activity and are recommended for verifying nitrogen fixation
156 activity (Martínez-Hidalgo et al. 2014b).

157 Phosphorous, another essential macronutrient, is usually found as insoluble forms in the
158 soil—organic and inorganic phosphates. Hence, phosphate-solubilizing capabilities are a key
159 PGP trait and easily detected by plate assays. Numerous *in vitro* analyses have been described
160 (Peix et al. 2001), and published methods generally take into consideration the existence of
161 acidic, alkaline, and neutral phosphatases. However, they rarely consider phytases (myo-inositol
162 hexakisphosphate phosphohydrolase), which breaks down organic phosphates. Phytates are
163 serious pollutants in certain agricultural areas (Rodríguez et al. 2006), and a number of bacteria,
164 e.g., *Bacillus* species, have phytase genes. In addition, most Gram-negative bacteria break down
165 mineral phosphate by secreting gluconic acid. Several bacterial mechanisms used to solubilize
166 phosphate have been described (Rodríguez and Fraga 1999, Rodríguez et al. 2006).

167 Iron is another indispensible nutrient that is difficult for many plants to obtain, especially
168 those that do not produce their own siderophores. Siderophores are "iron-carrier" molecules that
169 can give adaptive advantages to the plant if the sequestered iron is released by the bacteria and
170 used by the plant. Siderophores may also act as plant pathogen defense factors because bacterial
171 siderophores are better at binding Fe^{3+} than fungal pathogens. Siderophore activity can also be
172 detected through plate assays (Alexander and Zuberer 1991).

173 Microbes also synthesize plant hormones, including indole acetic acid (IAA),
174 gibberellins, cytokinins, and others (Lugtenberg and Kamilov 2009, Glick 2012). Perhaps the
175 best way to detect phytohormones is through spectrophotometric analyses rather than bioassays
176 such as the Salkowski test for IAA, which often results in false positives. For example, we
177 identified incomplete IAA-synthesis operons in the *Bacillus simplex* genome, but no IAA was

178 detected by LC-MS/MS-MRM (Maymon et al., 2015). However, genes for the synthesis of
179 polyamines, which also regulate plant growth, were found, and peaks for spermine, spermidine
180 and putrescine were detected (Maymon et al. 2015). Spermidine, also synthesized by *B. subtilis*
181 OKB105, promotes plant growth by inhibiting ethylene synthesis in plant root cells (Xie et al.
182 2014).

183 Many beneficial microbes also produce volatiles such as 2,3-butanediol, and/or other
184 elicitors, e.g., proteins (flagellin); cell wall or membrane fragments (LPS); antibiotics (2,3-
185 diacetylphoroglucinol, phenazine), as well as siderophores that trigger induced systemic
186 resistance (ISR) to a broad range of parasites and pathogens (Van Loon and Bakker 2005;
187 Pieterse et al. 2014) (Fig. 2). For example, the actinomycete *Micromonospora*, which was
188 isolated from alfalfa root nodules, generates an ISR in tomato against *Botrytis cinerea* (Martinez-
189 Hidalgo et al. 2015). Microbial molecules prime the plant and trigger its innate immunity, which
190 results in the expression of genes for the synthesis of endogenous phytohormones including
191 salicylic acid, jasmonic acid, and ethylene, thereby reducing infection and disease. Volatiles are
192 also reported as being nematicidal or anti-fungal.

193 In summary, the number of molecules involved in beneficial microbe-plant
194 communication overall is staggering and a recent review describes many of them (Chargas et al.,
195 2018).

196

197 **Impact of Bioinoculants on Soil Microbiome**

198 Although microbial fertilizers have been released into the market and the strains used in
199 them studied in depth, the effects of adding large numbers of foreign microorganisms to

indigenous soil microbiomes are under-investigated. It is important to initiate studies to creating a data set that gives the microbial baseline of soil prior to inoculation as well as afterwards (Fig. 1). In this way, we can assess the importance of microbiome shifts and how they affect soils and crops (van Elsas et al. 2015). Careless additions could affect soil health negatively, altering soil performance in nutrient cycling and the capability to promote plant growth (van Elsas et al. 2015).

To differentiate the indigenous bacterial communities from inoculants, several methods that aim to obtain more quantitative and statistically robust data have been developed (Kowalchuk et al. 2004). Semenov et al. (2014) described taking measurements on the so-called normal operating range in different soils at undisturbed states at different times. This strategy defined a reference (the normal operating range of a soil) and used a mathematical approach to evaluate how much divergence a soil shows from this unaltered state. Other strategies include the study of soil microbial biomass and enzyme activity, which respond very quickly to changes in soil management (García-Ruiz et al. 2008).

Experiments with turmeric (*Curcuma longa*), a crop that exhausts the soil, revealed that the use of *Azospirillum lipoferum* and *Bacillus megaterium* combined with organic manure could increase microbial biomass compared with adding chemical fertilizers. Also improved was the amount of total N mineralized as well as oxidoreductase enzyme production, an indicator of microbiological activity. In this study, the soil quality was improved by the use of inoculants (Dinesh et al. 2010).

Another indirect form of microbial activity measurement is cellulolytic activity. A study by Zhao et al. (2005) showed the differences in cellulolytic activities in soil depending on the biofertilizer used. For biofertilizers that contain a mixture of different bacteria, results show that

certain inoculants cause a shift in cellulose degradation in PGPB-inoculated fields versus uninoculated fields, suggesting that the type of biofertilizers or inoculants used could affect the microbial community structure.

A more direct way of monitoring changes in soil over time in response to an inoculum is through metagenomic sequencing (Fig. 1). The same strategy of isolating eDNA via the cultivation-independent method described earlier needs to be followed, but it will be essential to have samples taken prior to the addition of both biological and abiotic amendments. Such an approach is a research area that will need a great deal of development over the next decade.

Biosafety Concerns

Mutualists or Pathogens? Greater use of PGPB as supplemental inoculants or total replacements for chemical pesticides and herbicides is key to sustainability, but questions of efficacy and biosafety must be addressed. For example, a number of bacterial species potentially harmful to mammals, including humans, have been isolated from plant rhizospheres. Many bacteria with plant growth-promoting traits belong to genera such as *Burkholderia, Enterobacter, Ochrobactrum, Pseudomonas, Serratia, Klebsiella*, and *Ralstonia*, and are phylogenetically related to species that are virulent or are opportunistic human pathogens (Berg et al. 2005). Such relationships are not to be taken lightly because the possibility exists that some of these bacteria might cause nosocomial infections and disease in immunocompromised patients (Baldwin et al. 2007, LiPuma 2010). Strains with PGPB activity that are related to the *B. cepacia* or *B. cenocepacia* lineages are commonly isolated from soil and also from root nodules (Martínez-Hidalgo and Hirsch 2017).

The genus *Pseudomonas* has for many years been used in commercial inoculants and a member of that genus, *P. aeruginosa* is a dangerous human pathogen. It is a common cause of respiratory tract infection in people with cystic fibrosis, a serious hereditary lung disease. Other commonly found pseudomonads in clinical samples and in soil include *P. fluorescens*, *P. putida*, *P. pseudoalicaligens*, *P. sturtzeri*, and *P. putrefaciens* (Ortega-Calvo and Saiz-Jimenez 1998, Baum et al. 2009). Commercial phenotypic tests are not always able to differentiate among the different species. In a study where Spilker et al. (2004) tested 66 pseudomonads from sputum from various laboratories across the world, they showed that 38 of them were initially misidentified using phenotypic traits. Using genus- and species-specific PCR assays and 16S rDNA sequencing, these authors reported that many of the strains were identified as *P. aeruginosa*. Among the isolates, they also detected *P. fluorescens, P. ludensis, P. pseudoalcaligenes, P. stutzeri*, *and P. synxantha.* Thus, where molecular techniques are not available, the identification of pseudomonads can be challenging, strongly suggesting that the probability of human infection could increase.

The rhizosphere and some plant parts such as leaves have been shown to also house opportunistic human pathogens including *P. aeruginosa* (Berg et al. 2005). A study by Kumar et al. (2013) using *recN* sequencing, multilocus sequence typing, and comparative genome hybridization showed that a *P. aeruginosa* strain isolated from black pepper in India initially did not cluster with *P. aeruginosa* strains that originated from clinical isolates. However, the same strain later proved to be resistant to many antibiotics, grew at high temperatures, and was toxic to mammalian cells. Other researchers have published on additional *P. aeruginosa* strains. For example, Wu et al. (2011) found a strain that was easily cleared from a mouse with acute lung infection. Together, these studies highlight the need for taking any potential PGPR through a

rigorous biosafety protocol. Interestingly, while regulatory frameworks for biosafety are said to be in place, most government publications on the topic are unclear on the topic of inoculants or do not define what a bioinoculant is or regulate them using outdated lists of genera that do not represent current knowledge on biosafety, if they are mentioned at all. The latter is a concern because the use of molecular methods to better differentiate bacterial taxa has resulted in genus name changes for many species, e.g., *Pseudomonas cepacia* is now *Burkholderia cepacia*, *Pseudomonas maltophila* is *Stenotrophomonas maltophila*, and others. The name changes and related evidence are not readily disseminated to many government agencies. Thus, similar to dietary supplements, where the risk of toxicity or contamination from an unwanted source is possible because of the lack of standardized quality control (Coutinho Moraes et al. 2015), "agricultural amendments" also require rigorous testing for safety as well as efficacy.

Although for the majority of the rhizosphere microbiomes investigated, information on how they impact plant growth is incomplete, many studies (Glick et al. 1997, Rodriguez and Fraga 1999, Bloemberg and Lugtenberg 2001, Vessey 2003) reported a positive impact of pseudomonads on plant growth. The mechanisms of plant growth by these organisms are well researched and documented (Compant et al. 2005, Compant et al. 2010, Glick 2012).

Table 1 shows several examples of genera that have both pathogenic and mutualistic representatives. Because of their potential health risk, the use of the *Burkholderia cepacia* complex (Bcc) in the field has been restricted (U.S. Environmental Protection Agency 2003); see also Chiarini et al. 2006 for a description of the Bcc). Eberl and Vandamme (2016) have discussed these topics in great depth with reference to *Burkholderia*, and efforts are being made to separate the pathogenic *Burkholderia* species from the beneficial ones (see Estrada-de los Santos et al. 2016, 2018) based not only on phylogeny, but also on physiology and the absence or

presence of factors associated with virulence. Earlier, Gyaneshwar et al. (2011) showed that the plant-associated and nodulating *Burkholderia* (now *Paraburkholderia*, Sawana et al. 2014) have a lower G+C content than the pathogenic species. Whether this is coincidental or meaningful is difficult to evaluate at this time.

The genus *Ochrobactrum* is similar to *Burkholderia* in that several strains induce nitrogen-fixing nodules on legume roots (Willems 2006) whereas others appear to have PGP capabilities, but lack nodulation ability (Tariq et al. 2014). However, the symbionts, *O. lupini* and *O. cytisi*, are closely related to the opportunistic human pathogen *O. anthropi* (Trujillo et al. 2005, Zurdo-Piñeiro et al. 2007). Other studies show that human pathogenic strains of *O. anthropi* form a subpopulation that differs from the plant-associated strains (Romano et al. 2009), but more testing is needed.

Rhodococcus is another example of a genus where some species can be either plant pathogens or PGPB. Some members of the genetically diverse genus *Rhodococcus* are pathogenic and cause fasciations and hyperplasias when certain virulence genes are expressed (Creason et al. 2014, Putnam and Miller 2007). An example of a PGPB strain is *Rhodococcus erythropolis*, which promotes pea growth especially at low temperatures and in heavy-metal contaminated soils (Trivedi et al. 2007). This *Rhodococcus* species, isolated from *Hedera helix,* is important for phytoremediation (Stevens et al. 2017) as well as plant-growth promotion via plant hormones (Francis et al. 2010). However, some studies show that this same species contains strains that can cause septicemia or encephalitis in immunocompromised patients (Park et al. 2011, Bagdure et al. 2012). Efforts are being made towards an effective way of distinguishing the plant pathogens from beneficial bacteria. Given that molecular methods potentially can be used to distinguish pathogenic strains from beneficial ones (Savory et al.

2017), one goal would be to employ such methods routinely to address this issue in the future.

Another feature used to distinguish non-pathogenic from pathogenic strains is that the latter grow at human body temperature and the former do not (Berg and Martinez 2015; Eberl and Vandamme 2016). Growth of a strain at 37 °C is a definite concern and accordingly, such isolates should not ever be employed as PGPBs. The risk of opportunistic infections is far too great, especially for immunocompromised patients, and points to the need for exclusion of certain taxa from consideration as bioinoculants. Further study is warranted.

Given these examples, it is clear that a deeper understanding of the molecular, physiological, and biochemical characteristics of PGPB is needed. Microorganisms are currently classified into different risk groups based on their safety of use to avoid human health risks. Only microbial strains included in Risk Group 1 (Europe) or Biosafety Level (BSL) 1 (USA) are regarded as safe and utilizable as bioinoculants. However, this classification should not be considered as the only valid reference to determine the potential risk of a novel or established microorganism.

In addition, mutualistic as well as potential pathogenic characteristics of a microbial strain are often clustered into pathogenicity or symbiotic islands, the genes of which are responsible for either the synthesis of virulence factors or the mutualistic interaction of the strain with a particular host (Dobrindt et al. 2004). Hence, a good first start is to sequence the genomes of potential PGPB microbes and determine whether pathogenicity islands or genes are present. This strategy is extremely helpful in determining the potential risks of a microbial inoculant. Furthermore, whole genome comparisons between potentially pathogenic and mutualistic members of a single genus, as described for *Rhodococcus*, will provide critical information about the potential avirulence or virulence of a strain. Also, studies testing whether pathogens and

commensals/mutualists have the ability to take up and more importantly, maintain genes conferring each other's behaviors may also need to be performed. Cases where there are definite blocks to gene exchange between the different strains/species might be a benchmark for using a particular strain in agriculture.

Species of *Micromonspora* follow many of the trends indicated by Levy et al. (2018) such as increased genome size in root and nodule-associated species (data not shown). However, this correlation is not strict because non-ecto- and endo-rhizospheric species (*sensu* Carro et al. 2018), such as *M. pallida* DSM 45599^T and *M. carbonaceae* DSM 43148^T (7762816 and 7941928 bp, respectively) have larger genomes than either *M. coriariae* DSM 44875^T (6929687 bp) or *M. lupini* Lupac 08 (7321224 bp), which are nodule isolates. However, similar to the findings of Levy et al. (2018), the genomes of *Micromonospora* spp. are replete with a large number of genes involved in carbohydrate metabolism (Carro et al. 2018).

Biosafety tests for bioinoculants. Before the use of bioinoculants can expand further into routine field applications, concrete regulation and testing with a system of assessment of the biosafety of PGPB strains with respect to human, animal, other plant life, and the environment is needed (Berg 2009, Selvakumar et al. 2014). As mentioned earlier, thorough strain characterization is essential along with tests for pathogenicity and toxicity to eliminate strains that pose even a minimal risk. In the lab, *Caenorhabditis elegans* has been used as a model organism to obtain insight into whether certain bacterial strains of *Burkholderia, Pseudomonas, Serratia,* and *Stenotrophomonas* were or were not harmful to the nematodes (Aballay and Ausubel 2002, Zachow et al. 2009, Angus et al. 2014). Additional tests include the use of insect and other animal hosts as well as plants (Fig. 1) (Vílchez et al. 2016). Plant tests are usually performed with the host of a known pathogen. In the case of plant pathogenic *Burkholderia*,

Allium cepa bulb scales have been used to screen for *B. cepacia* strains that cause disease (Jacobs et al. 2008). Tests to determine the disease potential of various microbes have also been carried out on the non-host *Nicotiana benthamiana* (Wei et al. 2007, Savory et al. 2017).

Ecological toxicity must also be considered because a wide range of micro- and macroscopic organisms could be affected by inoculating novel PGP strains (Stephens and Rask 2000, Köhler and Triebskorn 2013). Vílchez et al. (2016) proposed the Environmental and Human Safety Index (EHSI) that assesses the biosafety of the bacterial strains used as bioinoculants. EHSI is based on a panel of assays on model organisms for all trophic levels and has two primary advantages: it avoids the high economic cost of testing the environmental impact of bioinoculants and does not employ assays on vertebrates. This economic factor helps primarily small industries that cannot afford the large-scale series of tests that large multinational companies undertake. Nevertheless, some vertebrate testing may be required depending on the potential risks of the species in question (Fig. 1).

Many microbes are already viewed as non-pathogenic (Risk Group 1/BSL1), including most species of *Rhizobium* and allied genera as well as *Azospirillum* and *Azotobacter* species, which fix nitrogen and also exhibit numerous PGPB traits. Rhizobial species and *Azospirillum* are well represented among the commercial inoculants such as Monsanto BioAg (Monsanto 2015a) or Seedland (Seedland 2013). Moreover, many *Bacillus*, *Paenibacillus*, and *Brevibacillus* species are commonly employed for their PGP ability and may also be used as biocontrol agents. *Bacillus* species are frequent PGPB partners with rhizobia or mycorrhizal fungi to establish effective tripartite symbioses with plants (Francis et al. 2010, Schwartz et al. 2013). A number of *Bacillus* species are already available as bioinoculants (Monsanto 2015b). Nonetheless, some *Bacillus*, *Paenibacillus*, and *Brevibacillus* species are animal pathogens,

383 namely *B. anthracis*, *P. larvae*, and *B. laterosporus* (Francis et al. 2010, Grady et al. 2016,
384 Marche et al. 2017), and hence should be avoided in any consideration of their use as PGPB.

385 Actinobacteria, such as the genus *Micromonospora*, some *Streptomyces* species, and
386 *Frankia*, which is a nitrogen-fixing genus that nodulates certain non-legume trees and shrubs,
387 e.g., *Casuarina*, *Alnus*, and *Ceonothus* (Froussart et al. 2016) are good candidates for
388 bioinoculants. Although *Micromonospora* strains are not associated with biological nitrogen
389 fixation (Martínez-Hidalgo et al. 2014b), they are common inhabitants of both legume and
390 actinorhizal nodules, and frequently in high numbers (Trujillo et al. 2015). In addition,
391 *Micromonospora* strains are important agents for biocontrol and plant growth promotion
392 (Martínez-Hidalgo et al. 2014a, 2015). Like the Firmicutes, Actinobacteria have been co-
393 inoculated with nitrogen-fixing rhizobia onto legumes to enhance the mutualistic interaction
394 (Solans et al., 2009, Benito et al. 2017). So far, no human disease-causing isolates have been
395 detected in the genus *Micromonospora*, nor have any plant pathogens been described, which
396 strongly suggests that this BSL1 genus consists of biologically safe microorganisms.
397 Interestingly, only 1% of *Streptomyces* species are plant pathogens (Wanner and Kirk 2015), and
398 to our knowledge, only one human pathogen, *Streptomyces somaliensis*, has been described in
399 the literature; its genome has been sequenced (Kirby et al. 2012).

400 Rigorous studies of the efficacy as well as the potential risks of novel microbes in
401 sustainable agriculture must be pursued, especially in light of the effort to replace chemical
402 pesticides and fertilizers. Nevertheless, another problem may surface when an inoculant reaches
403 the market, and this is related to whether the farmer is willing to buy the product. Besides the
404 lack of communication between academic scientists and farmers, there are several issues that
405 may keep farmers from using inoculants. The most pressing is that chemical fertilizers provide

406 almost instantaneous positive results, whereas inoculants need more time and are not always
407 consistent in their beneficial effects in the field (Parnell et al. 2016). Also, the knowledge level
408 farmers need to correctly apply bioinoculants is higher than with chemical fertilizers. The
409 procedures for application are new and the benefits from that application may be unclear or
410 different from the readily visible effect of chemicals, a risk that some farmers may not be willing
411 to take (Tabassum et al. 2017).

412

413 **Future Prospects**

414 A new era in plant-microbe interactions has begun. In the past, the one plant-one
415 microbe model was very effective in understanding the complicated genetic interactions that
416 occurred between two organisms. Now it is important to elucidate how diverse microbes either in
417 small clusters or large consortia interact with their plant hosts, their environment, and the
418 indigenous microbial communities. Also, we need to learn more about what differentiates a
419 beneficial microbe from a pathogen.

420 A recent large-scale genomic comparison of plant-associated bacteria (comprising
421 endophytes, root-adhered (rhizoplane or rhizosphere), soil, and non-plant-associated (NPA)
422 microbes) has given us clues as to the characters that will help in this endeavor. Levy et al.
423 (2018) found that the dominant bacteria associated with plants are Actinobacteria, Bacteriodetes,
424 Firmicutes and Proteobacteria, all of which had been suggested as dominant phyla by earlier
425 studies. Interestingly, bacteria in these phylogenetic groups have much larger genomes than the
426 NPA microbes, as well as more genes coding for enzymes involved in carbohydrate metabolism
427 (Levy et al. 2018). Another intriguing difference was that the NPA microbe genomes had more
428 mobile genetic elements (phages and transposons) than the plant-associated group, even though

429 the NPA microbes had smaller genomes. Analyses of these and other differences between the
430 plant-associated bacteria and the NPA, especially in the genera that consist of both beneficial and
431 pathogenic species, may not only prove to be useful in designing agricultural microbiomes, but
432 may also result in a better delineation of the differences between pathogenic and beneficial
433 microbes.

434 Culturing new varieties of PGPB is also an extremely important priority for the future
435 because unless the bacteria can be grown and developed into commercial inoculants, new players
436 in plant-microbe interactions will remain small in number. Efforts are being made in this
437 direction, but it will take a dedicated, as well as well-funded effort, to bring more scientists into
438 pursuing this goal. Building on data obtained from basic studies in plant-microbe interactions,
439 scientists now need to develop a diverse toolset for not only preserving soil health, but also for
440 growing crops sustainably. It will take a dedicated assemblage of scientists to develop microbial
441 consortia as critical inputs into agriculture to ensure that our environment remains not only
442 productive but also healthy.

443

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Figure Description

Figure 1. An overview of efficacy and biosafety evaluation methods for plant growth-promoting bacteria. A soil sample from a natural habitat is diluted (A) and plated on selective and non-selective media (B). After incubation (short- and long-term), individual isolates are plate-purified and identified by 16s rRNA sequencing (C). Identified bacteria are subjected to multiple biochemical assays, such as phosphate solubilization (illustrated), along with quantitative mass spectrophotometry studies to investigate the presence of plant hormones, elicitors, and other compounds and determine their potential as PGP molecules. Isolates are then used as bioinoculants on plants to confirm growth enhancement (D). A thorough biosafety assessment is applied based on phylogenetic, physiological, and molecular testing for the

912 presence or absence of virulence factors. Pathogenicity and toxicity are determined by testing
913 the isolated strains on various model organisms such as mice, nematodes, and plants (E). DNA
914 fingerprinting (also illustrated in E.) is often used as a molecular tool to discriminate between
915 pathogenic and non-pathogenic strains. The ecological effects of using the bioinoculants on the
916 endogenous environment are assessed by metagenomic analyses of the soil microbiome pre- and
917 post-inoculation (A and F).
918
919 **Figure 2**. The interactions of plants and their microbial community as well as the abiotic and
920 biotic factors associated with soil that highly influence plant growth. Plants, microbes, and soil
921 chemistry are all linked together by their requirement for water. In addition, the interactions
922 between the microbial community and the various elicitors and volatiles they produce that trigger
923 ISR, (in this case against white flies), as well as rhizodeposition and decompositon/nutrient
924 recycling brought about by the plant-microbe collaboration all affect plant growth. The various
925 gases diffusing in soil are starting or end points for microbial metabolism. Modified from an
926 unpublished drawing prepared by Drora Kaplan.
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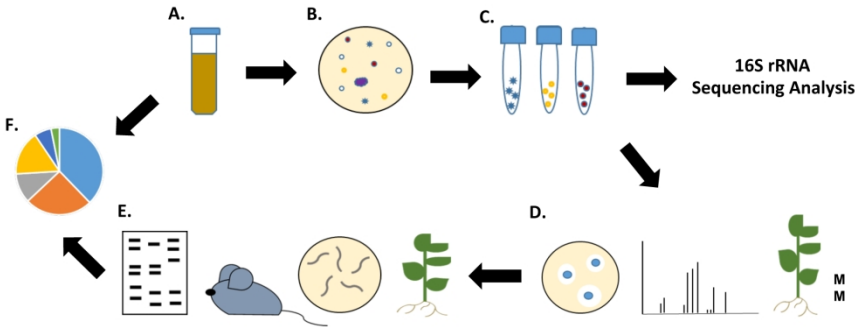
Table 1: Important PGPR genera and their potential health risks.

Bacteria	Uses as PGPR	Health risk
Acinetobacter baumannii	Rokhbakhsh-Zamin et al. (2011)	Peleg et al. (2008)
Achromobacter xylosoxidans	Dawwam et al. (2013)	Duggan et al. (1996)
Bacillus cereus group	Guttmann and Ellar (2000); Liu et al. (2015)	Kotiranta et al. (2000)
Bacillus simplex	Schwartz et al. (2013)	Angus et al. (2014)
Burkholderia cepacia	Dinesh et al. (2014), Dinesh et al. (2015)	da Costa Capizzani et al, (2017); Mali et al. (2017)
Burkholderia cenocepacia	Ho et al. (2015)	Scoffone et al. (2017)
Enterobacter cloacae	Nelson (1988), Singh et al. (2017)	Sanders and Sanders (1997), Davin and Pages (2015)
Enterobacter sp.	Selvakumar et al. (2014) and refs. therein	Selvakumar et al. (2014) and refs. therein
Klebsiella pneumoniae	Pramanik et al. (2017);	Clegg and Murphy (2016)
Micromonospora sp.	Martínez-Hidalgo et al. (2014, 2015)	ND
Ochrobactrum anthropi (Ribotype B)	Chakraborty et al. (2009)	Teyssier et al. 2005
Ochrobactrum intermedium (ribotype A)	Paulucci et al. (2015)	Teyssier et al. 2005
Other Ochrobactrum (Ribotype C)	Hahm et a.l (2012)	Teyssier et al. 2005
Pantoea agglomerans	Mishra et a.l (2011)	Dutkiewic et al. 2016
Phyllobacterium spp.	Flores-Félix et al. (2015)	Swings et al. 2006
Pseudomonas putida	Patten and Glick (2002)	Fernández et al. (2015)
Pseudomonas stutzerii	Lim et al. (1991), Yan et al. (2008), Islam et al. (2015)	Shalabi et al. (2017)
Ralstonia mannitolilytica	Grönemeyer et al. (2012)	Ryan and Adley (2014)
Ralstonia pickettii	Paul et al. (2013)	Ryan and Adley (2014)
Serratia marcescens	Lavania et al. (2006)	Marin et al. (2017)
Stenotrophomonas maltophila	Islam et al. (2015)	Berg and Martinez (2015); Ribbeck-Buschet al. (2005)
Stenotrophomonas rhizophila	Alavi et al. (2013)	Berg and Martinez (2015)
Streptomyces somaliensis/sudanensis	Qin et al. (2015)	McNeil and Brown (1994); Quintana et al. (2008)

ND: none determined. No pathogenic representatives have been found in the literature. Green: No pathogenic strains have been found for the species based on some of the tests described herein. Yellow: opportunistic strains have been isolated from diseased humans. Red: Pathogen of importance for human health.



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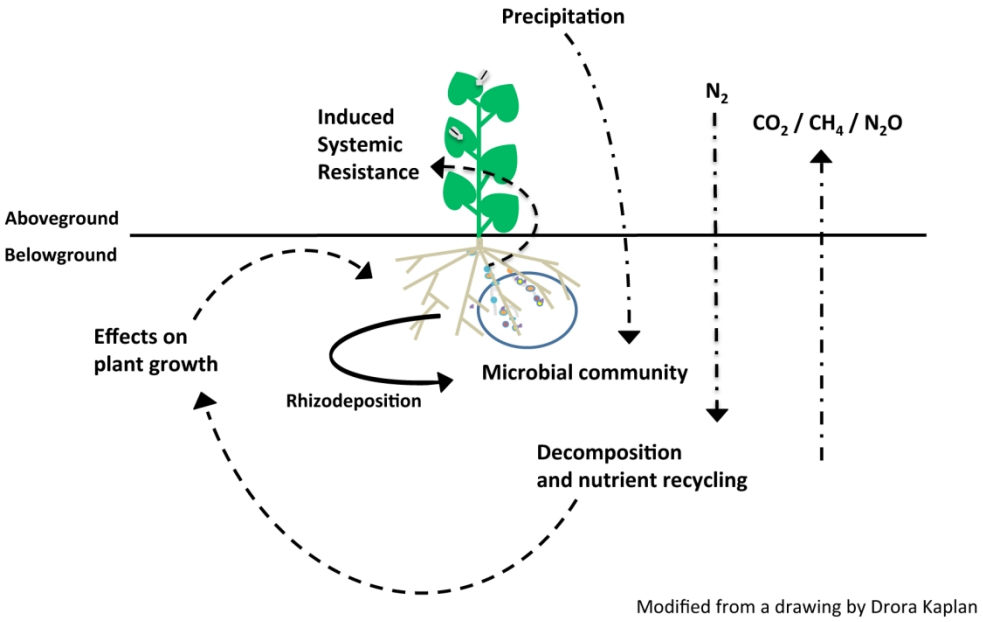

Figure 2. The interactions of plants and their microbial community as well as the abiotic and biotic factors associated with soil that highly influence plant growth. Plants, microbes, and soil chemistry are all linked together by their requirement for water. In addition, the interactions between the microbial community and the various elicitors and volatiles they produce that trigger ISR, (in this case against white flies), as well as rhizodeposition and decompositon/nutrient recycling brought about by the plant-microbe collaboration all affect plant growth. The various gases diffusing in soil are starting or end points for microbial metabolism. Modified with permission from an unpublished drawing prepared by Drora Kaplan.

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